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Transgenic Plants
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TRANSGENIC PLANTS – ADVANCES AND LIMITATIONS

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http://dx.doi.org/10.5772/1409
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First published in Croatia, 2012 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

Additional hard and PDF copies can be obtained from orders@intechopen.com

Transgenic Plants - Advances and Limitations
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p. cm.

ISBN 978-953-51-0181-9

eBook (PDF) ISBN 978-953-51-5229-3

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Meet the editor

Yelda Özden Çiftçi is an Associate Professor in the Department of Molecular Biology and Genetics at Gebze Institute of Technology, Kocaeli, Turkey. Her studies focus on many aspects of plant biotechnology including molecular marker-based characterization, development of in vitro propagation, medium and long-term conservation (cryopreservation) techniques, assessment of genetic fidelity of micropropagated/conserved plantlets. In recent years, she is also involved in Agrobacterium-mediated transformation of woody plants in collaboration with Vigo University, Vigo, Spain. She has authored or co-authored more than 50 papers, reviews, book chapters and congress proceedings.

Contents

Preface

"Green revolution" aided to develop enormous number of improved varieties especially in wheat and rice. Following this revolution, traditional and molecular breeding that benefited from either the desirable genes available naturally or induction of mutation in economically valuable species, provided also improved varieties in tree species. However, with the advent of transgenic technology, it became possible to introduce foreign genes from other plant species that are cross-incompatible and/or even from bacteria, fungi, viruses, mice, and humans. Thus, the scientific community realized the importance of genetically modified (GM) crops, not especially for supplementation of enough food to the growing population, but also for decreasing the usage of pesticides and other crop protective chemicals.

Today, GM crops are cultivated in USA, Argentina, Brazil, Canada, China, India, Paraguay, South Africa, Germany, Spain etc. and numerous studies revealed that cultivation of GM crops is safe for the environment and usage as food, at least for approved plants. However, there is still a public concern on GM crops in a number of countries especially in European Union. The main concerns involve cross-pollination between GM crops and wild species, the use of especially antibiotic resistance marker genes, the introduction of possible allergens into the food chain, generation of adverse effect on non-target organisms. But, all of these concerns caused improvements of the technology such as development of new marker systems as phosphomannose isomerase (PMI) and marker-free plants and also production of cisgenic plants. Moreover, biosafety regulations are also carried out very carefully to prevent its potential side-effects.

As emerging studies carried on transgenic plants, this book tried to address many aspects of GM plants including its application on different plant species (i.e., wheat, fruit trees and sorghum) together with its usage for crop improvement (i.e., insect and virus resistance, enhancement of quality etc.) and metabolomic studies (i.e., usage for gene discovery and production of biopharmaceuticals). In addition, the risk assessment and economical implications of GM crops are also discussed. Thus, this book is structured into four sections namely, i) Application, ii) Crop Improvement, iii) Metabolomics, and iv) Biosafety. All of those sections include general and research papers that are written by scientist who have experience in transgenic technology. I would like to thank to all of the Authors not only for making this book a valuable

recent resource for various aspects of transgenic crops, but also for enabling this Open Access publication to reach many scientist, teachers, and students working in that field. Finally, I would also like to thank InTech Publishing Company, especially Publishing Process Managers Mr Marko Rebrovic and Ms Silvia Vlase, and the Technical Editor of the book.

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Part 1

Application

1

Agrobacterium-Mediated Transformation of Wheat: General Overview and New Approaches to Model and Identify the Key Factors Involved

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1. Introduction

Wheat is the world's second largest crop, supplying 19% of human calories; the largest volume crop traded internationally and grown on approximately 17% of the world's cultivatable land (over 200 million hectares) (Jones, 2005; Atchison et al., 2010). However, probably due to climate change, some adverse environmental conditions have caused a downward trend in world wheat production (FAO, 2003; 2011). In this context, developing new higher yielding wheat varieties more tolerant or resistant to abiotic and/or biotic stress, using all available plant biotechnology technologies available, should be considered as the major challenge.

The scientific community has made considerable efforts to understand and improve the goal of the integration of an exogenous T-DNA in the genome of a host plant cell and, subsequently, the regeneration into a whole plant. The most extended method for plant genetic transformation uses the *Agrobacterium* bacteria as the biological vector to transfer exogenous T-DNA into the plant cell. Although, *Agrobacterium*-mediated transformation became widely available for the routine transformation of most crops, cereals initially have been recalcitrant to this system, since these crops were not naturally susceptible to *Agrobacterium* sp (Potrykus, 1990, 1991). However, by the mid-1990s, improvements in technological development in *Agrobacterium*-mediated genetic transformation led to the desirable transformation of wheat (Cheng et al., 1997; Peters et al., 1999; Jones et al., 2007). These results "open the avenue" by avoiding the usage of gene direct transfer methods, such as biolistic, which is widely found more disadvantageous compared to *Agrobacterium*-mediated transformation (Jones, 2005; Jones et al., 2007; Khurana et al., 2008).

Developing an appropriate method for genetic *Agrobacterium*-mediated transformation is a highly complex task, because it is essential to understand the effect of all the factors

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influencing the T-DNA delivery into the tissue from which whole plant can be regenerated. After plant regeneration, further analyses were required to check the integration and stability of the T-DNA and to obtain the final transformation efficiency parameter. Artificial intelligence technologies are very successful in establishing relationships, in complex processes, between multiple processing conditions (variables or factors) and the results obtained, using networks approaches. Recently, several studies have demonstrated the effectiveness of artificial neural networks and neurofuzzy logic in modelling and optimizing different plant tissue culture processes. Neurofuzzy logic is a useful modeling tool that has been introduced to help the handling of complex models and to data mining. Data mining can be defined as the process of discovering previously unknown dependencies and relationships in datasets. It is a hybrid technology combining the strength and the adaptive learning capabilities from artificial neural networks (ANNs) and the ability to generalize rules of fuzzy logic. Neurofuzzy logic technology generates understandable and reusable knowledge in the way of IF (conditions) THEN (observed behavior) rules helping the researchers to understand the process or the phenomena they are studying (Gallego et al., 2011).

In this chapter we overview the recent advances in *Agrobacterium*-mediated transformation of the wheat, but we also proposed the utility of artificial intelligence technologies as a modeling tool used to understand the complex cause–effect relationships between the most common parameters used in *Agrobacterium*-mediated transformation of the cereals too. That information should help cereal researchers to gain in knowledge on the transformation process, which means determining the factors that favour the interaction between *Agrobacterium* and cereal plants in order to improve the transfer of T-DNA and afterwards to regenerate whole plants from transformed cells, improving final transformation efficiency. Moreover, in a near future, this technology could be easily adapted to the rest of cereals or even any crop.

2. Agrobacterium-mediated transformation: Main factors

From the early 1990s many efforts were carried out in order to achieve stable transformation of wheat via *Agrobacterium*-mediated transformation (Bhalla et al., 2006; Vasil, 2007). This methodology presents several advantages over other approaches including the ability to transfer large segments of DNA with minimal rearrangement of DNA, fewer copy gene insertion, higher efficiency and minimal cost.

Several factors were identified as influencing the efficiency of T-DNA delivery: primary source materials; *Agrobacterium* strains; plasmids vectors; *Agrobacterium* density; medium composition; transformation conditions such as temperature and time during pre-culture, inoculation and co-culture; surfactants or induction agents in the inoculation and co-culture; and antibiotics or selectable markers, among others (Jones et al., 2005; Bhalla et al., 2006; Opabode, 2006; Kumlehn & Hensel, 2009).

2.1 Plant material

A summary of the different plant sources reported as main factors for *Agrobacterium*-mediated transformation of wheat can be found in Table 1. Wheat recalcitrance to *in vitro* culture is one of the most important crucial steps for *Agrobacterium* mediated transformation

protocols and directly correlated with the wheat source material. It was assessed that *in vitro* regeneration can be highly influenced by different factors such as plant growth regulators. In fact, auxins, polyamines and cytokinins were considered as essential to enhance the efficiencies on target explant and genotype (Khanna & Daggard, 2003; Przetakiewicz et al., 2003; Rashid et al., 2009).

2.1.1 Wheat genotype

Transformation and regeneration of the infected explants are highly genotype-dependent, the plant genotype has been revealed as a major factor influencing transformation efficiency. Indeed, the largest transformation efficiency compared to any other commercial wheat germplasm was reported when the highly regenerable wheat breeding line "Bobwhite" was used (Table 1).

The *Triticum aestivum* Spring "Bobwhite" is the most representative cultivar representing over 25% of the data reported of *Agrobacterium*-mediated transformation of wheat (Table 1), becoming "the genotype model" (Fellers et al., 1995; Sears & Deckard, 1982; He et al., 1988). It has a good response in tissue culture with a high rate of callus induction and regeneration (Janakiraman et al., 2002) making it a suitable cultivar for transformation, since a high ratio for both transformation and regeneration can be achieved. However, it would be highly desirable to transform genotypes other than the model ones (Kumlehn & Hensel, 2009) with much better agronomical and grain quality traits.

Other *T. aestivum* lines, cultivars or varieties such as "Turbo" (Hess et al., 1990); "Millewa" (Mooney et al., 1991); "Chinese" (Langridge et al., 1992); "Kedong 58", "Rascal" and "Scamp" (McCormac et al., 1998); "Lona" (Uze et al., 2000); "Baldus" (Amoah et al., 2001); "Fielder" (Weir et al., 2001); "Florida" and "Cadenza" (Wu et al., 2003); "Vesna" (Mitic et al., 2004); "Veery-5" (Khanna & Daggard, 2003; Hu el al., 2003) and so on (see the complete list in Table 1) were also tested.

Finally, some other commercial *Triticum sp* (different to *T.* aestivum) such as *Triticum dicoccum* (Chugh & Khurana, 2003), *Triticum durum* (Patnaik et al., 2006) or *Triticum turgidum* (Wu et al., 2008; Wu et al., 2009; He et al, 2010) were also being successfully used for Agrobacterium-mediated wheat transformation (see Table 1).

2.1.2 Target explants

The primary source of material is one of the main constraints for *Agrobacterium*-mediated wheat transformation. Regeneration is performed from highly regenerant tissues with active cell division. In these tissues embryogenic calli are induced and regeneration leads to the recovery new formed transgenic plants. Two types of explants are typically used for the recovery of fertile transgenic plants: immature inflorescences and the scutellum of immature zygotic embryos. Although other explants (Table 1) have been used for the same purpose such as reproductive-derived material (Hess et al., 1990; Liu et al., 2002), seeds (Zale et al., 2004); leaf (Wang & Wei, 2004) or shoot meristems (Ahmad et al., 2002), none of them were capable of reliably production of fertile adult transgenic wheat adult plants.

Wheat (Variety / Cultivar)	Explant Type	Strain / Plasmid	Promoter / Reporter Gene	Promoter / Selectable gene	Transformation efficiency (%)	Reference / Remarks
Triticum aestivum						
Turbo (Spring)	SPK	C58C1 / pGV3850:1103neo	-	*nos / nptII*	1	Hess et al., 1990 / No regeneration
Millewa (Spring)	IE	C58C / pGV3850:1103neo	-	*nos / nptII*	1 – 2 (based on kanamycin selection)	Mooney et al., 1991 / Gene integration was not demonstrated
Chinese (Spring)	SPK	LBA4404 / pPCV6NFHyg A281 / pPCV6NFHyg C58C1 / PCV6NFHyg GV3101 / pPCV6NFHyg	*CaMV35S / gus*	*nos / nptII* *CaMV35S / hpt*	0.8 – 4.7 (based on kanamycin selection)	Langridge et al., 1992 / No regeneration
Bobwhite (Spring)	IE PCIE IEdC	ABI / pMON18365	*CaMV35S / gus*	*CaMV35S / nptII*	1.12 (IE) 1.56 (PCIE) 1.55 (IEdC)	Cheng et al., 1997 / Salt strength test, surfactants & explants types
Bobwhite (Spring)	SDS	EHA105 / pIG121Hm	*CaMV35S / gus*	*nos / nptII* *CaMV35S / hpt*	28 foci/seed (*GUS*)	Trick et al., 1997 / Sonication test. Transient GUS expression
Rascal (Spring) Scamp (Spring) Kedong 58 (Winter)	IEdC	EHA101 / pBECKS.red	*CaMV35S / gus* *CaMV35S / gfp* *CaMV35S / Lc/C1*	*nos / nptII*	40 - 70 (based on reported genes)	McCormac et al., 1998 / *gfp* and *Lc/C1* gene reporters
Chinese (Spring)	MSdC IEdC	GV3101 / pMVTBP GV3101 / pNFHK1 GV3101 / p35S*GUS*INT	*CaMV35S / gus*	*CaMV35S / nptII*	1.2 - 2.2 (*GUS*)	Peters et al., 1999 / Use of modular vector
Several (Chinese)	IEdC	AGL1 / pUNN2	-	*ubi1 / nptII*	3.7 – 5.9	Xia et al., 1999 / Stable transformation
Bobwhite (Spring) Lona (Spring)	PCIE	LBA4404 / pBin9UG EHA105 / pBin9UG C58C1 / pBin9UG LBA9402 / pBin9UG	*ubi1 / gus*	-	20 foci/callus (*GUS*)	Uze et al., 2000 / Several factors studied for transformation
Baldus (Spring)	lNFdC	AGL1 / pAL154-pAL156 AGL1 / pAL155-pAL156 AGL1 / pSoup-pAL186	*ubi1 / gus*	*ubi1 / bar*	14 – 64 (*GUS*)	Amoah et al., 2001 / Inflorescence tissue. Sonication and vacuum infiltration
Fielder (Spring)	PCIE	AGL0 / pTO134	*CaMV35S / gfp*	*CaMV35S / bar*	1.8 PCIE	Weir et al., 2001 / Several factors studied for transformation
Nongda 146 (Spring)	IE PCIE	AGL1 / pAL155-pAL156	*ubi1 / gus*	*ubi1 / bar*	90 (*GUS*)	Ke et al., 2002/ Transient GUS expression
Sohag 2 (Durum)	SPK	LBA4404 / pBI-P5CS	*CaMV35S / gus*	*nos / nptII*	0.9	Sawahel and Hassan, 2002 / *In planta* transformation
Sourav (Spring) Gourav (Spring) Kanchan (Spring) Protiva (Spring)	IE ME IEdC MEdC	EHA105 / pCAMBIA1301	*CaMV35S / gus*	*nos / nptII*	75 – 85 (IE) 60 - 65 (ME) 80 – 87 (IEdC) 67 - 73 (MEdC)	Sarker & Biswas, 2002 / Transient GUS expression
Bobwhite (Spring)	PCIE IEdC	ABI / pMON18365	*CaMV35S / gus*	*CaMV35S / nptII* *CaMV35S / aroA:CP4*	3.1 (PCIE -glyphosate) 6.1 (PCIE-paromomy.) 10.5 (EC-paromomycin)	Cheng et al., 2003 / Large scale experiments
Bobwhite (Spring)	PCIE	C58C1 / pPTN115	*CaMV35S / gus*	*CaMV35S / nptII*	0.5 – 1.5	Haliloglu and Baenziger (2003) / Several factors were studied
Bobwhite (Spring)	PCIE	ABI / pMON30120 ABI / pMON30174 ABI / pMON30139	-	*act1 / aroA:CP4* *CaMV35S / aroA:CP4* *ScBV / aroA:CP4*	4.4	Hu et al., 2003 / Large-scale production. Roundup ready wheat
Veery5 (Spring)	IEdC	LBA4404 / pHK22 LBA4404 / pHK21	*ubi1 / gus*	*ubi1 / bar*	1.2 – 3.9	Khanna and Daggard, 2003 / Use of spermidine in regeneration
Bobwhite (Spring)	PCIE EC	n.d / PV-TXGT10	-	*act1 / aroA:CP4* *CaMV35S / aroA:CP4*	-	Zhou et al., 2003 / Roundup ready wheat
Florida (Winter) Cadenza (Winter)	IE	AGL1 / pAL154-156	*ubi1 / gus*	*ubi1 / bar*	0.3 – 3.3	Wu et al., 2003 / Several factors studied for transformation
CPAN1676 (Bread) PBW343 (Bread)	MSdC	LBA4404 / pCambia3301	*CaMV35S / gus*	*CaMV35S / bar*	6.7 – 8.7	Chugh & Khurana, 2003 / Herbicide Resistance. Use of basal segment calli as target tissue
Vesna (Spring)	IE	LBA4404 / pTOK233 AGL1 / pDM805	*CaMV35S / gus* *act1 / gus*	*ubi1 / bar* *nos / nptII* *CaMV35S / hpt*	0.13 (LBA) 0.41 (AGL)	Mitic et al., 2004 / Use of super-binary vectors. Only PCR test
Kontesa (Winter) Torka (Winter) Eta (Winter)	IE	AGL1 / pDM805 EHA101 / pGAH LBA4404 / pTOK233	*CaMV35S / gus* *act1 / gus*	*ubi1 / bar* *nos / nptII* *CaMV35S / hpt*	1 (AGL1) 0.2 – 8.1 (EHA101) 0.2 – 2.3 (LBA4404)	Przetakiewicz et al., 2004 / Use of super-binary vectors and auxins
Hesheng3 (Winter) Yan103 (Winter) Yanyou361 (Winter)	IEdC	GV3101 / pROK2-AtNHX1	-	*CaMV35S / nptII*	1.3 – 2.9	Xue et al., 2004 / Survival tests in saline conditions. Field trial
Shannong 9956049 (Winter)	IEdC	LBA4404 / pROK2	-	*nos / nptII*	1.18	Bi et al., 2006 / Insect resistance
Yan361 (Winter) Yan2801 (Winter) H11 (Winter)	SDS	EH105 / pBLG	-	*nos / nptII*	8.62 – 11.2	Zhao et al., 2006 / Powdery Mildew resistance
HD2329 (Bread) CPAN1676 (Bread) PBW343 (Bread)	ME MEdC	LBA4404 / pBI101 LBA4404 / pCAMBIA3301	*act1 / gus* *CaMV35S / gus*	*nos / nptII* *CaMV35S / bar*	1.6	Patnaik et al., 2006 / Genotypic independence
Shiranekomugi	SDS	LBA4404 / pIG121Hm LBA4404 / pBI-res used LBA4404 / pBI-res2 used	*CaMV35S / gus*	*nos / nptII* *CaMV35S / hpt*	33 (PCR) 75 (Southern) 40 (plasmid rescue)	Supartana et al., 2006 / TE is referred to t1 progeny instead of inoculated explants
Yangmai158 (Winter)	PCIE	EHA105 / pCAMBIA3300	-	*CaMV35S / bar*	-	Yu and Wei, 2008 / Insect resistance
Een1 (Winter)	SDS	LBA4404 / n.d.	*CaMV35S / gus*	*CaMV35S / nptII*	3 – 31 (GUS)	Yang et al., 2008 / Use of seedling ages and inoculation time
Crocus (Spring)	SPK	C58C1 / pDs(Hyg)35S AGL1 / pBECKSred	*CaMV35S / Lc/C1*	*nos / nptII* *nos / hpt*	0.44	Zale et al., 2009 / *In planta* transformation
Certo (Winter)	IE	LBA4404 / pSB187	*ubi1 / gfp*	*CaMV35S / hpt*	2 – 10	Hensel et al., 2009 / Detailed protocols for transformation
EM12 (Chinese)	PCME	LBA4404 / pBI121	*CaMV35S / gus*	*nos / nptII*	0.27 - 2.5	Ding et al., 2009 / Optimization of transformation protocol

Wheat (Variety / Cultivar)	Explant Type	Strain / Plasmid	Promoter / Reporter Gene	Promoter / Selectable gene	Transformation efficiency (%)	Reference / Remarks
Triticum aestivum						
Bobwhite (Sring) Yumai66 (Winter) Lunxuan208 (Winter)	ME, PCME	C58C1 / pUbiGN	*ubi1 / gus*	*nos / nptII*	0.06 – 0.89	Wang et al., 2009 / Use of mature embryos
Inqilab-91 (Bread)	ME	EHA101 / pIG121Hm	*CaMV35S / gus*	*nos / nptII* *CaMV35S / hpt*	6.25 – 15.62	Rashid et al., 2010 / Effect of AS & bacterial culture density
Triticum turgidum						
Durum (ofanto)	IE	AGL1 / pAL156-pAL154 AGL1 / pAL156-pAL155	*ubi1 / gus*	*ubi1 / bar*	0.6 – 9.7 (pAL154) 2.1 – 3.9 (pAL155)	Wu et al., 2008 / Super-binary vectors. First time durum transf
Durum (Stewart)	IE	AGL1 / pAL156- pAL154	*ubi1 / gus*	*ubi1 / bar*	2.8 - 6.3	He et al, 2010 / Effect super-binary vectors, AS & picloram
Triticum dicoccum						
DDK1001 (Emmer)	MSdC	LBA4404 / pCambia3301	*CaMV35S / gus*	*CaMV35S / bar*	6.9	Chugh & Khurana, 2003 / Herbicide Resistance. Use of basal segment calli as target tissue
Triticum durum						
PDW215 (Pasta)	ME	LBA4404 / pCAMBIA3301	*CaMV35S / gus*	*CaMV35S / bar*	3	Vishnudasan et al., 2005 / Nematode resistance
PDW215 (Pasta) PDW233 (Pasta) WH896 (Pasta)	ME MEdC	LBA4404 / pBI101	*act1 / gus* *CaMV35S / gus*	*nos / nptII* *CaMV35S / bar*	1.28	Patnaik et al., 2006 / Genotypic independence

Table 1. Summary of wheat materials, *Agrobacterium* strains and vectors, and marker genes used to investigate wheat transformation. Explant type: IE (immature embryo); PCIE (pre-cultured immature embryo); IEdC (immature embryo derived calli); ME (mature embryo); PCME (pre-cultured mature embryo); MEdC (mature embryo derived calli); INF (inflorescence); INFdC (inflorescence derived calli); SPK (spikelet); SDS (seedling); MSdC (mature seed derived calli). Promoters: *CaMV35S* (cauliflower mosaic virus); *ubi1* (maize ubiquitin); *act1* (rice actin); *nos* (nopaline synthase gene); *ScBV* (sugarcane bacilliform virus). Reporter genes: *gus* (β-glucuronidase); *gfp* (green fluorescent protein); *Lc/C1* (anthocyanin-biosynthesis regulatory). Selectable gene: *nptII* (neomycin phosphotransferase II) and *hpt* (hygromycin phosphotransferase) antibiotic resistance and *bar* (phosphinothricin acetyltransferase) and *aroA:CP4* (5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)) herbicide resistance.

By far, the main target explant used to transform wheat was from immature embryos (IE). Concretely, the immature scutellum was used, a specialised tissue that forms part of the seed embryo, and it was recommended that embryo isolation was performed 11-16 days post-anthesis (Jones, 2005). Freshly isolated IE, pre-cultured IE or IE derived callus had been widely included in experiments to obtain transgenic wheat plants. Cheng et al. (1997) reported, for the first time, the success of *Agrobacterium*-mediated transformation in wheat using IE (freshly isolated and pre-cultured) and embryogenic calli producing fertile transgenic plants despite the experiments being limited to small-scale. Later, many attempts were carried out by several authors (McCormac et al., 1998; Xia et al., 1999; Uze et al., 2000; Ke et al., 2002, Sarker & Biswas, 2002) but no stable transgenic plants were reported until Weir et al. (2001), who confirmed results obtained previously by Cheng et al. (1997), transformed pre-cultured immature embryos, 9 day old. Large-scale experiments were carried out using immature embryos as the initiation tissue for both genetic transformation and plant regeneration (Cheng et al., 2003; Hu et al., 2003; Vasil, 2007; Jones et al., 2007; Rashid et al., 2009).

Immature inflorescences were also easier to isolate and can be collected earlier from younger plants in comparison to immature embryos. However, these explants present more specific-genotype requirements for its *in vitro* culture regeneration (Jones, 2005 and references therein). Seeds were also used as started explant for wheat in plant transformation (Trick & Finer, 1997; Supartana et al., 2006; Zhao et al., 2006; Yang et al., 2008; Razzaq et al., 2011) but only Supartana et al. (2006) and Zhao et al. (2006) demonstrated stable gene inheritance and integration in progeny by Southern blot analysis

(Table 1). Other initiation explants were also tested as tissue for wheat *Agrobacterium*-mediated transformation: mature embryo (ME) either freshly isolated, pre-cultured or derived calli (Sarker & Biswas, 2002; Vishnudasan et al., 2005; Patnaik et al., 2006; Ding et al., 2009; Wang et al., 2009; Rashid et al., 2010), inflorescence or inflorescence derived calli (Amoah et al., 2001) and mature seed derived calli (Peters et al., 1999; Chugh & Khurana, 2003). Mature embryos offer some advantage over the typically used immature embryos, as a low-cost procedure because immature embryos must be recollected from plants grown under a controlled environment, moreover the extraction of the embryos in a narrow developmental stage (i.e. 0.8–1.5 mm in diameter) is required (Wu et al., 2009; Wang et al., 2009).

In the early 1990s transgenic wheat materials were generated by inoculating florets with *Agrobacterium* at or near anthesis (Hess et al., 1990; Langridge et al. 1992) produced similar results since both failed to demonstrate gene integration in successive plant generations or successful plant regeneration (Table 1). Using the same protocol but changing the *Agrobacterium* strain and the plasmid construction, a floral dip efficient transformation of wheat was achieved by Sawahel & Hassan (2002). More recently (Zale et al., 2009) by performing transformation at an earlier stage of floral development than previously (i.e., Hess et al., 1990; Langridge et al. 1992; Sawahel & Hassan, 2002) successful transgene integration and expression were obtained when wheat ovules were used as target explants.

2.2 *Agrobacterium* and plasmids

It has been widely described in the literature that the combination of highly competent *Agrobacterium* strain with effective and suitable plasmid construction leading to improved successful wheat transformation efficiencies (Khanna & Daggard, 2003; Cheng et al., 2004). The most used *Agrobacterium* strains and plasmids are summarized in Table 1.

2.2.1 *Agrobacterium* strain

Cereals are not natural hosts for *Agrobacterium* and many studies have been carried out to match host strains with wheat genotypes (Jones et al., 2005). Mainly, only three strains of *Agrobacterium tumefaciens* are currently used in wheat transformation (Table 1) thus from the 41 reports reviewed: 44% used LBA4404, followed by C58C1 (24%) and AGL1 (24%). While other strains has been used with a less frequency (10%) including other *A. tumefaciens* strains such as: A281, GV3101, ABI, EHA101, EHA105, AGL0, M-21 and *A. rhizogenes* LBA9402 and Ar2626. Interestingly, most of those *Agrobacterium* strains share only two chromosomal backgrounds: the C58 type (C58C1, AGL1, GV3101, ABI, EHA101, EHA105 and AGL0) and TiAch5 (LBA4404) (Hellens et al., 2000; Jones et al., 2005).

The infection process of *Agrobacterium* include several chromosome-encoded genes involved in the attachment of bacteria to plant cells and Ti plasmid-encoded *vir* genes, that function in trans, helping the transfer and integration of T-DNA into the plant genome (Wu et al., 2008). Some of the above strains also contain a binary or helper plasmids, carrying further copies of virulence genes. Therefore, depending on agro construction, "standard or low virulent" strains as LBA4404 and C58C1 or "hyper-virulent strains" such as AGL have been designed to successful transformation of wheat.

Although rare, also some a-virulent *A. tumefaciens* mutant strain has also been used for wheat transformation studies as a reliable marker of transformation (Table 1). As an example, Supartana and co-workers (2006) employed the M-21 *Agrobacterium* mutant, in which the *iaaM* gene (tryptophan monooxygenase gene) - involved in IAA (indole acetic acid) biosynthesis in the T-DNA region - is destructed by transposon5 (Tn5) insertion. As a consequence, this mutant strain was capable of integrating its T-DNA into chromosomes of host plants, but no galls were produced. Wheat transformants obtained by the M-21 mutant strain were expected to synthesize a high cytokinin level (since all other genes including the *ipt* gene – involved in cytokinin biosynthesis in the T-DNA region – were intact and fully functional), resulting in a high altered phenotype due to hormone imbalance which can be easily detected (Supartana et al., 2006).

2.2.2 Plasmid and virulence

As stated previously, wheat is not a natural host for *Agrobacterium*, for this reason only a few genotypes (such as Bobwhite) can be transformed with standard strains, such as LBA4404 and binary vectors (Cheng et al., 1997; Hu et al., 2003). When other genotypes were tested, no successful transformation was obtained, only their virulence was increased by adding an extra binary plasmid (such as pHK21) with extra *vir* genes (Khanna & Daggard, 2003) that enhance the transformation.

Many other Ti vectors and helper plasmids, known as binary plasmids, which can include an extra copy of virulence genes in the namely "super-binary" vectors, have been incorporated in the selected *Agrobacterium* strain to enhance infection. Several combinations regarding virulence are possible: from a-virulent to hyper-virulent *Agrobacterium* strain.

The most common *Agrobacterium* strains used in wheat transformation below to hyper-virulent group and is the disarmed plasmid pTiBo542 from *A. tumefaciens* wild strain A281 harbouring additional virulence genes usually *vir* B, C and G, which confer the hyper-virulence character (Komari et al., 1990).

Two different constructs have been widely employed to carry extra *vir* region (Table 1): first, using the helper plasmid pAL155 which is a derivative of pSoup modified by the addition of *vir* G (Amoah et al., 2001; Ke et al., 2002; Wu et al., 2008); and second, using different plasmids as pAl154, pAL186 or pTOK233 carrying "15 kb Komari fragment" containing set of *vir* B, C and G (Amoah et al., 2001; Wu et al., 2003; Mitic et al., 2004; Przetakiewicz et al., 2004; Wu et al., 2008; Wu et al., 2009; He et al., 2010).

2.2.3 Promoters

Regarding the promoters (see Table 1), the most common were the constitutive "*CaMV35S*" (cauliflower mosaic virus) and "*ubi1*" (maize ubiquitin). Other promoters such as "*act1*" (rice actin promoter); "*nos*" (nopaline synthase gene) or "*ScBV*" (sugarcane bacilliform virus) (Hu et al., 2003) were also used with much less frequency.

A great challenge will be to identify specific promoters that would direct the expression of genes in a tissue-specific manner. This can be used not only with reporter genes in studies to optimize the *Agrobacterium*-meditated transformation protocols but also with agronomical importance genes, such as quality improvement, disease resistance or drought tolerance.

2.2.4 Reporter genes

Three reporter marker genes have been used to establish expression and/or integration of foreign DNA into wheat material (See Table 1).

The most usual one is *gusA* (*uidA*) gene encoding the enzyme β-glucuronidase (GUS); although *gfp* (green fluorescent protein) gene, (McCormac et al., 1998; Weir et al., 2001; Hensel et al., 2009) and *Lc/C1 (*anthocyanin-biosynthesis regulatory) genes, that results in the accumulation of anthocyanin so creating the "red cell" phenotype (McCormac et al., 1998; Zale et al., 2009), were also used.

2.2.5 Selectable and interest genes

Antibiotic and herbicide resistance is by far the most widely used selection system in *Agrobacterium*-mediated transformation of wheat (See Table 1). As the selectable marker gene, the most common one is "*nptII*" (neomycin phosphotransferase II) gene (Table 2), which confers resistance to kanamycin antibiotic, although "hpt" (hygromycin phosphotransferase) gene conferring hygromycin B resistance has been recently employed (Zale et al., 2009; Rashid et al., 2010), which may be due to cereals being more sensitive to hygromycin B than to kanamycin (Janakiraman et al., 2002 and references therein).

Selectable marker gene	Encoded enzyme	Selective agent	Mode of action
nptII	neomycin phosphotransferase II	Aminoglycoside antibiotics: -kanamycin -neomycin -hygromycin - G418 (geneticin) - paromomycin	Binds 30S ribosomal subunit, inhibits translation
hpt	hygromycin phosphotransferease	Aminoglycoside antibiotics: -hygromycin	Binds 30S ribosomal subunit, inhibits translation
bar (pat)	phosphinothricin acetyl transferase	Herbicides: -phosphinothricin (PPT) -glufosinate ammonium -bialaphos (tripeptide antibiotic)	Inhibits glutamine synthase
aroA:CP4	5-Enolpyruvylshikimate-3-phosphate synthase	Herbicides: -glyphosate	Inhibits aromatic acid biosynthesis (EPSPS)

Table 2. Selectable marker genes most commonly used in wheat *Agrobacterium*-mediated transformation.

The other most popular selectable gene is "*bar*" (also called "pat", phosphinothricin acetyl transferase) gene that confers herbicide resistance to phosphinothricin (PPT) and glufosinate ammonium, the active ingredient being the herbicide Basta® by Hoechst AG and Liberty by AgroEvo®, respectively (Table 2; Rasco-Gaunt et al., 2001). Also, other resistance marker genes for wheat transgenic plants selection have been described (Table 2), such as" *aroA:CP4*" (5-enolpyruvylshikimate-3-phosphate synthase) gene that confers tolerance to glyphosate, the active ingredient of the RoundupReady® herbicide (Zhou et al., 2003; Hu et al., 2003).

2.3 Transformation conditions

Many variables have been pinpointed, and extensively reviewed (Janakiraman et al., 2002; Sahrawat et al., 2003; Bhalla et al., 2006; Jones, 2005), as the key factors in the *Agrobacterium*-mediated transformation process of wheat. Here, those variables are listed in Table 3 under heading that describe the factor, the type or stage studied, the range tested and the optimal value proposed for the highest transformation efficiency together with the main references related. Latter on those data are discussed step by step and we divided the *Agrobacterium*-mediated transformation protocol in four separates stages: preculture, inoculation, coculture and selection.

Factors	Type	Range tested / Higher efficiency	Some references
Time	Pre-culture	From 4 to 21 days. Optimal conditions varied among source explants	Haliloglu & Baenziger, 2003; Weir et al., 2001; Ding et al., 2009; Amoah et al., 2001
	Inoculation	From 30 min to 12 h. Optimal conditions at 30 min and 3 h.	Yang et al., 2008; Wu et al., 2003; Ding et al., 2009
	Coculture	From 1 to 5 days. Optimal conditions at 3 days.	Wu et al., 2003; Uze et al., 2000
Temperature	Inoculation	From 22 to 28 °C. Optimal condition at 24-25°C	Wu et al., 2003; Wu et al., 2008; Mitic et al., 2004
	Coculture	From 21 to 27°C. Optimal condition at 24-25°C.	Amoah et at., 2001; Weir et al., 2001; Khanna & Daggard, 2003; Xue et al., 2004; Wu et al., 2008
Auxins	Picloram	From 1 to 10 mg/L. Optimal conditions around 2- 2.2 mg/L	Weir et al., 2001; Ding et al., 2009; He et al., 2010; Jones et al., 2005
	2,4 D	From 0,5 to 10 mg/L. Optimal conditions at 0,5 and 2 mg/L.	Cheng et. al, 1997; Hu et al., 2003; Razzaq et al., 2011
Surfactans	Pluronic F68	From 0.01 to 0.05 %. Optimal conditions at 0.02%	Cheng et al., 1997; Cheng et al., 2003; Khanna & Daggard, 2003; Zhou et al., 2003
	Silwet L-77	From 0.001 to 0.5 %. Optimal conditions at 0.01-0.02%.	Cheng et al., 1997; Wu et al., 2003; Zale et al., 2009; Haliloglu & Baenziger, 2003
Sugars	Maltose	From 40 to 80 g/L. Optimal conditions at 40	He et al., 2010
	Glucose	From 10 to 36 g/L. Optimal conditions at 10-20 g/L.	Cheng et al., 1997; Khanna & Daggard, 2003
Optical Density		From 0.5 to 2 Optimal conditions at 0.6	Sarker & Biswas, 2002; Amoah et al., 2001; Ke et al., 2002; Haliloglu & Baenziger, 2003; Bi et al., 2006
Phenolic inducers	Acetosyringone	From 100 to 400 µM. Optimal conditions at 100-200 µM.	Cheng et al., 1997; McCormac et al., 1998; Amoah et al., 2001; Wu et al., 2003; Patnaik et al., 2006; He et al., 2010
Salt strength		From 0.1 to 2. Optimal conditions at 0.1 – 1 MS salts strength	Cheng et al., 1997; Ding et al., 2009

Table 3. Summary of current published data on main factors with positive effect on wheat *Agrobacterium*-mediated transformation efficiency.

2.3.1 Preculture

Most reports on *Agrobacterium*-mediated transformation include a first stage called "preculture" to increase the transformation efficiency. For example, survival rate was higher in explants precultured before inoculation than in freshly isolated explants (Cheng et al., 1997). Moreover, Uze et al. (2000) reported the highest T-DNA delivery ratio, based on transient GUS assay, of immature wheat embryos "Bobwhite" when precultured during 10 days; Amoah et al. (2001) found that inflorescence tissue precultured during 21d had the highest GUS activity and finally, Ding et al. (2009) obtained the best transformation rate when mature embryos were precultured for 14 days. However, other authors (Jones et al., 2005) described a successful protocol without pre-culture period or special inoculation treatments.

Some plant growth regulators, such as synthetic auxins picloram (4-amino-3, 5, 6-trichloropicolinic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid), are commonly added to the preculture medium to increase regeneration and the recovery of transgenic explants. Przetakiewicz et al. (2004) demonstrated the promotion effect of 2,4-D for obtaining a higher number of transgenic plants than picloram, whereas, picloram promotes a higher regeneration frequency than 2, 4-D in other report (Ding et al., 2009). Taken into account those results, picloram and 2,4-D or both together have been widely employed in wheat transformation via *Agrobacterium* (Table 3).

2.3.2 Inoculation

The second step of any *Agrobacterium* mediated process is the inoculation of wheat explants in an *Agrobacterium* suspension during a quite variable period of time: 30 minutes to 12 hours (see references in Table 3) and several factors have been proposed as key for inoculation such as included as the most important inoculation stage such as: time, temperature, media strength or *Agrobacterium* optical density as well as some inducers of stable transformation, such as acetosyringone, sugars, auxins or surfactans.

Several authors (Amoah et al.; 2001; Yang et al., 2008) have described a direct relationship between increase of inoculation time and decrease in transformation efficiency after 2-3 h and there is a general consensus that the optimal time of inoculation for T-DNA delivery (Jones et al., 2005; Wu et al., 2008; Ding et al., 2009) should be around 3 h.

Although in the literature reviewed (Table 3), a wide range of inoculation temperatures have been tested: 22 – 28ºC (Peters et al., 1999; Cheng et al., 2003; Mitic et al., 2004; Supartana et al., 2006) however, no clue on the optimal ones or significant differences has been clearly reported. Moreover, most reports do not indicate the inoculation temperature and it is assumed that room temperature has been applied (c.a. 25ºC).

The use of surfactants and phenolic inducers in the media were widely assessed by different researchers (Table 3). Surfactants, like pluronic acid F68 and Silwet L-77, were first studied by Cheng et al. (1997) finding that either Silwet or pluronic enhance transient GUS expression, especially on the immature embryos because it is believed that the surface-tension-free cells favour the *A. tumefaciens* attachment. Several studies reported an optimal concentration for Silwet around 0.01% (Wu et al., 2003; Jones et al., 2005) and for pluronic around 0.02% (Cheng et., 1997). On the contrary, other authors (Haliloglu & Baenziger, 2003) have described that the presence of a surfactant in the inoculum medium makes no

difference in terms of T-DNA delivery efficiency, even when concentrations as higher as 0.05% of Silwet have been used.

Acetosyringone was always pointed out to be the key factor in T-DNA delivery in a range of concentration from 100 to 400 μM (McCormac et al., 1998; Xue et al., 2004; He et al., 2010). Its presence, at 200 μM concentration, clearly increased transformation efficiency (Wu et al., 2003; Amoah et al., 2001).

The addition of some sugars, like maltose or glucose to the inoculation medium was essential to achieve efficient T-DNA delivery; in fact T-DNA delivery efficiency was significant reduced in the freshly isolated immature embryos when acetosyringone and glucose were absent in the inoculation media (Cheng et al., 1997, Wu et al., 2003).

Agrobacterium optical cell density at 600 nm around 0.5-0.6 (Cheng et al., 2003; Haliloglu & Baenziger, 2003; Bi et al., 2006); close to 1.0 (Khanna & Daggard, 2003; Jones et al., 2005) or even higher, such as 1.3 (Amoah et al., 2001) during inoculation were found to be crucial for transformation efficiency. However when *Agrobacterium* is inoculated at high density or when is cocultured with the explant at high temperatures or for long period conditions an overgrowth can occurs promoting the death of the explants. Several antibiotics can be used after coculture and the selection stage to control *Agrobacterium* overgrowth or to eliminate it completely, such as timentin (Hensel et al., 2009, Wu et al., 2009), carbenicillin (Cheng et al., 1997) and cefotaxime (Bi et al 2006, Chugh & Khurana, 2003).

2.3.3 Coculture

The third stage of any wheat *Agrobacterium*-tumefaciens transformation protocol starts, after the removal of excess of bacteria from the previous stage, when the explants are cocultivated for a period of 1-5 days (Table 3) in dark conditions at 23 -27ºC. Again, during this period virulence inductors such as acetosyringone, osmoprotectors such as proline, carbon sources such as sugars, and plant growth regulators are added to the medium

Several studies have focused on time, temperature and media composition variables as important factors, during cocultivation stage, to transform wheat successfully. For example, Wu et al. (2003) found that a long cocultivation time (5d) promoted a reduction on the capacity of the transformed immature embryos to form embryogenic callus and regenerate when cocultivation was assessed for 1–5 days. Short periods (2-3 days) have been proposed as optimum for high transformation efficiency (Cheng et al., 1997; Amoah et al., 2001; Wu et al., 2003; Ding et al., 2009).

Also, the temperature during the cocultivation period could play an important role. Weir and coworkers (2001) obtained 83.9 and 81.4% of GFP expression at 21 and 24ºC, respectively and concluded that transient GFP expression is not significantly affected by co-cultivation temperature. Although, an elegant assay demonstrated that coculture at two temperatures (1d at 27ºC and 2d at 22ºC) reduced the damage to the soft callus tissue due to the common overgrowth of *Agrobacterium* during coculture (Khanna & Daggard, 2003). More information about it can be found in 2.3.2 section.

As stated previously for inoculation condition, the addition of acetosyringone 200μM is also critical in the coculture media to increase the efficiency on T-DNA delivery (Cheng et al., 1998; Wu et al., 2003).

Finally, it has been described (Table 3) that the salt strength in both, the inoculation and co-culture media, had a significant influence on the T-DNA delivery. For example, transient GUS expression was higher on freshly isolated immature embryos when one tenth-strength MS salts were used than the full-strength MS salts (Cheng et al., 1997). Several medium strength 2x, 1x, 0.5x, and 0.1x media concentration were also assessed elsewhere (Khanna & Daggard, 2003) but no main conclusion has been drawn and MS media 1x has been generally employed in *Agrobacterium* mediated transformation of wheat (Weir et al., 2001; Ke et al., 2002; Sarker & Biswas, 2002; Wu et al., 2003; Patnaik et al., 2006; Ding et al., 2009)

2.3.4 Selection

Due to the most common selectable marker genes being *nptII, hpt* and *bar*, the most widely selected agents, to discriminate transformed explants , and not to transform explants, were kanamicyne, hygromycin and phosphinothricin (PPT) and their analogues G418 (geneticin) and paromomycin for *nptII* gen and Bialaphos when *bar* gene was used as selectable marker gene.

3. Agrobacterium-mediated genetic transformation: Time to model

As described in the previous section, plant genetic transformation is a really complex process to understand and, subsequently, to optimize. The reason behind this is the important number of variables (factors) involved in the whole process (plasmid or *Agrobacterium* strain, type of plant explant, preculture, inoculation, coculture and selection conditions, etc) together with the different scales of biological organization concerned (molecular, genetic, cellular, physiological and whole plant). Moreover, different kinds of data are generated in those studies: binary data (transformed- non transformed; alive–dead); discrete or categorical (number of GUS spots); continuous (length, weight, …); image data (GUS or GFP) or even fuzzy data (callus colour: brown, brownish, yellowish and so on).

Traditionally, the effect of those variables on genetic transformation studies and particularly, wheat *Agrobacterium*-mediated transformation, is determined by analysis of variance (ANOVA). According to statistical theory (Mize et al., 1999), only continuous data normally or approximately normally distributed should be analysed with ANOVA. Discrete and binomial data should be analysed using Poisson and logistic regression, respectively. This type of methodology makes, the analysis of the results complicated and specialized, the biologist often being helped by statisticians. Finally, although statistics can be used for making predictions, normally this feature is not used in plant transformation studies.

Because of these limitations, plant genetic transformation studies include, usually, a small number of variables at the same time. Often, one variable at a time is studied; for example to study the effect of a variable (eg. effect of acetosyringone) on a selected response (eg. GUS transient expression), the experiments are performed at different concentrations (0, 100, 200 and 300 μM) keeping the rest of the variables constant. This "one-factor at a time" procedure is time consuming and has clear limitations when the best conditions for *Agrobacterium*-meditated transformation of wheat need to be achieved. The main limitation is that this

procedure ignores the possible interactions between variables (the addition of acetosyringone can have a positive or negative interaction with any other variable kept constant during a particular experiment).

Finally, this kind of methodology enables the researcher to select the best combination of factors between the performed experiments and not to predict the best possible combination of factors or, in other words, to optimize the whole procedure.

The *Agrobacterium*-mediated transformation process is difficult to describe accurately by a simple stepwise algorithm or a precise formula and require a network (multivariable) approach using computational models. For developing a model several steps need to be followed: first, a clear identification of the process (including all kind of variables/factors) to be simulated, controlled and/or optimized; secondly, the selection of variables, and the definition of what the model is for; thirdly, the creation of the database with the most accurate and precise data of each variable and the selection of the type of model and finally, the model validation, to check if the distances between the observed and predicted data is low enough (Gallego et al., 2011).

To establish the key factors affecting the quality of an *Agrobacterium*-mediated transformation process an Ishikawa diagram can be developed (Fig. 1) using data from literature (Tables 1, 2 and 3). This cause-effect diagram helps in identifying the potential relationships among several factors, and provides an insight into the whole process. The main factors (causes) can be selected and grouped into major categories such as plant material, *Agrobacterium*, transformation conditions and selection conditions.

Initially both *Agrobacterium* characteristics (strain, plasmid, extra virulence gene, promoters, reporter and selectable marker gene) and plant material (genus and species, variety/cultivar/line and type of explant) should be defined. Within the transformation conditions (preculture, inoculation and coculture) several variables as process conditions (temperature and time); chemical properties as media composition (type, strength, vitamins, sugars, plant growth regulator (PGR) such as synthetic auxins) and/or transformation inductors (acetorysingone and surfactans) should be considered and interrelated. Finally, selection conditions (antibiotics and/or herbicides) need to be established.

From this diagram, it can be deduce that there are an enormous amount of variables involved in the transformation process. Moreover, variables of different types: numerical data (temperature, time, etc.) or nominal (strain, explant, etc.) should be considered. Once the key or main variables (inputs) are identified, their effects over the defined parameters (outputs) should be studied by the appropriate experimental design or model.

Different models and/or networks have been used to integrate all kind of biological components (Yuan et al., 2008). Both networks and model have become more and more accurate (and better at predicting outcomes of the complex biological process) by using new experimental and modelling tools (Giersch, 2000). Recent studies have pointed out the effectiveness of different artificial intelligence technologies, such as artificial neural networks (Gago et al., 2010a, 2010b, 2010c) combined with genetic algorithms and neurofuzzy logic (Gago et al., 2010d; 2011) in modelling and optimizing the complex plant biology process (Gallego et al., 2011).

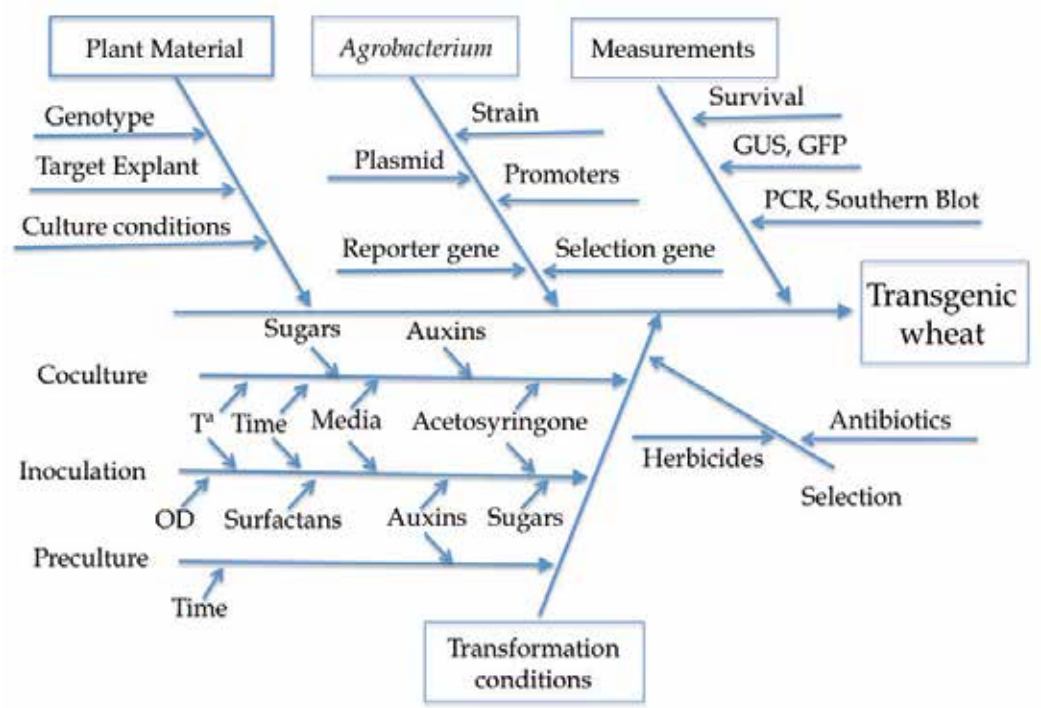

Fig. 1. Ishikawa diagram identifying the potential key variables of a wheat *Agrobacterium*-mediated transformation process.

4. Artificial Intelligence: A novel approach to model, understand and optimize cereals genetic transformation

Artificial intelligence approaches are based on the use of computational systems that simulate biological neural networks. They have been used not only for many industrial and commercial purposes since the 1950s (Russell & Norvig, 2003) but they have also been applied to fields more often related to biology, such as agricultural, ecological and environmental sciences (Jimenez et al., 2008; Huang, 2009). More detailed information about these technologies (Rowe & Roberts, 2005), and their applications to plant biology (Prasad & Dutta Gupta, 2008; Gallego et al., 2011) can be found elsewhere. Herein, we will briefly describe some relevant aspects of three of those technologies: Artificial Neural Networks (ANNs), genetic algorithms and neurofuzzy logic, which have been employed in plant science for modelling and optimizing different processes, in order to facilitate the understanding of its future applicability in cereal genetic transformation studies.

4.1 Artificial neural networks

Artificial Neural Networks (ANNs) are computational systems inspired in the biological neural systems. Information arrives to biological neurons through the dendrites. The neuron soma processes the information and passes it on via axon (Figure 2). In a similar way, ANNs use the processing elements called "artificial neurons", "single nodes" or

"perceptrons", that is, simple mathematical models (functions). Every perceptron receives information (inputs) from "neighbouring" nodes, then processes the information (either positive or negative) by multiplying each input by their associated weight (it is a measure of the strengths of the connection between perceptrons) giving a new result, which is adjusted by a previously assigned internal threshold (to simulate the output action), and produces an output to be transmitted to the next node. The perceptrons are organized into groups called layers. By connecting millions of perceptrons complex artificial neural networks can be achieved. The most used network architecture is called "multilayer perceptron" and consists in three simple layers: input, hidden and output layer (Rowe & Roberts, 2005).

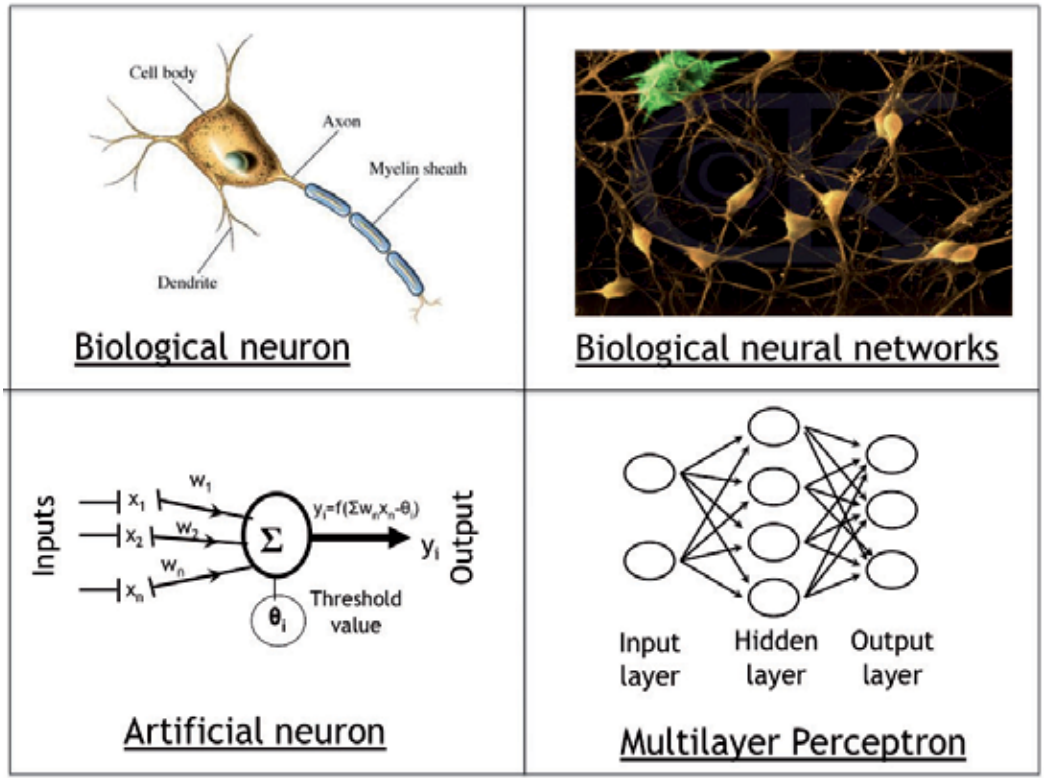

Fig. 2. Comparative schemes of biological and artificial neural system. X= input variable; W= weight of i_n input; θ= internal threshold value; f=transfer function.

Advantageously, while most conventional computer programs are explicitly programmed for each process, ANNs are able to learn, using algorithms designed to optimize the strength of the connections in the networks. For the network to learn it is necessary to use an example dataset (a collection of inputs and related outputs). Between 60 and 80% of the total data are chosen randomly, to perform the "training". In this process ANNs are able to search for a set of weight values that minimize the squared error between the data predicted by the model and the experimental data in the output layer. Furthermore, almost all the rest of the data set (10-20%) is used to "test" the model. Performance and predictability of the

model can be demonstrated by statistical parameters like the correlation coefficient (R^2) and the f value of the ANOVA of the model. Values of both training and test sets over 75% and f values over the f critical value for the corresponding degrees of freedom are indicative of high predictability and good performance (Colbourn & Rowe, 2005; Shao et al., 2006). Validation of the model can be performed by using a set of unseen data (validation data set) After a validation of the model, the ANNs is able to quickly predict accurately the output for a specific never tested combination of inputs or, in other words to answer "what if" questions, saving costs and time. Predictions using ANNs technology have been demonstrated to be more accurate than ones derived from experimental design and traditional statistic methods (Landín et al., 2009; Gago et al., 2010a). In conclusion, the ANNs approach could be useful to data processing, modeling, predicting and optimizing wheat genetic transformation.

ANNs have also some limitations related to the difficulties of interpreting the results when large data sets are used (several inputs and outputs are fitted in the model) and a large number of 2D surface plots or even 3 D graphs are generated by the model. In this case, ANNs can be coupled with other artificial intelligence technologies, such as genetic algorithms or fuzzy logic, creating hybrid systems that help to handle complex models and/or to data mining (Colbourn, 2003).

Sometimes the objective of modelling a specific process is not to predict new results (outputs), such as, when wheat *Agrobacterium*-mediated transformation is used to estimate the transformation efficiency when more amount of acetosyringone is added in the coculture stage. Probably for most researchers the main question could be "how to get" the maximum transformation efficiency, and more generally in those cases the objective is to find the combination of inputs that will provide the "optimum/best/highest" output in other words: optimize the process. This can be achieved combining ANNs and genetic algorithms.

4.2 Generic algorithms

Genetic algorithms (GA) are also a bio-inspired artificial intelligence tool, specially design to select the best solution of a specific problem (optimization). They are based on the biological principles of genetic variation and natural selection (mutation, crossover, selection or inheritance), mimicking the basic ideas of evolution over generations. In a simple way: when combined with ANNs, the genetic algorithms randomly generate a set of inputs and their corresponding predicted outputs using the ANNs model, called "set of candidate solutions" to the problem. Candidate solutions are then selected according to their fitness to previous established criteria; the best ones are used for evolving new solution populations to the problem, using crossover and mutation. After few generations the optimum should be reached because the most suitable candidates have more chance of being reproduced. Using this approach, complex micropropagation processes have been modelled by ANNs and successfully optimized by genetic algorithms (Gago et al., 2010a, 2010b).

4.3 Neurofuzzy logic

Neurofuzzy logic is a hybrid system technology that combines the adaptive learning capabilities from ANNs with the generality of representation from fuzzy logic (Shao et al.,

2006). Fuzzy logic is also an artificial intelligence tool especially useful in problem solving and decisions making, helping with the understanding of the complex cause-effect relationships between variables. When coupled with ANN, it becomes a powerful technique in handling complex models by generating comprehensible and reusable knowledge through simple fuzzy rules: IF (condition) THEN (observed behaviour). This kind of rules facilitates the understanding of a specific process, in a semi-qualitative manner, in a similar way to how people usually analyse the real world (Babuska, 1998; Gallego et al., 2011 and references therein). Many times words are more important for making decisions, drawing conclusions or even solving problems than a collection of accurate data (Fig. 3). Human knowledge is normally built on linguistic tags, and not on quantitative mathematical data, even though sometimes words are imprecise or uncertain.

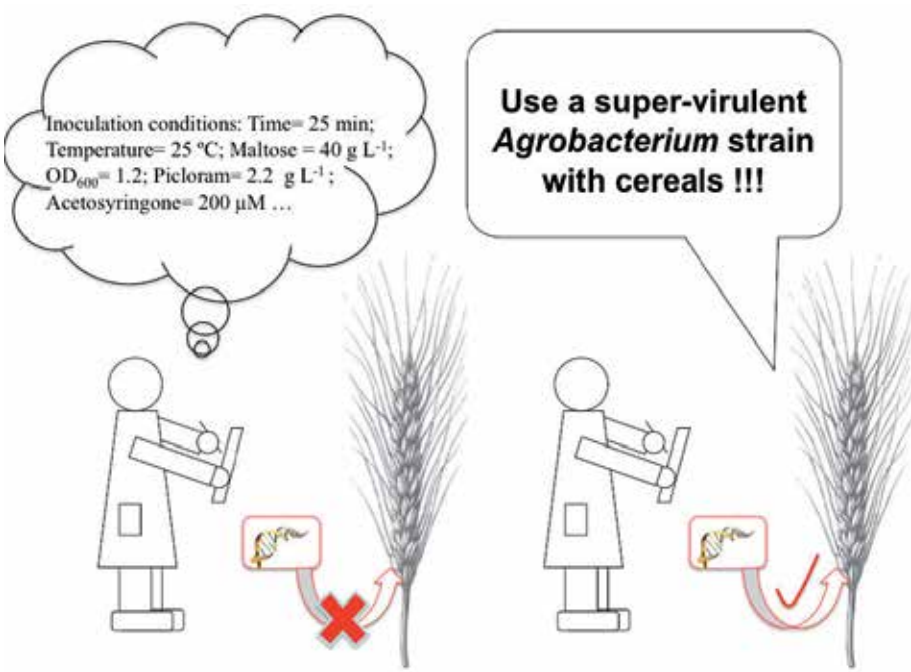

Fig. 3. Precision versus significance in the real world of researchers in the plant genetic transformation field.

The major capabilities of fuzzy logic are the flexibility, the tolerance with uncertainty and vagueness and the possibility of modelling non linear functions, searching for consistent patterns or systemic relationships between variables in a complex dataset, data mining and promoting deep understanding of the processes studied by generating comprehensible and reusable knowledge in an explicitly format (Setnes et al., 1998; Shao et al., 2006; Yuan et al., 2008). The neurofuzzy logic approach has been recently applied in modelling plant processes, such as *in vitro* direct rooting and acclimatization of grapevine (Gago et al., 2010d) or to gather knowledge of media formulation using data mining in apricot (Gago et al., 2011). In those cases, the authors found higher accuracy in identifying the interaction effects among variables of neurofuzzy logic than the traditional statistical analysis.

Moreover, neurofuzzy logic showed a considerable potential for data mining and retrieved knowledge from very large and highly complex databases.

5. Future perspectives

Agrobacterium-mediated transformation of wheat is a complex process although can be understood easily. It involves different scales of biological organization (genetic, biochemical, physiological, etc.) and many factors that influence the process. The storm of information generated by the analysis carried out during those processes would be useless if they could not be analysed together. Nowadays, artificial intelligence technologies give us the opportunity to handle a huge amount of biological data generated during the transformation process, with many advantages over traditional statistics. Artificial Intelligence technologies can solve common problems plant researchers associate to analysing, integrating variable information, extracting knowledge from data and predicting what will happen in a specific situation.

Different artificial intelligence approaches could be used for modeling, understanding and optimizing any *Agrobacterium*-mediated transformation procedure, either for wheat, cereals, fruit trees or any other biological process, giving results at least as good as, and less time consuming, those obtained by traditional statistics . More specifically, ANNs combined with genetic algorithms could predict the combination of variables (inputs) that would yield quality transformed wheat plants.

As a starting point a database can be obtained from historical results in the literature that can be modelled to find the more important variables affecting the *Agrobacterium*-mediated transformation procedure (data mining). On this knowledge, new experiments can be designed and performed and their results added to the database to fulfil the optimization processes (Gago et al., 2010a, 2011).

Great efforts have been made to improve the *Agrobacterium*-mediated transformation process, although the its full optimization is still far from being reached. In the future the application of modelling tools, such as those described here, could add a new insights into discovering the interactions between the variables tested and into understanding the regulatory process controlling molecular, cellular, biochemical, physiological and even developmental processes occurring during wheat *Agrobacterium*-mediated transformation.

6. Acknowledgments

We also want to thank Ms. J. Menis for her help in the correction of the English version of the work. This work was supported by Regional Government of Xunta de Galicia: exp.2007/097 and PGIDIT02BTF30102PR. PPG and ML thanks to Minister of Education of Spain for funding the sabbatical year at Faculty of Science, University of Utrecht, Netherlands.

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2

Recent Advances in Fruit Species Transformation

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1. Introduction

Rapid increase of human population together with global climate variability resulted in increased demand of plant based food and energy sources (Varshney et al., 2011). Fruits and nuts have essential role to enhance quality of humankind life since a diet based on cereal grains, root and tuber crops, and legumes is generally lacked a wide range of products such as fiber, vitamin, provitamins or other micronutrients and compounds exist in fruit and nut species (Heslop-Harrison, 2005). According to last FAOSTAT statistics, totally about 594.5 million t fruit crops (except melons) were produced in the world in 2009 (http://faostat.fao.org). Because an increase demand exists in global food production, many economically important fruit crops production need to be improved, however, conventional breeding is still limited due to genetic restrictions (high heterozygosity and polyploidy), long juvenile periods, self-incompatibility, resources restricted to parental genome and exposed to sexual combination (Akhond & Machray, 2009; Malnoy et al., 2010; Petri et al., 2011). Thus, there is an urgent need for the biotechnology-assisted crop improvement, which ultimately aimed to obtain novel plant traits (Petri & Burgos, 2005).

Plant genetic engineering has opened new avenues to modify crops, and provided new solutions to solve specific needs (Rao et al., 2009). Contrary to conventional plant breeding, this technology can integrate foreign DNA into different plant cells to produce transgenic plants with new desirable traits (Chilton et al., 1977; Newell, 2000). These biotechnological approaches are a great option to improve fruit genotypes with significant commercial properties such as increased biotic (resistance to disease of virus, fungi, pests and bacteria) (Ghorbel et al., 2001; Fagoaga et al., 2001; Fagoaga et al., 2006; Fagoaga et al., 2007) or abiotic (temperature, salinity, light, drought) stress tolerances (Fu et al., 2011); nutrition; yield and quality (delayed fruit ripening and longer shelf life) and to use as bioreactor to produce proteins, edible vaccines and biodegradable plastics (Khandelwal et al., 2011).

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Currently, public concerns and reduced market acceptance of transgenic crops have promoted the development of alternative marker free system technology as a research priority, to avoid the use of genes without any purpose after the transformation protocol as selectable and reporter marker genes. Typically, it is employed for the selection strategy that confers resistance to antibiotics and to herbicides (Miki & McHugh, 2004; Manimaran et al., 2011). A large proportion of European consumers considered genetically modified crops as highly potential risks for human health and the environment. European laws are restrictive and do not allow the deliberate release of plant modified organism (Directive 2001/18/EEC of the European Parliament and the Council of the European Union). Under these premises, great efforts have also been realized to develop alternative marker free technologies in fruit species. Recently, it was demonstrated in apple and in plum, that transgenic plants without marker genes can be recovered and confirmed its stability by molecular analysis (Malnoy et al., 2010; Petri et al., 2011). In 2011, for first time it was described authentically "cisgenic" plants in apple cv. Gala (Schouten et al., 2006a,b; Vanblaere et al., 2011).

Efficient regeneration systems for the generation of transgenic tissues still appear as an important bottleneck for most of the species and cultivars. In the literature, different protocols were described to transform fruit cells using various DNA delivery techniques, however the attempts generally focused on transformation via *Agrobacterium* or microprojectile bombardment. In this chapter, a detailed application of these techniques in fruit transformation is summarized together with usage of proper marker and selection systems and *in vitro* culture techniques for regeneration of the transgenic plants.

2. Techniques used to transform fruit species

Improvement of the plant characteristics by transfer of selected genes into fruit plant cells is possible mainly through two principal methods: *Agrobacterium*-mediated transformation and microprojectile bombardment (also called "biolistic" or "bioballistic"). Soil-borne Gram negative bacteria of the genus *Agrobacterium* infect a wound surface of the plants via a plasmid called Ti-plasmid containing three genetically important elements; *Agrobacterium* chromosomal virulence genes (*chv*), T-DNA (transfer DNA) and Ti plasmid virulence genes (*vir*) that constitute the T-DNA transfer machinery. Since Ti plasmid encodes mechanisms of integration of T-DNA into the host genome, it is used as a vector to transform plants.

Since direct gene transfer procedures involve intact cells and tissues as targets, in some species breaching of the cell wall is needed in order to enable entrance of DNA to cell (Petolino, 2002). This is accomplished by making some degree of cell injury or totally enzymatic degradation of the cell wall. Advantages of microprojectile bombardment can be summarized as i) transfer of multiple DNA fragments and plasmids with co-bombardment, ii) unnecessity pathogen (such as *Agrobacterium*) infection and usage of specialized vectors for DNA transfer (Veluthambi et al., 2003). Although microprojectile bombardment eliminates species-dependent and complex interaction between bacterium and host genome, stable integration is lower in this technique in comparison to *Agrobacterium*-mediated transformation (Christou, 1992). Moreover, the existence of truncated and rearranged transgene DNA can also lead transgene silencing in the transgenic plants (Pawlowski & Somers, 1996; Klein & Jones, 1999; Paszkowski & Witham, 2001). On the other hand, other important requirement for this technique is that the explants or target cells have to be

physically available for the bombardment (Hensel et al., 2011). Also, it was described that transgenic explants regenerated can be chimeric (Sanford et al., 1990). Nevertheless, application of both of the techniques for the transfer of foreign DNA results in "transient" or "stable" expression of the DNA fragment. In the following sections, recent advances in genetic transformation of fruit species via *Agrobacterium*-mediated and direct gene transfer techniques are presented.

2.1. *Agrobacterium*-mediated gene transfer

2.1.1 A complex relationship

In the decade of the 80´s, the first reports were published related to the introduction of foreign DNA in plant genome thanks to the Ti plasmid of *Agrobacterium tumefaciens* (De Block et al., 1984; Horsch et al., 1985). After more than 25 years, *Agrobacterium*-mediated gene transfer is still the most used method for fruit species transformation including apple, almond, banana, orange, grapevine, melon etc. (Table 1; Rao et al., 2009).

Plant transformation by using *Agrobacterium* has some advantages since the technique is relatively simple; transfer and integration of foreign DNA sequences with defined ends (left and right borders of T-DNA) is precise; stable transformation is high; transgene silencing is typically low and long T-DNA sequences (>150 kb) can be transferred (Veluthambi et al., 2003). However, it is still far from to be a routine transformation application in plants because of its host-range restrictions (Gelvin, 1998).

The initial drawback of *Agrobacterium*-mediated transformation method is the host-range restrictions. However, the bacterium and the target tissue can be manipulated to overcome this obstacle (Trick & Finer, 1997). These authors proposed a new approach to facilitate *Agrobacterium* penetration into plant tissues, the sonication assisted *Agrobacterium*-mediated transformation (SAAT) method. This method consists the use of ultrasounds to produce cavitations on and below the plant surfaces and into the membrane cells, wounding the tissues to enhance *Agrobacterium* infection (Trick & Finer, 1997, 1998).. Also, this method can be combined with vacuum infiltration to promote bacteria agglutination around the tissues to increase the *Agrobacterium* infection as it was demonstrated in kidney bean (Liu et al., 2005) and in woody plants as *Eucalyptus* (Villar et al., 1999; Gallego et al., 2002; Gallego et al., 2009). Today, application of these modifications solely or in combination with other approaches has made it possible to transfer foreign DNA via *Agrobacterium* even to monocots (Hiei et al., 1994; Ishida et al., 1996; Hensel et al., 2011) which were initially transformed with direct gene transfer methods since *Agrobacterium* is not a natural host. Following its first successful usage in soybean and Ohio buckeye (Trick & Finer, 1997), SAAT was also applied recently to fruit species including orange (Oliveira et al., 2009); banana (Subramanyam et al., 2011) and grapevine (Gago et al., 2011). In the last paper the developed efficient methodology that combined SAAT with vacuum infiltration allowed to obtain reporter gene expression in different newly formed organs such as stems, petioles and leaves. Expression was related to vascular tissues due to the *EgCCR* promoter of *Eucalyptus gunnii* and demonstrated that its activity is conserved and fully functional in grapevine as it was shown by *uidA* (GUS) and *gfp* reporter marker genes. Transgenic grapevine lines were verified by Southern blot analysis in five randomly chosen transgenic lines showing simple integration patterns in four lines with different band length indicating

independent transformation events into the grapevine genome. We also applied the optimized protocol to pistachio nodes to reveal out if this method of transformation and vascular-specific promoter of eucalyptus, also works in this species. Histological observations of GFP activity presence in vascular bundles and leaves (Fig. 1) together with PCR amplification of 858 bp fragment of *nptII* and 326 bp of *uidA* (Fig. 2) genes confirmed not only gene integration but also showed that SAAT in combination with vacuum infiltration and vascular specific promoter could also be used for pistachio transformation. With PCR amplification, four out of five GFP+ putative transgenic shoots showed the amplified bands of *nptII* and *uid*A genes (Fig 2).

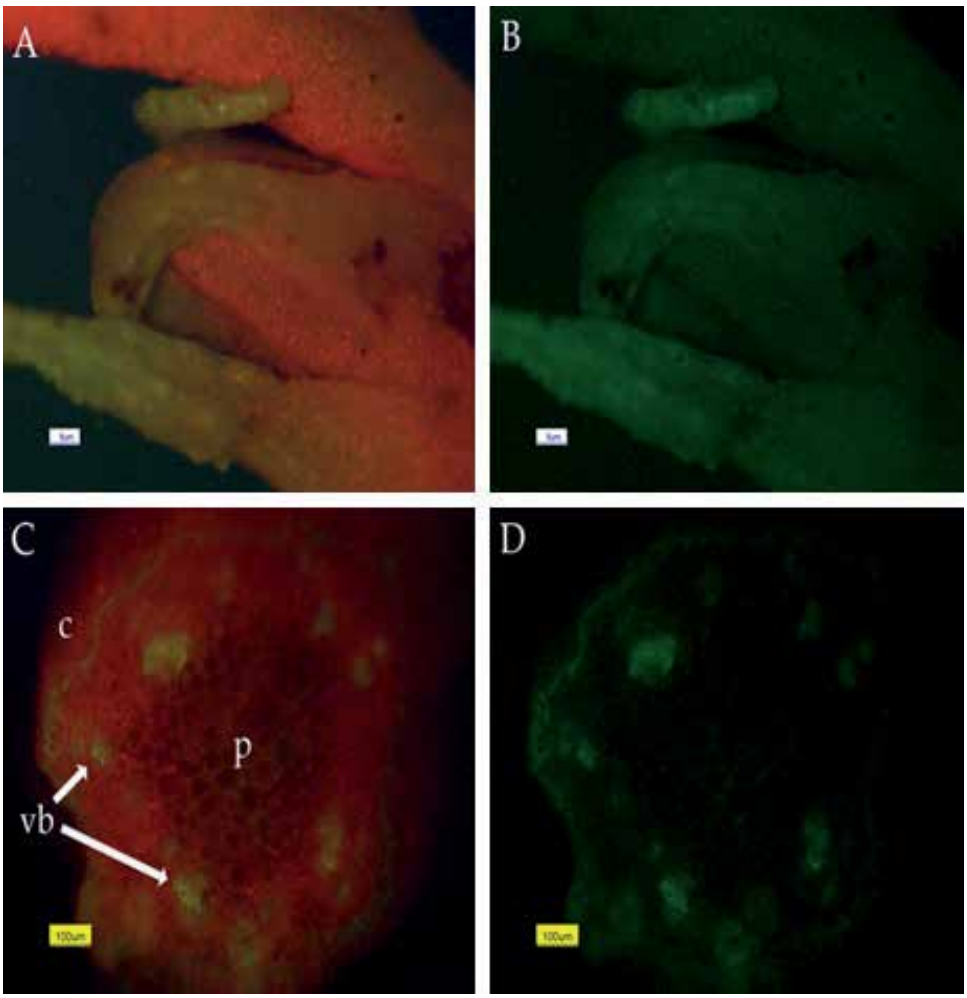

Fig. 1. Expression of GFP in pistachio transformed with *EgCCR-GFP-GUS* construct. Fluorescent images of different tissues and organs were taken 6 months after *Agrobacterium*-mediated transformation. GFP fluorescence in shoot apex (A-B, bars represent 5µm) and transverse section of the transgenic microshoot (C-D, bars represents 100µm) were carried out using a 480/40 nm exciter filters, and two-barrier filter >510 nm (wide range) and 535/550 nm (specific filter for GFP fluorescence). (Abbreviations: vascular bundles (vb), pith, p; cortex, c).

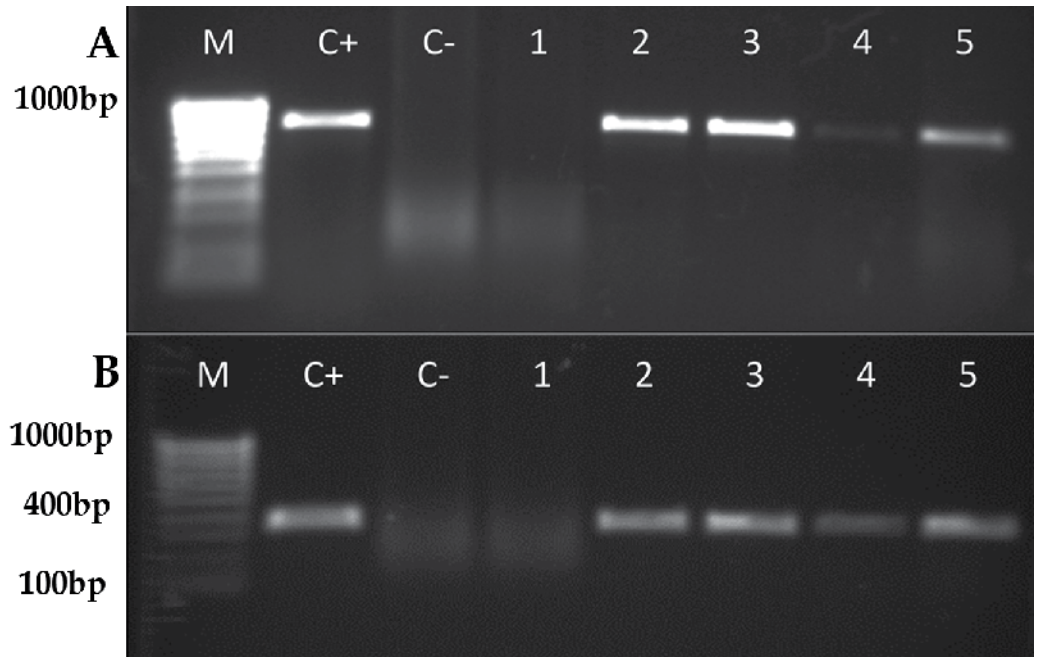

Fig. 2. Analysis of putative transgenic pistachio plants by PCR amplification using primers designed for 858 bp fragment of the *nptII* gene (A) and for a fragment of 326 bp of *uid*A gene (B). (M: DNA 1000 bp ladder, C+: positive control, C-: untransformed plant, T1-T5 putative transgenic shoots lines).

Agrobacterium-mediated transformation is highly genotype dependent for many plants (Pérez-Piñeiro, 2012) but also for fruit species. Different reports described that some cultivars were completely found to be as highly recalcitrant for transformation process whereas others are completely successful (Galun & Breiman, 1998; Petri & Burgos, 2005). This problem is widely described in different fruit species as apricot, grapevine and others (López-Pérez et al., 2008; Wang, 2011). López-Pérez and collaborators (2009) described that grapevine cultivars "Crimson Seedless" and "Sugraone" obtained different transformation efficiencies depending on the optical densities tested. Transformation of hypocotyls obtained from germination of mature seeds and nodal explants of apricot cultivars Dorada, Moniquí, Helena, Canino, Rojo Pasión and Lorna resulted in different transformation efficiencies (Wang, 2011). Some authors pointed out that one of the main goals of plant genetic engineering must be the development of genotype-independent transformation procedures, however due to this highly complex plant-pathogen interaction it will be very difficult to achieve this with the currently available technologies (Petri & Burgos, 2005).

Species	Aim	Plasmid	Transgenes	References
Apple				
Malus x domestica	Method optimisation	pBIN6	*nptII, nos*	James et al., 1989
M. x domestica	Investigation of early events in transformation	pDM96.0501	*sgfp, gusA,nptII*	Maximova et al., 1998

Species	Aim	Plasmid	Transgenes	References
M. x domestica	Investigation of influence of rolA gene on shoot growth	pMRK10	*rolA, nptII*	Holefors et al., 1998
M. x domestica	Scab resistance	p35S-ThEn42, pBIN19ESR	*ech42, nptII*	Bolar et al., 2000
M. x domestica	Resistance to fireblight	pLDB15	*attE, nptII, gusA*	Ko et al., 2000
M. x domestica	Improve rooting ability	pCMB-B	*rolB, nptII, gusA*	Zhu et al., 2001
M. x domestica	Scab resistance	pBIN(Endo+Nag)	*ech42, nag70, nptII*	Faize et al., 2003
M. x domestica	Self-fertility	pGPTV-KAN	*S_3RNase, nptII*	Broothaerts et al., 2004
M. x domestica	Method optimisation Enhance rooting	pCMB-B	*rolB, nptII, gusA*	Radchuk & Korkhovoy, 2005
M. x domestica	Method optimisation	pNOV2819	*pmi, nptII, gusA*	Degenhardt et al., 2006
M. x domestica	Investigation of function of ARRO-1 in adventitious rooting	pK7GWIWG2 (II)	*ARRO-1, nptII*	Smolka et al., 2009
M. x domestica	Stability of scab resistance	pMOG402.hth.gus.intron	*Hth, nptII, gusA*	Krens et al., 2011
M. x domestica	Development of selection system	pCAMBIAVr-ERE-GUS	*VrERE, gusA*	Chevreau et al., 2011
M. x domestica	Transformation without selectable marker gene	pPin2Att.35SGUSint+.n pPin2MpNPR1.GUS−.n ptII		Malnoy et al., 2010
Almond				
Prunus dulcis	Method optimisation	pBI121mgfp-5-ER pNOV2819	*nptII pmi*	Ramesh et al., 2006
Avocado				
Persea americana Mill.	Method optimisation	pMON9749, pTiT37-SE	*nptII, gusA*	Cruz-Hernandez et al., 1998
Banana				
Musa spp.	Method optimisation (Agro + SAAT+ Vacuum infiltration)	pCAMBIA1301	*hptII, gusA*	Subramanyam et al., 2011
Musa spp.	Resistance to Fusarium wilt	pBI121-PFLP	*pflp, nptII*	Yip et al., 2011
Blueberry				
Vaccinium spp.	Method optimisation	p35SGUS-int	*gusA*	Cao et al., 1998

Species	Aim	Plasmid	Transgenes	References
Blueberry				
V. corymbosum L.	Method optimisation	pBISN1	*nptII, gusA*	Song & Sink, 2004
Grapevine				
Vitis vinifera	Method optimisation	Nr	*gusA, nptII*	Nakano et al., 1994; Gago et al., 2011
V. rootstocks	Resistance to viruses and crown gall	pBIN19 pGA482G	*mutant virE2, nptII GLRaV-3cp*	Xue et al., 1999
V. vinifera	Resistance to fungal pathogens	pBI121	*nptII rice chitinase gene*	Yamamoto et al., 2000
V. vinifera	Fungal resistance	pGJ42	*chitinase, rip, nptII*	Bornhoff et al., 2005
V. vinifera	Method optimisation	pGA643	*nptII, GFLVCP*	Maghuly et al., 2006
V. vinifera	Method optimisation	Nr	*egfp, nptII*	Dutt et al., 2007
V. vinifera	Resistance to powdery mildew	pGL2	*ricechitinase gene, hgt*	Nirala et al., 2010
V. vinifera	Method optimisation	pBin19-sgfp	*nptII, sgfp*	Pérez-López et al., 2008
V. vinifera	Method optimisation	pSGN	*nptII, egfp*	Li et al., 2006
V. vinifera	Method optimisation	pCAMBIA2301	*nptII, gusA*	Wang et al., 2005
Grapefruit				
Citrus paradisi	Resistance to Citrus tristeza virus	pGA482GG	*CP, RdRp, gusA, nptII*	Febres et al., 2003
C. paradisi	Resistance to Citrus tristeza virus	pGA482GG	*CP, gusA, nptII*	Febres et al., 2008
Kiwifruit				
Actinidia spp.	Hairy root induction	A722,C58, ICMP8302, ICMP8326, ID1576, LBA 4404, A4T	*gusA, nptII*	Atkinson et al., 1990
Actinidia spp.	Method optimisation	pLAN411, pLAN421	*gusA, nptII*	Uematsu et al., 1991
A.deliciosa	Improved rooting	pBIN19	*nptII, rol A,B,C*	Rugini et al., 1991
A. eriantha	Method optimisation	pART27-10	*gusA, nptII*	Wang et al., 2006
A. deliciosa	Manipulation of plant architecture	pBI121	*İpt*	Honda et al., 2011

Species	Aim	Plasmid	Transgenes	References
Mango				
Magnifera indica L.	Method optimisation	pTiT37-SE::pMON9749	*nptII, gusA*	Mathews et al., 1992
M.indica L.	Methodoptimisation	pGV3850::1103	*nptII*	Mathews et al., 1993
M.indica L.	Mediate ethylene biosynthesis	pBI121	*nptII, gusA antisense ACC oxidase, antisense ACC synthase*	Cruz Hernandez et al., 1997
M. indica L.	Rooting enhancement	Nr	*rol B*	Chavarri et al., 2010
Melon				
Cucumis melo	Resistance to ZYMV, TEV, PVY	FLCP core AS	*nptII, ZYMV coatpr.*	Fang & Grumet, 1993
C. melo	Salt resistance	pRS655	*nptII, gusA, hal1*	Bordas et al., 1997
C. melo	Resistance to ZYMV	pBI-ZCP3'UTR	*nptII, ZYMV coatpr.*	Wu et al., 2009
Nectarberry				
Rubus arcticus	Method optimisation	pFAJ3001	*gusA*	Kokko & Kärenlampi, 1998
Orange				
Citrus sinensis	Method optimisation (Agro + SAAT+ vacuum infiltration)	pGA482GG	*gusA, nptII*	Oliveira et al., 2009
C. sinensis	Research on expression of Mt-GFP	pBI. mgfp4.coxIV	*Mt-gfp*	Xu et al., 2011
C. sinensis	Influence of methylation on gene expression	pBIN.mgfp5-ER	*gfp, nptII*	Fan et al., 2011
C. sinensis	Modification of gibberellin levels	pBinJIT-CcGA20ox1-sense pBinJITCcGA20ox1-antisense	*nptII, CcGA20ox1 nptII, CcGA20ox1*	Fagoaga et al., 2007
C. sinensis	Resistance to fungi	pBI121.*P23*	*nptII, PR-5*	Fagoaga et al., 2001
C. aurantifolia	Resistance to virus	pBin19-sgfp	*nptII, sgfp, p23*	Fagoaga et al., 2006
Poncirus trifoliate	Enhanced salt tolerance	pBin438	*nptII, AhBADH*	Fu et al., 2011
Papaya				
Carica papaya	Resistance to PRSV	pRPTW	*PRSV replicase gene, neo*	Chen et al., 2001

Species	Aim	Plasmid	Transgenes	References
Pear				
Pyrus communis	Alter growth habit	pGA-GUSGF	*rolC, gusA, nptII*	Bell et al., 1999
P. communis	Method optimisation	pPZP pME504	*gusA, nptII*	Yancheva et al., 2006
P. communis	Method optimisation	PBISPG	*nptII, gusA*	Sun et al., 2011
Peanut				
Arachis hypogaea	Production of edible vaccines for *Helicobacter pylori*	pBI121.Oleosin-UreB	*ureB, nptII*	Yang et al., 2011
A. hypogaea	Improvement of salt and drought resistance	pGNFA-(pAHC17)	*AtNHX1*	Asif et al., 2011
A. hypogaea	Production of vaccines for Peste des petits ruminants (PPR)	pBI121	*Hn*	Khandelwal et al., 2011
Plum				
Prunus armeniaca	Method optimisation	pBIN19-sgfp	*nptII, gfp*	Petri et al., 2004
P. armeniaca	Method optimisation	pBIN19-sgfp, p35SGUSint	*nptII, gfp/nptII, gusA*	Petri et al., 2008
P. domestica	Transformation of marker free plants	pCAMBIAgfp94(35S) / pGA482GGi ihpRNAE10´	*nptII, gfp, gusA, ppv-cp*	Petri et al., 2011
P. domestica	New selection system with hygromycin	pC1381, pC1301, pC2301	*gusA, hpt, nptII*	Tian et al., 2009
P. domestica	Control of PPV infection	pGA482GG	*nptII, gusA, PRVcp*	Scorza et al., 1995
P. salicina	Method optimisation	pCAMBIA2202	*nptII, gfp*	Urtubia et al., 2008
Pomegranate				
Punica granatum	Method optimisation	pBIN19-sgfp	*nptII, gfp*	Terakami et al., 2007
Strawberry				
Fragaria spp.	Method optimisation	pBI121	*nptII, gusA*	Barcelo et al., 1998
Fragaria x ananassa Duch.	Modulation of fruit softening	pBI121	antisense of *endo-β-1,4-glucanase*	Lee & Kim, 2011
White mulberry				
Morus alba	Method optimisation	pBI121	*nptII, gusA*	Agarwal & Kanwar, 2007

Table 1. Some important reports on genetic transformation of fruit species via *A. tumefaciens* or *A. rhizogenes*.

Some abbreviations: *AtNHX1*: a vacuolar type Na^+/H^+ antiporter gene; *gfp:* green fluorescent protein coding gene; *hal1:* yeast salt tolerance gene; *hpt*: hygromycin phosphotransferase coding gene; *ipt*: isopentyl transferase gene; *neo*: neomycin phosphorate transferase coding gene; *nos*, nopaline synthase coding gene; *nptII*, neomycin phosphtransferase II coding gene; *pmi*: phosphomannose isomerase coding gene; *ppv*: Plum pox virus; *prsv*: papaya ringspot virus; *pvy*: potato virus Y; *tev*: tobacco etch virus; *gusA (uidA)*: β-glucuronidase coding gene; *UreB*: antigen gene; *zymv*: zucchini yellow mosaic virus.

2.2 Direct gene transfer

Direct gene transfer techniques include microprojectile bombardment, microinjection, electroporation, and usage of whiskers. Among them, microprojectile bombardment is an alternative technique of *Agrobacterium*-mediated transformation since its physical nature overcomes biological barriers and enables naked DNA delivery directly into host genome or alternatively into mitochondria and chloroplasts. In this technique, plasmid or linearized DNA-coated metal microparticles (gold or tungsten) at high velocity is bombarded to intact cells or tissues (Sanford et al. 1987; Klein et al. 1987; Sanford, 1988). Furthermore, biological projectiles such as bacteria (i.e., *E. coli, Agrobacterium*), yeast and phage associated with tungsten can also be used in microprojectile bombardment (Bidney, 1999; Kikkert et al. 1999).

Microprojectile bombardment was developed in the 1980s for transformation of plants which were recalcitrant to *Agrobacterium*–mediated transformation (Paszkowski et al., 1984) such as agronomically important cereals. Following the development of the first particle delivery system (Sanford et al. 1987; Sanford 1988), different effective devices such as PDS-1000/He, Biolistic® particle delivery system; particle inflow gun; electrical discharge particle acceleration; ACCELL™ technology and microtargeting bombardment device were also evolved to improve transformation capacity. Among them, PDS-1000/He, Biolistic® particle delivery system (BIO-RAD), which is a modified version of Sanford's system, is the most used system for biolistic transformation due to its efficient and relatively simple application and acquisition of reproducible results between laboratories (Taylor & Fauquet, 2002). Particle inflow gun can be an alternative to other biolistic systems due to its very low cost and it was used successfully in banana transformation (Becker et al., 2000). Electrical discharge particle acceleration, ACCELL™ technology uses high voltage electrical discharge into a droplet water to generate shock waves and project microprojectiles to different cell layers of target tissues (McCabe & Christou, 1993). Microtargeting bombardment device was designed for shoot meristem transformations (Sautter, 1993) but it is not widely used for plant transformation. All of the microprojectile bombardment systems are not depend on any plant cell type but target cells which will be bombarded need to be physically accessible (Hensel et al., 2011).

Particle bombardment were carried out not only to optimize plant transformation but also to transfer gene constructs encoding for various antimicrobial peptides or proteins for fungal resistance against to *Fusarium oxysporum* f. sp. cubense and *Mycospaerella fijiensis* or preharvest and postharvest diseases *Verticillium theobromae* or *Trachysphaera fructigen* (i.e., Remy et al., 2000; Sagi et al., 1998; Tripathi, 2003), virus (i.e., Fitch et al., 1992; Tennant et al., 1994; Gonsalves et al., 1994; Scorza et al., 1996), pest (i.e., Serres et al., 1992) and herbicide tolerance (i.e., Zeldin et al., 2002). This technique has been applied to transformation of various fruit species including banana, cranberries, citrus, grapevine, melon, papaya and peanut (Table 2).

Species	Aim	Transfer system	Plasmid	Transgenes	References
Apple					
Malus x domestica	Method optimisation	PEG-mediated	pKR10	*Gfp*	Maddumage et al., 2002
Banana					
Musa spp.	Method optimisation	Particle bombardment	pUbi-BtintORF1 pBT6.3-Ubi-NPT pUbi-BTutORF5 pBT6.3-Ubi-NPT pUGR73 pDHkan	*nptII, gusA, BBTV*	Becker et al., 2000
Musa spp.	Tolerance to Sigatoka leaf spot	Particle bombardment	pYC39	*ThEn-42, StSy, Cu, Zn-SOD*	Vishnevetsky et al., 2011
Musa spp.	Resistance to virus	Particle bombardment	pAB6, pAHC17,pH1	*gusA, bar,ubi, BBTV-G-cp*	Ismail et al., 2011
Cranberry					
Vaccinim macrocarpon	Method optimisation & Pest control	Particle bombardment	pTvBTGUS	*nptII, gusA, Bt*	Serres et al., 1992
V. macrocarpon	Herbicide resistance	Particle bombardment	pUC19	*bar, aphII*	Zeldin et al., 2002
Grapevine					
Vitis vinifera	Method optimisation	Biolistic	pBI426	*nptII, gusA*	Hebert et al., 1993
V. vinifera	Method optimisation	Particle bombardment & Agro	pGA482GG	*nptII, gusA TomRSV-CP*	Scorza et al., 1996
V. vinifera	Method optimisation	Biolistic	pSAN237	*nptII, magainin,PGL*	Vidal et al., 2003
V. vinifera	Comparison of minimal cassette with standard circular plasmids	Biolistic	pSAN168, pSAN237	*Magainin, nptII*	Vidal et al., 2006
Kiwifruit					
Actinidia spp.	Method optimisation	PEG 4000	pDW2	*Cat*	Oliveira et al., 1991
Actinidia spp.	Method optimisation	Electroporation	pB1121, pTi35SGUS	*gusA, nptII*	Oliveira et. al., 1994
A. deliciosa	Method optimisation	PEG 4000	p35SGUS	*gusA*	Raquel & Oliveira, 1996

Species	Aim	Transfer system	Plasmid	Transgenes	References
Melon					
Cucumis melo	Protection against	Particle bombardment İnfection & Agro	pGA4822GG/ CP	*nptII, gusA, CMV-WLCP*	Gonsalves et al., 1994
Papaya					
Carica papaya	PRV resistance	Particle bombardment	pGA482GG	*PRV, nptII*	Fitch et al., 1992
C. papaya	PRV resistance	Particle bombardment	pGA482GG	*nptII, gusA, cpPRVHA*	Tennant et al., 1994
C. papaya	Control of PRSV	Particle bombardment	pGA482GG	*cpPRSV-pHA5, nptII, gusA*	Cai et al., 1999
C. papaya	Method optimisation	Particle bombardment	pCAMBIA1303 pML202	*hpt, nptII, mgfp5'*	Zhu et al., 2004
C. papaya	Use of PMI/Man	Particle bombardment	pNOV3610	*Pmi*	Zhu et al., 2005

Table 2. Some important reports on genetic transformation of fruit species via direct gene transfer.

A successful protocol was studied very recently in banana cv. Williams apical meristems with microprojectile bombardment of a new construct pRHA2 plasmid containing *bar* and coat protein of banana bunchy top nanovirus (*BBTV-cp)* genes that encoded the viral coat protein by using Biolistic™ PDS-1000/He system, 650 psi helium pressures and 5 µg DNA/shot for acquisition of virus resistance (Ismail et al., 2011). After bombardment, 62% of apical meristems were survived on the selective medium and 80% of explants produced shoots in the following first subculture and all shoots were rooted (Ismail et al., 2011). In addition to those disease-based studies, others were also carried out in order to develop efficient transformation protocols via biolistic transformation (Sagi et al., 1995; Becker et al., 2000). Among them, Sagi and co-workers (1995) reported the transformation of embryogenic cell suspensions of cooking banana 'Bluggoe' (ABB genome) and plantain 'Three Hand Planty' (AAB genome) via particle bombardment. Then, Cavendish banana cv. Grand Nain embryogenic suspension cells were co-bombarded with the plasmid containing *npt*II selectable marker gene under the control of *BBTV* promoter or the cauliflower mozaic virus (*CaMV*) 35S promoter, the β-glucuronidase (*gus*A) reporter gene and *BBTV* genes under the control of the maize polyubiquitin promoter by using particle inflow gun and stably integration was obtained in all of the tested transformed plants (Becker et al., 2000). Very recently, microprojectile bombardment was also applied to induce tolerance to Sigatoka leaf spot caused by *Mycosphaerella fijiensis* in banana by transferring endochitinase gene of *ThEn-42* from *Trichoderma harzianum* together with the grape stilbene synthase gene (*StSy*) under the control of 35 S promoter and the inducible PR-10 promoter, respectively (Vishenevetsky et al., 2011). Moreover, in order to improve scavenging of free radicals generated during fungal attack, the superoxide dismutase gene (*Cu, Zn-SOD*) of tomato was also included to this gene cassette under the control of ubiquitin promoter. Both PCR and Southern blot analysis confirmed the stable integration of the transgenes and 4-year field trial showed that several transgenic banana lines had improved tolerance not only to Sigatoka but also other fungus such as *Botrytis cinerea.* Gene transfer via microprojectile

bombardment was also carried out in American cranberry (*Vaccinium macrocarpon*) firstly to increase productivity by transferring *Bacillus thuringiensis* subsp. Kurstaki crystal protein gene (*Bt*) for pest resistance (Serres et al., 1992), and latter on, by *bar* gene to confer tolerance to the phosphinothricin-based herbicide glufosinade (Zeldin et al., 2002). Although preliminary bioassays for efficiency of the *Bt* gene against an important lepidopteran demonstrated no consistently effective control in former, stable transmission and expression of herbicide tolerance was observed in both inbred and outcrossed progeny of cranberry trans clone in latter.

In tangelo (*Citrus reticulata* Blanco × *C. paradisi* Macf.) cv. Page embryogenic suspension cells were bombarded with tungsten coated plasmid containing *gusA* and *nptII* genes (Yao et al., 1996). Following to bombardment, 600 transient and 15 stable transformants were obtained and integration of the interest genes confirmed by PCR and Southern blot analyses. A large of kanamycin-resistant embryogenic calli showed also GUS activity. In another study, Kayim and associates (1996) bombarded tungsten-coated plasmid (pBI221.2) containing the *gusA* gene into lemon cv. Kütdiken nucellar cells by biolistic device and expression of the *gusA* gene was histochemically confirmed.

Feronia limonia L. is important fruit tree because of its edible fruits. It is suitable for cultivation in semi-arid tropics and also can be used for reforestation and wasteland reclamation projects (Sing et al., 1992; Purohit et al., 2007). *Feronia limonia* L. hypocotyl segments were also bombarded with tungsten-coated plasmid pBI121 having *gusA* reporter gene driven by *CaMV35S* promoter and *nptII* as a selective marker under control of *nos* promoter using Biolistic™ PDS-1000/He particle delivery system at different rupture disc pressures (1100 and 1350 psi) and target distances (6 and 9 cm) (Purohit et al., 2007). This study revealed that 1100 psi/6 cm and 1350 psi/9 cm were the optimal bombardment condition with supplying a maximum 90% of GUS transient expression.

In grapevine, the initial transformation studies via microprojectile bombardment were performed for method optimization with transferring *nptII* and *gus* genes as selective and reporter marker genes, respectively (Hebert et al., 1993; Kikkert et al., 1996; Scorza et al., 1996). Later, Vidal and co-workers (2003) studied the efficiency of biolistic cotransformation in grapevine for multiple gene transfer of *nptII* and antimicrobial genes (*magainin* and *peptidyl-glycine-leucine*). The stable transformation was confirmed by *gus* gene expression, followed by PCR and Southern blot analyses of *nptII* and antimicrobial genes showed. Three years later, same research group (Vidal et al., 2006) reported the efficient biolistic transformation of grapevine by using minimal gene cassettes, which are linear DNA fragments lacking the vector backbone sequence.

Papaya is economically important and preferred another fruit species because of its nutritional and medicinal properties grown in tropical and subtropical regions (Tripathi et al., 2011). Papaya ringspot virus (PRSV) is major limiting factor in papaya production in Hawaii (Gonsalves, 1998; Fuchs & Gonsalves, 2007). First PRSV resistant papaya plants (cv. SunUp) were obtained by PDS/1000-He particle bombardment device of cv. Sunset with the transformation vector pGA482GG/cpPRV4 containing the *prsv* coat protein (*CP*) gene (Fitch et al., 1992). The PRSV resistant papaya has been commercialized, reached to end user and improved papaya is now under production in Hawaii (Tripathi et al., 2008). This study was followed by other reports mainly on improvement of PRSV tolerance in papaya via

microprojectile bombardment-based transformation (Tennant et al., 1994; Cai et al., 1999; Guzman-Gonzalez et al., 2006). The deployment of transgenic papayas has showed that virus CP protein supplies durable and stable resistance to homologous strains of PRSV (Fermin et al., 2010). Moreover, no ecological influence of transgenic papayas on adjacent non-transgenic papaya trees, microbial flora and beneficial insects was evident (Sakuanrungsirikul et al., 2005). However, political and social factors have negatively affected the technology in Thailand (Davidson, 2008).

Although there are various wild peanut species having disease resistance traits, hybridization between wild and cultivars is difficult due to self-incompatibility, low frequency of hybrid seed production and linkage drag (Stalker & Simpson, 1995) and because of that genetic transformation is a practical tool to improve disease resistant cultivars. Singsit and associates (1997) transformed peanut somatic embryos with gold-coated plasmid constructs containing both *Bacillus thuringiensis cryIA*(c) and *hph* genes driven by *CaMV35S* promoters by PDS 1000 He biolistic device for resistance lepidopteran insect larvae of lesser cornstalk borer. The embryogenic cell lines showed hygromycin resistance and integration of *hph* and *Bt* genes were confirmed by PCR and/or Southern blot analyses in regenerated plants and a progeny. 18% CryIA(c) protein of total soluble protein was detected by ELISA immunoassay in the hygromycin resistant plants. Production of peanut stripe virus (PStV) resistant peanut is another attempt for biotechnologists since the virus negatively affects seed quality and yield in Asia and China (Higgins et al., 1999). Somatic embryos of peanut cv. Gajah and cv. NC-7 were transformed by co-bombardment of *hph* gene and one of two forms of the *PStV* coat protein genes and both of the transgenic plants showed high level resistance to the homologous virus isolate (Higgins et al., 2004). Transfer of anti-apoptotic genes originated from mammals, nematods or virus into plants is another approach for enhancement of plant resistance against to biotic and abiotic stresses (Chu et al., 2008a). With this aim, peanut cv. Georgia Green embryogenic callus was bombarded with anti-apoptotic *Bcl-xL* gene by microprojectile bombardment. Although Bcl-xL protein was detected in four transgenic lines, just one transgenic line (25-4-2a-19) had stable protein expression and showed tolerance to 5μM paraquat (commercial herbicide) (Chu et al., 2008a). Around 0.6% of total population in USA is affected of IgE-mediated allergic reaction following to peanut consumption (Sicherer et al., 2003). To produce hypoallergenic peanut, peanut cv. Georgia Green embryogenic cultures were also transformed via microprojectile bombardment and silenced peanut allergens (Ara h 2 and Ara h 6) by RNA interference. Expression of these allergens was not decreased effectively but, binding of IgE to the two allergens, significantly declined (Chu et al., 2008b).

Apart from microprojectile bombardment, electroporation (Oliviera et al., 1994) and PEG-mediated transformation were also carried out in apple (Maddumage et al., 2002) and kiwifruit (Raquel & Oliveira, 1996) in order to optimize transformation protocol by transferring *gusA*, *gfp* and/or *nptII*.

3. Markers and selection of transformants

3.1 Reporter genes

Reporter genes or non-selectable marker genes are commonly used components of the plasmid constructs allowing the verification of transformation and the detection of the

putative transformed cells. In many fruit transformation studies, histochemical analyses of transformed cells are visualized by using β-glucuronidase (GUS) expression as a reporter gene (Jefferson, 1987; Table 1). This enzyme is encoded by *E. coli uidA* (*gusA*) gene and histochemical localization of the gene expression is detected in subcellular levels (Daniell et al., 1991). High levels of GUS is not toxic for plant and the enzyme is very stable in cells, however, the assay is destructive to plants (Miki & McHugh, 2004). *gusA* generally co-transformed with other selective marker genes to enable the selection of transformants. The gene *gfp* encodes for the protein green fluorescent protein (GFP) (Chalfie et al., 1994). This is one of the mostly used reporter marker gene in fruit transformation protocols for monitoring transformed cells *in vivo* and in real time just by application of UV-light for the excitation of the fluorescent protein. GFP has not any cytotoxic effect on transformed plant cells (Stewart, 2001; Manimaran et al., 2011). *In vivo* detection may permit the manual selection of transformed tissues with focusing in the areas where the signal is more brightly. Fusion of GFP with other proteins of interest provides precise visualizing of intracellular localization and transport in transformed plant (Miki & McHugh, 2004; Manimaran et al., 2011). In some fruit species, it is reported that chlorophyll red autofluorescence can mask GFP expression making the detection really difficult or even impossible in species as apricot, peach and plum (Billinton & Knight, 2001; Padilla et al., 2006; Petri et al., 2008; Petri et al., 2011). However, it was described as an efficient reporter gene in other woody fruit plants, such as citrus (Ghorbel et al., 1999) and peach (Pérez-Clemente et al., 2004). These contrary results confirm the highly variability of the reporter *gfp* gene which is described by Hraška and co-workers (2008). Other reporter gene, luciferase (*luc*) (Gould & Subramani, 1988) also let the monitorization of the transgene putative cells in living tissues, however, it is not so widely employed as the *gfp* (van Leeuwen et al., 2000; Miki & McHugh, 2004).

3.2 Selection systems, a critical step

Selection of transformed regenerants is a critical step in any transformation procedure (Burgos & Petri, 2005). Selection systems can be classified as positive or negative, and conditional or non-conditional. Positive selection systems are those that promote the growth of transformed cells and tissues, by the contrary, negative selection systems are those that promote the death of the transgenic cells. Both systems can be conditioned by an external substrate to perform their activity. Currently, negative selection systems are used in combination with positive selection systems to eliminate transformed cells with incorrect molecular programmed excision of the T-DNA (Schaart et al., 2004; Vamblaere et al., 2011). Typically, in positive conditional selection systems the selectable marker gene encodes for an enzyme conferring resistance to some specific toxic substrate that enable the growth of the transformed cell, tissues and inhibiting or killing non transformed tissues (more information in the comprehensive review of Miki & McHugh, 2004). In the literature approximately 50 selection marker genes are described for genetic plant transformation, however, just only three genes of positive conditional selection system (*nptII* and *hpt*, resistance to the antibiotics kanamycin and hygromycin, respectively, and *bar* gene encoding resistance to herbicide phosphinothricin) are commonly employed in more than 90% of the papers (Miki & McHugh, 2004). These three selectable genes are also the most used ones to transform fruit species as it can be seen in Table 1 and Table 2. *Escherichia coli nptII* gene (also known *neo*) encoded protein (neomycin phosphotransferase, NPTII) inactives aminoglycoside antibiotics such as kanamycin, neomycin, geneticin (G418), and

paramomycin that inhibit protein translation in the transformed cells (Padilla & Burgos, 2010). Hygromycin B is another aminoglycoside antibiotic that inhibits protein synthesis with a broad spectrum activity against prokaryotes and eukaryotes and especially it is very toxic in plants. *Escherichia coli hpt* (*aphIV*, *hph*) gene codes for the hygromycin phosphotransferase to detoxify hygromycin B by phosphorylation via an ATP-dependent phosphorylation of a 7''-hydroxyl group and it is generally used as another selection marker gene when *nptII* was not effective in plant transformation studies (Twyman et al., 2002; Miki & McHugh, 2004).

Similar to antibiotics, herbicides have different specific target sites in plants. The resistance can be achieved by various mechanisms such as usage of natural isozyme or generation of enzyme mutagenesis or detoxification of the herbicides by metabolic processes. Phosphinothricin (PPT; ammonium glufosinate) is an active component of commercial herbicides formulations and analogous to glutamate, the substrate of glutamate synthase. In plants, this enzyme catalyzes the conversion of glutamate to glutamine by removing ammonia assimilation from the cell. Inhibition of the enzyme results in ammonia accumulation and disruption of chloroplast and finally cell death due to photosynthesis inhibition (Lindsey, 1992; OECD, 1999). In plant transformation studies, as herbicide resistance selection marker gene, *pat* from *S. viridochromogenes* (Wohlleben et al., 1988) and *bar* gene from *S. hygroscopicus* (bialophos resistance; Thompson et al., 1987) encoding the enzyme phosphinothricin N-acetyltransferase (PAT) are extensively used for resistance to PPT. PAT converts PPT to a non-herbicidal acetylated form by transferring the acetyl group from acetyl CoA to the free amino group of PPT (Miki & McHugh, 2004).

Currently, an alternative to these highly employed "toxic" approaches conditional positive selection markers based on the promotion of a metabolic advantage to transformed cells are used. Some authors mentioned that this kind of selection can improve considerably the selection of the transformants, since others such as antibiotics generally cause considerable necrosis (produced by the death of non-transformed cells) that often inhibits regeneration from adjacent tissues (Petri & Burgos, 2005). Previously, results obtained with this approach demonstrated higher yields than when the toxic selective agents were employed, and seems to be broadly applicable to crop plants (Miki & McHugh, 2004). Some of the most widely used are the *AtTPS1*/glucose (Leyman et al., 2006); *galT*/galactose (Joersbo et al., 2003); xylose isomerase (Haldrup et al., 1998); D-aminoacid/*dao1* (Alonso et al., 1998) and the *pmi*/mannose (Joersbo et al., 1998). Probably, one of the most used one in fruit species is the gene *pmi* that encodes the enzyme phosphomannose isomerase (EC 5.3.1.8) that catalyzes the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. This enzyme is present in bacteria as *E. coli* and also, in humans, however it is not present in plants, as exception of soybean and other legumes. Using a media with mannose as the unique carbon source, only transformed cells can grow and develop. Glycolysis is inhibited due to the accumulation of mannose-6-phosphate converted from mannose by hexokinase with preventing cell growth and development in non-transformed cells (Miki & McHugh, 2004). Sensitivity to the toxic effect of mannose-6-phosphate is different between species, and can be avoided by combining with other sugars such as sucrose, maltose and fructose (Joersbo et al., 1999). Diverse fruit trees were selected with this system, alone or in combination with sucrose, i.e., 12 g/L mannose and 5 g/L sucrose in orange (Ballester et al., 2008); 30 g/L mannose without any sugar more in papaya (Zhu et al., 2005); 2,5 g/L

mannose and 5 g/L sucrose in almond (Ramesh et al., 2006) or 1-10 g/L mannose and 5-30 g/L in apple (Degenhardt et al., 2006). In *Citrus sinensis*, the best results were obtained when 13 g/L mannose as unique source of carbon was added into the selection media. Mannose combined with other sugars promoted reduction in transformation efficiencies and escapes (Boscariol et al., 2003). Apricot cv. Helena and Canino required the lower combination of mannose with sucrose (1,25 g/L mannose and 20 g/L sucrose) in comparison with other woody fruit trees to obtain the most effective selection procedure. Moreover, safety assessments were revealed that there is no any adverse effect of the enzyme on mammalian allergenicity and toxicity (Reed et al., 2001).

Other selective strategies were developed as positive non-conditional systems, or in other words, using selectable marker genes that "promote" plant regeneration. Currently, there is more information about the genetic and biochemical control of organogenesis than embryogenesis for plant regeneration. Because of this, commonly genes related with cytokinins synthesis are employed for shoot organogenesis. More efforts are required to discover molecular mechanisms of embryogenesis to use these strategies in species highly dependent on embryogenesis regeneration to develop transgenic plants. Genes as *cki1* or the most employed isopentenyl transferase *ipt* gene encoding the enzyme IPT, catalyze the synthesis of isopentyl-adenosine-5-monophosphate, which is the first step in cytokinin biosynthesis (Miki & McHugh, 2004). This gene modify the endogenous balance between cytokinins and auxins, stimulating cell division and differentiation of the cells that promote an altered morphology, development and physiology of transgenic plants (Sundar & Sakthivel, 2008). Some authors observed that the *ipt* gene improved transformation efficiency in apricot leaf explants in comparison with the selection through *nptII* (López-Noguera et al., 2009).

3.3 A differential transgene expression: Constitutive versus specific promoters

Currently, an important debate is carrying out about the risks of the "unpredictable" behavior and recombinogenic potential of constitutive promoters (Gittins et al., 2003) and to avoid the public concerns about the risks of ubiquitous transgene expression in crops.

Commonly, most of the fruit species have been transformed with plasmidic constructions harbouring constitutive or ubiquitous promoters, as the Cauliflower Mosaic virus 35S (*CaMV35S*). In this sense, different authors described that constitutive expression may be harmful for the host plant, causing sterility, retarded development, abnormal morphology, yield penalty, altered grain composition or transgene silencing (Cai et al., 2007 and references therein) and its expression level is dependent on the cell type, the developmental stage and on the perception of environmental triggers (Hensel et al., 2011). Moreover, under constitutive promoters reporter and selectable marker, and genes of interest are expressed continuously in all tissues without any temporal control. In this sense, specific-promoters appear as an alternative approach to avoid the undesirable side effects of constitutive promoters and to target transgene expression in a spatial or temporal specific way (Gago et al., 2011; Hensel et al., 2011).

Recently, vascular specific promoter *EgCCR* from *Eucaliptus gunnii* was checked in pistachio in this study as mentioned above as well as other fruit species such as kiwifruit and grapevine (Paradela et al., 2006; Gago et al., 2011) and results demonstrated that this promoter is conserved and fully functional in these species. Vascular promoters can drive

resistance to biotic or abiotic stresses related with vascular tissues. Specific promoters could be useful to synchronize transgene activity spatially and/or temporally to control with more accuracy the pathogenic process (Gago et al., 2011).

3.4 Alternative transformation systems: Transgenics without marker genes

A highly desirable approach to promote public acceptation for future commercialization of transgenic plants and products is focused on the elimination of marker genes from transformed plants or the direct production of marker-free transgenics (Kraus, 2010). These newly and promising approaches are highly dependent on previously established highly efficient regeneration protocols that may be based on organogenesis or embryogenesis (Petri et al., 2011). There are various technologies such as homologous recombination, co-transformation, site-specific recombination (Cre/loxP site specific recombination system, R/RS system, FLP/FRT system etc) or marker elimination by transposons to remove selective marker genes (Hao et al., 2011; Manimaran et al., 2011). However, there are still few marker-free fruit species transformation protocols.

Strawberry leaf explants were transformed with site-specific recombinase for the precise elimination of undesired DNA sequences and a bifunctional selectable marker gene used for the initial positive selection of transgenic tissue and subsequent negative selection for fully marker-free plants (Schaart et al., 2004).

MAT (multi-auto-transformation) (Ebinuma et al., 1997) combined with the *Agrobacterium* oncogene *ipt* gene, for positive selection with the recombinase system R/RS for removal of marker genes acting as "molecular scissors" after transformation were used as alternative approach in citrus plants (Ballester et al., 2007; 2008). Also, in apricot (López-Noguera et al., 2009) a similar strategy was used. Regeneration of apricot transgenic shoots was significantly improved to non-transformed plants (regenerated in non-selective media). Moreover, it was significantly higher in comparison with previous published data using resistance to kanamycin mediated by *nptII* gene. The lack of *ipt* differential phenotype promoted difficulties to assess the excision of the marker genes, that require periodic assays. Complete excision of marker genes ranged from 5 to 12 months, however, only 41% of the regenerated transgenic shoots R-mediated recombination occurs correctly. In *Citrus sp.,* it was also reported that anomalous excision of marker genes promoting failures in the expression of the reporter genes (Ballester et al., 2007, 2008).

Apple (Malnoy et al., 2010) and pineapple sweet orange (Ballester et al., 2010) transformation using ''clean'' binary vector including only the transgene of interest were carried out to create marker-free transformants. Very recently, melon (*C. melo* L. cv Hetao) was transformed with a marker-free and vector-free antisense 1-aminocyclopropane-1-carboxylic acid oxidase construct via the pollen-tube pathway and transgenic lines are choosen by PCR without using any selectable marker agent (Hao et al., 2011).

In plum (*Prunus domestica*), transformation was carried out without reporter or selectable marker genes using a high-throughput transformation system (Petri et al., 2011). Previously, authors checked the efficiency of the regeneration of transformed shoots using conventional constructs harbouring reporter marker such as *gusA* and *gfp,* and *nptII* gene. Transformation efficiency varied from 5.7-17.7%. Using a marker free construct, the intron-hairpin-RNA (ihpRNA) harbouring the Plum Pox Virus coat protein (*ppv-cp*) gene, these authors

regenerated five transgenic lines confirmed by Southern blot. It is important to take into account that this kind of free marker strategy is widely dependent on highly yields in regeneration systems.

3.5 Cisgenesis, the P-DNA technology and multigene transformation

Other relevant advance in fruit species transformation was the proposal made by Schouten and coworkers (2006), the "cisgenesis". This term means the use of recombinant DNA technology to introduce genes from crossable donors plants, isolated from within the existing genome or sexually compatible relative species for centuries therefore, unlikely to alter the gene pool of the recipient species. Cisgenesis includes all the genetic events of the T-DNA as introns, flanking regions, promoters, and terminators (Vanblaere et al., 2011).

This methodology proposes to transfer the own plant DNAs, the P-DNAs. The use of this technology requires the construction of whole plant derived vector from the target species. Within the target species genome, it must be a DNA fragment with two T-DNA border-like sequences oriented as direct repeats ideally about 1-2 kb apart with suitable restriction sites for cloning of a desirable gene.

In the last years, different works were considered to step towards introducing regulatory elements and genes of interest from crossable donor plants, however with some foreign elements as marker genes in species as melon and apples (Benjamin et al., 2009; Joshi, 2010; Szankowski et al., 2009). Up to 2011 there is no any report of real "cisgenesis" plantlets, in agreement with Schouten et al. (2006) definition of the topic. In 2011, Vanblaere and coworkers developed apple cv. Gala cisgenic plants by expressing the apple scab resistance gene *HcrVf2* encoding resistance to apple scab. Marker-free system was employed for the development of three cisgenic lines containing one insert of the P-DNA after removing by recombination with using chemical induction. These lines were not observed different from non-transformed cv. Gala plants.

Cisgenic plants are essentially the same as the traditionally bred varieties, and they might be easier to commercialise than the "problematic" transgenic plants (Schouten et al., 2006; Rommens et al., 2007). Critical opinions to these proposals also were clearly exposed, the uncontrolled P-DNA integration into the plant target genome can cause mutations or affect to the expression of other native genes, altering the behaviour of that cisgenic plants in an unpredictable manner (Schubert & Willims, 2006; Akhond & Machray, 2009). Recently, interesting approaches are being proposed for genome editing using ZFNs (Zinc finger nucleases) that can promote induction of double-strand breaks at specific genomic sites and promote the replacement of native DNA with foreign T-DNA (Weinthal et al., 2010).

The multigen transfer (MGT) methodology consist in introducing more than one gene at once. Commonly, most of the transgenic plants are generated by introducing just one single gene of interest, but now MGT are being developed to obtain more ambitious phenotypes as the complete import of metabolic pathways, whole protein complex and the development of transgenic fruit species with various new traits simultaneously transferred (Naqvi et al., 2009). In this sense, this technology would be highly desirable for commercial fruit species cultivars to obtain new traits related with large fruit size, high coloration of the fruit epidermis, flesh firmness and virus resistance (Petri et al., 2011) at the same time without the need of several rounds of introgressive backcrossing.

4. *In vitro* culture techniques for the recovery of transgenic plants

Plants are complex, diverse organisms and have adapted evolutionarily to almost every ecological niche on the planet. Development of successful transformation protocol depends on a reliable and highly efficient regeneration system. Explant types are highly variable since it depends on the selected organogenetic process optimized for each species. Commonly, the genetic transformation protocols of fruit species employed explants such as ovules, anthers, seedlings, zygotic and somatic embryos, cotyledons, epicotyles, hypocotyles, leaf pieces, roots, meristems (Fagoaga et al., 2007; Lopez- Perez et al., 2008; Petri et al., 2008; Husaini, 2010; Malnoy et al., 2010; Bosselut et al., 2011; Petri et al., 2011; Gago et al., 2011). Typically, it is recommended that those tissues have high and active cell division to enhance the regeneration of the transgenic lines (Mante et al., 1991; Schuerman & Dandekar, 1993; Wang, 2011). Ideally, fruit species transformation must be done with somatic tissues such as leaves and roots to transform varieties already well known and accepted in the market by the consumers. Recently, some authors also proposed the possibility of the use of transgenic seedlings to develop new fruit varieties through subsequent cross-breeding. These transgenic seedlings can add new traits impossible to obtain in the species genome-pool (Petri et al., 2011).

Organogenesis was the strategy selected in different species to develop most of the known and efficient regeneration protocols for fruit species, concretely for fruit trees (Petri et al., 2011). Almond (Costa et al., 2006); apple (Smolka et al. 2009; Lau & Korban, 2010; Vanblaere et al., 2011); banana (Subramanyam et al., 2011); fig (Yancheva et al., 2005); kiwifruit (Tian et al., 2011); peach (Padilla et al., 2006); strawberry (Mercado et al., 2010); peanut (Asif et al., 2011); watermelon (Huang et al., 2011) and pear (Sun et al., 2011) are some examples of transformed cultivars for some fruit species that the transformed tissues were regenerated via organogenesis. Since organogenesis protocols are developed for many different fruit species, it is easier to adapt the regeneration system into genetic transformation methods (Frary & Eck, 2005). However, some risks also are assumed in using this regeneration system. Generally, it is considered that the origin of the new adventitious shoots is based on the involvement of few cells (George et al., 2008), enhancing the risks of chimera development.

Somatic embryogenesis that leads the formation of an embryo from somatic cells is another procedure to regenerate fruit transformants such as banana (Vishnevetsky et al., 2011); papaya (Zhu et al., 2001); grapevine (Nirala et al., 2010) and mango (Chavarri et al., 2010). Regeneration from transformed embryos can be achieved via direct germination or shoot organogenesis and the method is useful for large-scale and rapid propagation of transformants. In grapevine most of the approaches are being performed by using embryogenic cultures from different tissues such as zygotic embryos, leaves, ovaries and anther filaments to provide cells amenable to gene insertion and regeneration (Mezzetti et al., 2002; Dutt et al., 2007; López-Noguera et al., 2009). However, these techniques are highly genotype dependent and for many cultivars they have been difficult to obtain successful results (Dutt et al., 2007). Moreover, it is considered that anther filaments, as commonly employed in grapevine for embryogenic calli, are laborious, cultivar-dependent, depend on availability of immature flowers and may affect strongly the phenotype of the regenerated plantlets (Mezzetti et al., 2002). However, it is really interesting to take into account that regeneration from somatic embryos and secondary somatic embryos are currently assumed that they are derivatives of single cell origin.

In the decade of the 90´s some unsuccessful efforts were reported to transform meristems from micropropagated shoot tips due to high explant mortality and uncontrolled *Agrobacterium* overgrowth after coculture stages (Ye et al., 1994; Druart et al., 1998; Scorza et al., 1995). Mezzetti and co-workers (2002) described in grapevine the development of meristematic bulk tissues, a highly aggregate of meristematic cells produced after three months in increased concentrations of BA (N^6-benzyladenine) and the removal of the apical meristem. After 90 days, under the previous conditions, these highly regenerative tissues produced easily adventitious shoots and can be transformed by *Agrobacterium*, being able to regenerate several transgenic lines. Other interesting approach was the genetic transformation of shoot apical meristems, previously subjected to a dark growth stage after wounding for transformation. Authors reported that 1% of shoot tips produced stable transgenic lines after weeks (Dutt et al., 2007). Ismail and co-workers (2011) transformed successfully banana apical meristems via microprojectile bombardment and regenerated 80.3% percent of the transformed meristematic tissues.

4.1 The chimeric question: Are my transgenic plants genetically uniform?

This is one of the most exciting questions that plant biotechnology researchers ask to themselves after all the long extensive, intensive and difficult labour needed to transform most of the fruit species. Some of the transformed regenerants can be chimeras, a mix of transformed and non transformed cells in the tissues, in other words, non genetically uniform organisms (Hanke et al., 2007). Recently, Petri and collaborators (2011) described that most of the known and efficient regeneration methods for fruit trees are based on organogenesis, where new adventitious shoot formation is originated from a determined number of cells. So, it comes hard to detect non chimeric and stable transgenic lines without using a selectable marker gene. Very recently, different authors using marker free technology as alternative systems or with genetically programmed marker excision reported the appearance of chimeric transformants in apple, strawberry, lime, citrus or plum (Domínguez et al., 2004; Schaart et al., 2004; Ballester et al., 2007; Malnoy et al., 2011; Petri et al., 2011).

Strawberry is highly sensitive to kanamycin selection, and it was described that selection of transgenic regenerants in these sensitive tissues can be associated with chimeric shoots containing transgenic and non-transgenic sections (Husaini, 2010). It was observed that increasing antibiotic concentration gradually avoid chimerisms in strawberry (Mathews et al., 1998; Husaini et al., 2010). Even under this strictly methodology some authors pointed out the inactivation events on the selection agent must be performed through the transformed cells, so, non transformed cells can develop and grown (Petri & Burgos, 2005; Wang, 2011). A useful methodology was also proposed for the quick and low-cost identification of chimeras by Faize and collaborators (2010) in tobacco and in apricot based in quantitative real-time PCR even in early developmental stages, and also let to monitor their dissociation.

5. Future perspectives and concluding remarks

Currently, most of the fruit genetic transformation protocols integrated the new genes randomly and in unpredictable copy numbers influencing negatively its expression. Also public concerns and reduced market acceptance of transgenic crops have promoted the

development of alternative marker free technologies in fruit species. For those reasons development of protocols to obtain transgenic fruits without marker genes and the use of the own plant DNA resources, such as "cisgenic" fruit plants, are the big challenges. ZFNs have also been succesfuly used to drive the replacement of native DNA sequences with foreign DNA molecules and to mediate the integration of the targeted transgene into native genome sequences.

Most of the fruit transgenic plants are generated by introducing just one single new character (gene of interest), however, some authors proposed that multigene transfer technology (MGT) needs to be developed to obtain new traits related at the same time. The combination of multiple traits can be a highly interesting approach as it could be applied to achieve resistance to several biotic or abiotic stresses and traits related to fruit quality such as large fruit size, high coloration of the fruit epidermis, increase flesh firmness to improve ripening without the need of several rounds of introgressive backcrossing.

The future of fruit genetic transformation is required of genotype-independent protocols, accuracy molecular tools to drive the T-DNA insertion and its expression, and efficiency and highly-yield selection and regeneration in vitro culture methodology. But *Agrobacterium*-mediated transformation procedure is a high non linear complex biological process, and its complexity can be understood with the composition of many different and interacting elements governed by non-deterministic rules and influenced by external factors. In this sense, the emergent technology dedicated to meta-analysis can be really useful to increase our understanding of fruit genetic transformation, making possible to identify relationships among several factors and extracting useful information generating understable and reusable knowledge (Gago et al., 2011; Gallego et al., 2011; Perez-Pineiro et al., 2012) Under these perspectives, modeling any fruit transformation procedure (*Agrobacterium*-mediated, biolistics, electroporation etc.) including the genetic engineering, *in vitro* plant tissue culture and regeneration stages will be improved for the next years.

6. Acknowledgements

The authors wish to thank to Dr. Mariana Landín and to Dr. María José Clemente for critical reading of this work. This work was supported by Regional Government of Xunta de Galicia: exp.2007/097 and PGIDIT02BTF30102PR. This study was also partially supported by # TBAG- 209T030 from TUBITAK—The Scientific and Technical Research Council of Turkey. PG thanks to Minister of Education of Spain for funding the sabbatical year at Faculty of Science, University of Utrecht, Netherlands.

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3

Green Way of Biomedicine – How to Force Plants to Produce New Important Proteins

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1. Introduction

Recombinant proteins can be expressed in transformed cell cultures of bacteria, yeasts, molds, mammals, plants, insects, or via transgenic plants and animals. Numerous factors influence quality, functionality, yield and protein production rate, so the choice of appropriate expression system is of primary importance. During last few years, plants have become an increasingly promising and attractive platform for recombinant protein production (Basaran & Rodriguez–Cerezo, 2008). Progress in recombinant DNA technology, plant transformation and *in vitro* regeneration techniques are major reasons why plants have emerged as efficient expression systems. Plant expression systems offer significant advantages over the other expression systems (Table 1). First of all, plants have a higher eukaryote protein synthesis pathway very similar to animal cells with only minor differences in protein glycosylation. Therefore, plant biosynthesis pathway ensures correct structure even in the case of highly complex proteins. In contrast to plants, bacteria are not able to carry out most of posttranslational modifications essential for eukaryotic proteins activity. There is no risk of contamination of recombinant proteins with human or animal pathogens (HIV, hepatitis viruses, prions), bacteria endotoxins or oncogenic DNA sequences (Sharma & Sharma, 2009).

Other advantages of the plant–based expression systems include: high scalability (in the case of field cultivation), low production cost of biomass (agriculture), in some cases low upstream costs (edible vaccines, purification process can be omitted), and what is most important - the ability to produce target proteins with desired structures and biological functions (Boehm, 2007). Recombinant proteins expressed in plants can be accumulated to a high level in seed endosperm, fruit or storage organs (e.g. tubers, roots) or secreted directly to the culture media. Because plant culture media contain no exogenous proteins, the recovery of recombinant proteins from a medium is expected to be much simpler and less expensive than the recovery from homogenized biomass (Cox et al., 2009).

Features	Transgenic plants	Plants viruses	Yeast	Bacteria	Mammalian cell culture	Transgenic animals
Cost/storage	Cheap	Cheap	Cheap	Cheap	Expensive	Expensive
Distribution	Easy	Easy	Feasible	Feasible	Difficult	Difficult
Gene size	Not limited	Limited	Unknown	Unknown	Limited	Limited
Glycosylation	Correct	Correct	Incorrect	Absent	Correct	Correct
Production costs	Low	Low	Medium	Medium	High	High
Production scale	Worldwide	Worldwide	Limited	Limited	Limited	Limited
Propagation	Easy	Feasible	Easy	Easy	Hard	Feasible
Protein folding accuracy	High	High	Medium	Low	High	High
Protein homogeneity	High	Medium	Medium	Low	Medium	Low
Protein yield	High	Very high	High	Medium	Medium-high	High
Safety	High	High	Unknown	Low	Medium	High
Scale up costs	Low	Low	High	High	High	High
Therapeutic risk	Unknown	Unknown	Unknown	Yes	Yes	Yes
Time required	Medium	Low	Medium	Low	High	High

Table 1. Comparison of features of recombinant protein production in existing systems (according to Fischer and Emans 2004; worked out /modified on the basis of Demain and Vaishnav 2009).

The usage of aquatic plants e.g. *Lemnaceae* seems to be a good solution. For example Rival et al. (2008) made studies on obtaining aprotinin from *Spirodela oligorrhiza* (duckweed). Their experiments show that significant amounts of recombinant aprotinin can be produced using *Spirodela* as a plant host. Whereas Cox and co-workers (2009) expressed human monoclonal antibody (mAbs) in *Lemna minor*. The micro-alga *Chlamydomonas reinhardtii* has recently been shown as a promising platform for foreign protein production (Muto et al., 2009). This photosynthetic single-celled plant possesses several interesting features in comparison to the majority of plants as it has a rapid doubling time (ca. 10 h); its homogenous culture is easily scaled up; it has a rapid sexual cycle (ca. 2 weeks) with stable and viable haploids. All these attributes make the time of petting a final product on a large-scale much shorter in comparison to higher plants (months or years). Growth in containment bioreactors allows to control conditions of farming as well as reduces the risk of contamination and loss of algae due to pathogens. It is worth mentioning that all three genomes of *C. reinhardtii* have been fully sequenced affording strong foundation for targeted genetic manipulation (Specht et al., 2010).

Feasible storage of recombinant proteins in desiccated plant parts excludes the requirement for its immediate isolation and lowers the risk of the loss of biological function during prolonged freezing of preparations. For example, antibodies or vaccines expressed in cereal seeds remain stable at ambient temperatures for years (Stoger et al., 2002). Until recently, low accumulation levels have been the major bottleneck for plant-made recombinant protein production. However, several breakthroughs have been done during past few years allowing for high accumulation levels. Mainly through chloroplast, vacuole, ER lumen transient expression, coupled with subcellular targeting and protein fusions (Sharma and Sharma, 2009). Viral transfection and agroinfiltration are promising alternative strategies ensuring increase in yields and speeding up the development of an expression platform (Gleba et al., 2005). On the other hand, plant–based expression systems are different from the mammalian host pattern of glycosylation. The occurrence has raised concerns regarding the potential immunogenicity of plant-specific complex N-glycans (α 1,3-fucose and β 1,2-xylose residue), which are present in the heavy chains of plant-derived antibodies (Gomord and Faye 2004). The above mentioned residues have been confirmed not only to induce immune response but also to make foreign proteins undergo a conformational change making them different from the native ones which results in decrease in their biological activity. However, some achievements in humanized glycosylation or removal of enzymatic pathway generating immunogenic residues on glycoproteins have been reported. Recently it has been shown that glycoengineered moss (*Physcomitrella patens*) can synthesize proteins carrying a humanized glycosylation pattern (Decker and Reski, 2008). A few years ago *Physcomitrella patens* platform was developed and commercialized as a contained tissue culture system for recombinant protein production in photo-bioreactors [Biotech GmbH (© greenovation)]. *P. patens* has some characteristic features which make it a suitable system for foreign protein production. Firstly, it grows rapidly under photoautotrophic conditions and secondly the moss protonema can release the desired protein into the medium. The moss remains productive in the system for a period of six months, in contrast to animal cell cultures (20 days) (Decker and Reski, 2008).

Other approaches to overcome undesirable glycosylation accommodate export of foreign proteins into subcellular compartments: ER lumen, where glycosylation characteristic of plants does not take place; cytosol, where glycosylation process is not found; or recombinant protein expression export into plastids (proteins do not undergo glycosylation there). According to several studies ER targeting gives higher yield of biologically active protein than cytosol targeting (referred by Boehm, 2007).

Potential disadvantages of transgenic plants include possible contamination with pesticides, herbicides, and toxic plant metabolites. Proteolytic degradation, post/transcriptional gene silencing, position effect and transgenic recombination are other obstacles affecting stability or expression level of transgenic plants (Basaran and Rodriguez–Cerezo, 2008).

The public concern about health and environmental risk associated with transgenic plants is being considered at different levels: inherent risk of transgene leakage into non-transgene crops or naturally occurring wild type species (transgene escape through pollen); transgene spread by seed or fruit dispersal; horizontal gene transfer by asexual means; unintentional exposure of non-targeted organisms (e.g. birds, insects or soil microorganism); elicitation of allergic response/reaction in people (Basaran and Rodriguez–Cerezo, 2008). There are some strategies which allow to alleviate these problems including usage of closed culture

facilities, such as greenhouses, hydroponic or suspension bioreactors or plastid transformation (as plastids are inherited through maternal tissues in most species and the pollen does not contain chloroplasts, hence the transgene cannot be transferred) (Basaran and Rodriguez–Cerezo, 2008).

From economical point of view, plants can be an alternative system for recombinant protein production (especially biopharmaceutical) in comparison to those exploiting mammalian or bacterial cell cultures. In this system a desired foreign protein can be produced at 2-10% of the cost of microbial fermentation system and at 0.1% of mammalian cell cultures, although it depends on the protein of interest, product field and a plant used. In general, the recombinant protein yields up to 1.5% of the total soluble protein (TSP). For example the content of antibodies does not exceed 0.35%-2% and vaccines- 0.01-0.4% of TSP (Basaran and Rodriguez–Cerezo, 2008). On the other hand, phytase from *A. niger* was obtained at the level 14% of the total tobacco soluble protein, but hirudin from *H. medicinalis* at 1% of canola seed weight and GUS from *E. coli* was produced in corn at 0.7% of TSP (Demain and Vaishnav 2009).

2. Expression strategies

Gene expression and synthesis of proteins is a complex multi-step process. For efficient expression of recombinant proteins in plants, it is essential to optimize every step of the process for the plant machinery. This includes the methods of plant transformation, the choice of a transgene promoter, improvement of transcript stability and the efficiency of its translation. After translation, the protein needs to be accumulated in plant cells or effectively secreted.

2.1 Stable nuclear transformation

The first step in plant transformation consists in the entrance of a desired genomic sequence into a plant cell. Stable nuclear transformation is caused by integration of the recombinant DNA in the nuclear genome. DNA can be transferred into the nuclear genome by either direct (e.g. biolistics) or indirect (e.g. *Agrobacterium*) methods, it depends on the plant species and the type of tissue (Thanavala et al., 2006).

In the stable nuclear transformation whole plants can be regenerated, eventually producing a seed stock or a plant tissue maintained in an aseptic culture. The advantage of this system is that the transgene is heritable, permitting the establishment of a seed stock for future use. Establishment and characterization of stable transgenic lines can be costly and time consuming. Large numbers of transgenic lines need to be screened and analyzed before a single optimal line can be selected for protein production (Ling et al., 2010). Other disadvantages are gene silencing and position effects.

Nuclear transformation has been employed and extensively studied in many plant species, however, it generally results in low expression of soluble foreign proteins (Yap & Smith, 2010).

Recombinant proteins can be targeted to different subcellular compartments in plant cells, such as cytostol, apoplast, endoplasmic reticulum, vacuole or chloroplast.

2.2 Transplastomics

Using particle bombardment or polyethylene glycol (PEG) treatment, DNA can be targeted into the chloroplast genome (Yusibov & Rabindran, 2008). Each cell contains a large number of plastids, ~100 chloroplasts per cell, and each of them contains about 100 genomes. Transplastomic lines vs. nuclear ones have significantly greater yield of foreign proteins (1-20% TSP) due to the high number of copies of the chloroplast genome and they offer major advantage in terms of transgene containment, as chloroplast genomes are predominantly maternally inherited, limiting out-crossing of the transgenic pollen. No transcriptional or post-transcriptional silencing effects have been observed in chloroplast transformation (Yap & Smith, 2010). Chloroplasts also support operon based on transgene allowing the expression of multiple proteins from a single transcript. There are two disadvantages of the chloroplast system – first: chloroplast transformation is not a standard procedure and is thus far limited to a relatively small number of crops, second: lack of some of the eukaryotic machinery for post-translational modification (Yusibov & Rabindran, 2008).

Gene integration in the plastid genome occurs by means of two homologous recombinant events mediated by a bacterial-like Rec A based system. Vectors include two 'targeting' regions flanking the selectable marker gene and a cloning site for insertion of the gene of interest. The targeting regions are between 1 and 2 kb in size and are plastid DNA sequences able to direct transgenic integration into plastome intergenic regions. Integration by homologues recombination in a preselected genome region enables insertion of only transgenic sequences and prevents uncontrollable variation in the expression of transgene. Strong promoters for plastid encoded polymerase (PEP) from the *rrn* operon and the *psbA* gene are used. Rregulatory sequences at the 5'-terminus must include a 5' untranslated region (UTR). Plastid transgene expression can be also achieved with the use of the T7 phage promoter and nuclear-encoded, plastid imported T7 RNA polymerase. In some cases protein accumulation was enhanced by translational fusion of a plastid gene N-terminal sequence with the protein of interest by including sequences downstream of the ATG start codon (downstream box) in the transgene 5'cassette that resulted in improved translation and/or protein stability. The 3'cassettes derived from 3'UTR of plastid genes generally function as inefficient terminators of transcription, but are important for plastid transcripts stability (Cardi et al., 2010).

2.3 Optimization of expression level

Increasing the transcription rate of stably transformed gene sequences is the most direct and efficient approach to increase protein expression. This is mainly achieved with the use of a strong constitutive or inducible promoter. Constitutive promoters directly drive the expression in all plant tissues and are independent of the production host developmental stage. The best known and most widely used constitutive promoter in plant biotechnology is derived from *Cauliflower Mosaic Virus (CAMV35S)*. It is more effective in dicots than monocots. Alternative constitutive promoters frequently used in plant cell transformation are the *ubiquitin* promoter, histone *H2B* promoter and the (*ocs*)*3mas* promoter (Hellwig et al., 2004). The *ubiquitin* promoter, isolated from a variety of plants including maize, *Arabidopsis*, potato, sunflower, tobacco and rice, has been frequently used to express biopharmaceuticals in plant cells. The (*ocs*)*3mas* promoter, constructed from octopine synthase (*osc*) and

mannopine synthetase (*mas*) agrobacterial promoter sequences , was used for the expression of *Hepatitis B* antigen in a soybean cell culture (Smith et al., 2002). Other constitutive promoters used for expression of foreign genes in transgenic plants include: tobacco cryptic constitutive promoter (Menassa et al., 2004), Mac promoter which is a hybrid mannopine synthetase promoter and cauliflower mosaic virus 35S promoter enhancer region (Dai et al., 2000), rice actin promoter (Huang et al., 2006), banana actin promoter (Herman et al., 2001), C1 promoter of cotton leaf curl Multan virus (Xie et al., 2003), nopaline synthase promoter (Stefanov et al., 1991).

Inducible promoters allow external regulation by chemical stimuli such as alcohol, steroids, salts, sucrose or environmental factors such as temperature, light, oxidative stress and wounding. Inducible expression is advantageous as this allows protein production to be separated from cell growth. The use of chemical inducible promoters in combination with the chemical responsive transcription factor can further restrict the target transgene expression to specific organs, tissues or even cell types (Zuo & Chua, 2000). The examples of inducible promoters and synthetic transcription activators are: the rice α-amylase 3D (*RAmy3D*) promoter, which is induced by sucrose starvation; the oxidative stress-inducible a peroxidase (*SWAPA2*); an estradiol-inducible chimeric XVE transcription activator and dexamethasone-inducible pOp/4v transcription activator (Xu et al., 2011), hydroxyl-3-methylglutaryl CoA reductase 2 promoter, which is inducible by mechanical stress (Cramer et al., 1996).

Tissue-specific promoters control gene expression in a tissue or in a developmental stage specific way. The transgen driven by such a promoter is expressed in a specific tissue leaving all the other tissues unaffected. It helps to force transgene expression in storage organs like seeds, tubers or fruits. Several of such promoters were tested: tuber specific patatin promoter (Jefferson et al., 1990), fruit specific E8 promoter (Jiang et al., 2007), arcelin promoter (Osborn et al., 1988), maize globulin 1 promoter (Rusell & Fromm, 1997), 7s globulin promoter (Fogher, 2000), rice glutelin promoter (Wu et al., 1988) and soybean P-conglycinin subunit promoter (Chen et al., 1986).

The optimization of promoters activity can be further improved by means of engineered DNA elements - enhancers, activators or repressors located up or downstream of the core promoter. Enhancers are shown to increase gene expression when placed proximally to the promoter, they bind activator proteins and promote RNA polymerase II placement at the TATA box. Transcription is also enhanced with flanking the transgene by nuclear scaffold/matrix attachment regions (S/MARs) important for structural organization of eukaryotic chromatin (Halweg et al., 2005).

The translational efficiency of a transgene is determined by proper processing (capping, splicing, polyadenylation, nuclear export) and mRNA stability. The 5′ and 3′ untranslated region (UTR) of the plant mRNA plays crucial roles in its processing (Cowen et al., 2007). The 5′-UTR is very important for 5′ capping and enables translation initiation, the 3′-UTR is indispensable in transcript polyadenylation which in turn influences the stability of mRNA (Chan and Yu, 1998). These untranslated sequences can be manipulated for the optimization of protein expression.

As the protein is synthesized, it undergoes several modifications before final delivery to its cellular destination. These modifications include enzyme involving glycosylation,

phosphorylation, methylation, ADP-ribosylation, oxidation, acylation, proteolytic cleavage and non-enzymatic modifications like deamidation, glycation, racemization and spontaneous changes in protein conformation (Gomord & Faye, 2004). Post-translational proteolysis can be effectively minimized by targeting the foreign proteins to sub-cellular compartments such as the endoplasmic reticulum (ER). Proteolysis is more likely to occur in the apoplast and cytosol. ER retrieval signal (e.g. KDEL, HDEL) retains the expressed protein in the ER lumen and has been used to improve foreign protein stability. The ER contains many molecular chaperones facilitating nascent proteins folding or assembly and it is regarded as an ideal compartment for accumulating many classes of foreign proteins (Nuttal et al., 2002).

Other strategies for proteolytic degradation reduction are: co-expression of recombinant protein and protease inhibitors, co-expression of protein co-factors or subunits, knockout mutations in the genes encoding specific proteolytic enzymes.

The recent advent of highly efficient transient expression systems has completely changed the concept and revolutionized plant made pharmaceutical research. Transient transformation implies the expression of foreign DNA which cannot be inherited but is still transcribed within the host cell in a transient manner. Transient gene expression provides a rapid alternative to the time consuming stable transformation methods. This approach uses the plant hosts - *Arabidopsis thaliana*, *Nicotiana tabacum*, *Nicotiana benthamina*, *Lactuca sativa*. Transient expression of recombinant proteins in plants is performed by the use of engineered plant viruses and/or *Agrobacterium* mediated DNA transfer (agroinfection/agroinfiltration). Fast and high level expression is the major advantage of the transient expression systems. Full expression of a gene of interest in agroinjected leaves may be achieved in 3-4 days after infiltration with *Agrobacteria*. This system is simple and experimental procedures do not require expensive supplies and equipment. Leaves of greenhouse grown plants are infiltrated using a syringe without a needle, vacuum infiltration or the wound and agrospray inoculation method (Medrano et al., 2009). Supplementation of the infiltration media with Silwet L-77, Tween-20, or Triton X-100 improves the efficiency of transformation. In the transient expression system one can use different virus types: Tobamoviruses, Potexviruses, Potyviruses, Bromoviruses, Comoviruses and Gemniviruses. Prolific production of any given protein using the plant virus approach results from the fact that a virus can infect a plant systemically by moving in its symplast. The *Agrobacterium* based method involves the injection or vacuum infiltration of whole plants or their parts with a suspension of bacteria harboring the construct of interest (Gómez et al., 2009). *Agrobacterium* delivered plant viral vectors use the RNA polymerase II mediated nuclear export route including 5′ end capping, splicing and 3′ end formation. Plant RNA viruses replicate in the cytoplasm and are not adapted to nuclear splicing machinery which recognizes and removes cryptic introns from viral RNA leading to its degradation. The *Agrobacterium* delivered so called 'first generation' TMV and PVX vectors have low production capacity and require coinjection of a plasmid encoding gene silencing suppressor such as tombusvirus p19 or potyvirus P1/HC-Pro (Komarova et al., 2010).

A major breakthrough in viral expression strategies was facilitated by the recent advent of deconstructed virus vectors. Originally reported for the TMV-based magnICON system developed by ICON Genetics GmbH merges advantages of *Agrobacterium*-mediated DNA

delivery and upgraded TMV based vectors where putative cryptic splice sites were removed and multiple plant introns inserted. Thus the basic idea is to amplify the foreign gene delivered by *Agrobacterium tumefaciens* to multiple areas of the plant allowing the virus to replicate and spread. In this process, bacteria start initial infection delivering the T-DNA encoded viral replicon to the nuclei of a large number of cells. Then, the transcripts are transported to the cytoplasm where the viral RNA amplification renders high yields of the desired protein (Gleba et al., 2005).

In conclusion, the two major strategies for expressing proteins in whole plants are transient expression with viral vectors and stable transformation where transgenes are targeted to either the nuclear or chloroplast genome. Stable transformation offers the advantage that protein production is scalable to large field production methods. However, this can be offset by low expression levels and the long time required for creating expressor lines stable across multiple generations. Today's most promising direction in the referred field is emerging from synthesis of genetically engineered agrobacteria, viruses and plants in one precisely tailored system where synthetic and system biology meet each other.

3. Overview of plant-derived medical recombinant proteins

3.1 Plant derived antibodies

Over the last few decades, medical biotechnology has led to major advances in diagnosis and therapy. At present most diseases can be detected at an early stage, and their treatment is more specific and potent. Biotechnological methods allow to identify the molecular mechanisms of a disease facilitating development of new diagnostic techniques and speeding up development of novel molecularly targeted drugs. One of the therapeutic strategies in the treatment of many diseases is the use of antibodies. Antibodies are a class of topographically homologous multidomain glycoproteins produced by the immune system and they display a remarkably diverse range of binding specificities. Since the first production of monoclonal antibodies by Kohler and Milstein in 1975 they have become an extremely important and valuable tool in medicine (Yarmush et al., 2003).

Constantly increasing demand for new and safe monoclonal antibodies forces development of high-performance production systems. Since the first report on antibody production in *N. tabacum* plants (Hiatt et al., 1989), plantibodies have been produced in various plant systems (Table 2).

Product	Disease/Pathogen	Plant	Promoter	Expression level	Organ	Reference
Human anti-rabies monoclonal antibody	Rabies	Tobacco	CaMV 35S promoter with duplicated upstream B domains	0.07% TSP	Leaves	Ko et al., 2003
Human monoclonal antibody	Hepatitis-B virus	Tobacco	CaMV 35S promoter with the omega sequence	0.2-0.6% TSP	Suspension cell cultures	Yano et al., 2004

Product	Disease/Pathogen	Plant	Promoter	Expression level	Organ	Reference
Full-length monoclonal mouse IgG1 (MGR48)	-	Tobacco	CaMV 35S, TR2' promotor	30–60 mg of fresh weight	Leaves	Stevens et al., 2000
Human-derived, monoclonal antibody	Anthrax	Tobacco	CaMV35S	-	Leaves	Hull et al., 2005
Anti-*Salmonella enterica* single-chain variable fragment (scFv) antibody	*Salmonella enterica*	Tobacco	EntCUP4, single and double-enhancer versions CaMV 35S	41.7 ug of scFv/g leaf tissue	Leaves	Makvandi-Nejad et al., 2005
Human anti-rabies virus monoclonal antibody	Rabies	Tobacco	CaMV 35S with duplicated upstream B domains (Ca2p), (Pin2p)	30 ug/g of cell dry weight	Cell suspension culture	Girard et al., 2006
BoNT antidotes	Botulinum neurotoxins (BoNTs)	Tobacco	CaMV35S	20-40 mg/kg	Leaves	Almquist et al., 2006
TheraCIM recombinant humanized antibody	Skin cancer	Tobacco	CaMV35S/ Agroinfiltration	1.2 mg/kg of leaves	Leaves	Rodríguez et al., 2005
Human monoclonal antibody 2F5	Activity against HIV-1	Tobacco	duplicated CaMV35S	2.9 ug/g fresh weight	Cell suspension	Sack et al., 2007
mAb BR55-2 (IgG2a)	Carcinomas, particularly breast and colorectal cancers	Tobacco	CaMV 35S	30 mg kg of fresh leaves	Leaves	Brodzik et al., 2006
LO-BM2, a therapeutic IgG antibody	Possible tool to prevent graft rejection	Tobacco	En2pPMA4	99 ug in the cell extract of a 100-ml culture, 12.81 ug. medium-associated antibody	Leaf and cell suspension culture	De Muynck et al., 2009
Monoclonal antibody H10 (mAb H10)	Tumour-associated antigen tenascin-C (TNC)	Tobacco	CaMV 35S with omega translational enhancer sequence from (TMV)	50–100 mg/kg fresh plant tissue	Leaves	Villani et al., 2009

Table 2. Plant derived antibodies.

3.2 Plant derived vaccines

Plants can be used to produce inexpensive and highly immunogenic vaccines. It is connected with heterologous expression of antigens. These are further purified to formulate injectable vaccine or are applied as edible vaccines. The latter idea is a very attractive alternative to injection, mostly because of low costs (no need for protein purification) and comfort of administration. However, there are some essential conditions which have to be satisfied. First of all, plants used for oral vaccine production should produce edible parts that can be consumed uncooked (antigens are often heat sensitive). Besides, these parts should be rich in protein because the antigen protein will constitute only a minor portion (0.01-0.4%) of TSP. Seeds seems to be a good choice because of antigen extended stability, even at ambient storage temperatures. As many studies revealed, vaccine antigens present in plant tissues were resistant to digestion in the gastrointestinal tract, on the other hand during this process they were release to elicite both mucosal and systemic immune responses (Sharma and Sood, 2011). Current progress in the matter is summarized in Table 3.

Vaccines	Disease	Plant	Promoter	Expression level	Organ	References
Subunit HAC1 and HAI-05	H1N1, H5N1 influenza	Tobacco	Not reported	HAC1 90 mg/ and HAI-05 50 mg/kg of plant biomass	Leaves	Shoji et al., 2011
VP1-capsid protein	FMDV (Foot and Mouth Disease Virus)	Tobacco	*psbA*	51% TSP	Leaves (Chloroplasts)	Lentz et al., 2010
TonB protein	Immunization against *Helicobacter infections*	*A. thaliana*	CaMV 35S	0.05% TSP	Entirely plant	Kalbina et al., 2010
Mycobacterial antigens Ag85B	Vaccine against tuberculosis	Tobacco	CaMV 35S	4 % TSP	Leaves	Floss et al., 2010
Surface protein 4/5 (PyMSP4/5)	Plasmodium	Tobacco	MagnICON® viral vector system	10% TSP or 1–2 mg/g of fresh weight	Leaves	Webster et al. 2009
TetC and PTX S1 antigens	DTP (diphtheria–tetanus–pertussis)	Tobacco Daucus carrota	CaMV 35S	Not reported	Leaves; Hypocotyls	Brodzik et al., 2009
HN glycoprotein	Newcastle Disease Virus (NDV)	Tobacco	P-RbcS	3µg of HN protein per mg of total leaf protein	Leaves	Gómeza et al., 2009
HBsAg	HBV (hepatitis B virus)	*Lactuca sativa*	CaMV 35S	Not reported	Shoots	Marcondes & Hansen, 2008

Vaccines	Disease	Plant	Promoter	Expression level	Organ	References
HPV-16 L1 protein	HPV (Human Papilloma Virus)	Tobacco	*psbA* promoter	24 % TSP	Leaves	Fernández-San Millán et al. 2008
16 E7 oncoprotein	HPV	Tomato; Potato	CaMV 35S	0.5 % of the cell protein-potato	Potato protoplast; leaves	Briza et al., 2007
G protein	Rabies virus	*Daucus carotta*	CaMV 35S	0.2–1.4% (TSP)	Carrot roots	Royas-Anaya et al., 2009
Capsid protein VP6	Rotavirus	Potato	P2	0.01%	Leaves, tubers	Yu & Landgridge, 2003

Table 3. Plant derived vaccines.

3.3 Plant derived biopharmaceuticals

Plants can be used to produce inexpensive biopharmaceuticals (Table 4).

Biopharmaceutical	Potential application	Plant	Promoter	Expression level	References
IL-10	Inflammatory and autoimmune diseases	Rice seeds	Glutelin B-1 promoter	2 mg pure IL-10	Fujiwara et al., 2010
Human transfferin	Receptor-mediated endocytosis pathway	Rice seeds	Glutelin 1 G-1 promoter	1% seed dry weight	Zhang et al., 2010
Glutamic acid decarboxylase (GAD65)	Autoimmune T1DM	Tobacco leaves	CaMV 35S	2.2% total soluble protein	Avesani et al., 2010
hGH, somatotropin	Growth hormone-treatment of dwarfism	*N. benthamiana*	CaMV 35S	60 mg per kilogram offresh tissue; 7%	Rabindran et. al., 2009;
Human erythropoietin (EPO)	Anemia, Renal failure	*N. tabacum*	CaMV 35S	0.05% of total soluble protein	Conley et al., 2009
Human serum albumin (HSA)	Deficiences	Tobacco, potato	Prrn; B33	11.1%TSP% (tobacco chloroplasts); 0.2%TSP (potato tuber)	Faran et al., 2002
Human lactoferrin (hLF)	Anti-inflammatory and immuno-modulation effects	Potato	Tandem promoter: P2& CaMV 35S	0.10% TSP	Chong et al., 2000
Enkephalins	Painkiller	Cress, *A. thaliana*	----------	0.10% seed protein	Daniell et al., 2001
Staphylokinase	Thrombolytic factor	*A. thaliana*	CaMV 35S	not reported	Wiktorek-Smagur et al., 2011

Table 4. Plant derived biopharmaceuticals.

3.4 Nutraceutical and non-pharmaceutical plant derived proteins

Antimicrobial nutraceutics, such as human lactoferrin and lysozymes, have now been successfully produced in several crops (Stefanova et al., 2008), and are commercially available (Table 5). Cobento Biotechnology (Denmark) has recently received approval for its *Arabidopsis* derived human intrinsic factor which is used against vitamin B12 deficiency and it is now commercially available as Coban. Other nutraceutical products are listed in Table 5.

Trypsin is a proteolytic enzyme that is used in a variety of commercial applications, including processing of some biopharmaceuticals (Sharma & Sharma, 2009). In 2004, the first plant derived recombinant protein product (bovine sequence trypsin; trade name – trypZean) developed in corn plant (Prodi Gene, USA) was commercialized. Avidin, a glycoprotein found in avian, reptilian and amphibian egg white, is primarily used as a diagnostic reagent. The plant optimized avidin coding sequence was expressed in corn and now it is available on the market. β-glucuronidase, peroxidase, laccase, cellulase, aprotinin were also developed and marketed (Basaran & Rodrigez-Cerezo, 2008).

Spider silk proteins, elastin and collagen, have been expressed in transgenic plants (Scheller et al., 2004). These are promising biomaterials for regenerative medicine.

Product name	Company name	Plant	Commercial name	Source
Avidin	Prodigene	Corn	Avidin	Obembe at al., 2011
β-glucuoronidase	Prodigene	Corn	GUS	Obembe at al., 2011
Trypsin	Prodigene	Corn	TrypZean	Obembe at al., 2011
Recombinant human lactoferrin	Meristem Therapeutic, Ventria Bioscience	Corn, Rice	Lacromin	http://www.meristemthera-peutics.com
Recombinant human lysozyme	Ventria Bioscience	Rice	Lysobac	http://www.ventria.com
Aprotinin	Prodigene	Corn, Tobacco	AproliZean	Obembe at al., 2011
Recombinant lipase	Meristem Therapeutic	Corn	Merispase	http://www.meristemthera peutics.com
Recombinant human intrinsic factor	Cobento Biotech AS	*Arabidopsis*	Coban	http://www.cobento.dk
Human growth factors	ORF Genetics	Barley	ISOkine™	http://www.orfgenetics.com
Food additive for shrimps	SemBioSys	Safflower	Immuno-spherte	http://www.sembiosys.com

Table 5. Transgenic plants based on products commercially available in the market.

4. Recombinant protein purification

4.1 Affinity chromatography

Isolation and purification of a biologically active protein from a crude lysate is often difficult and costly. Simple, cheap and more efficient strategies of its purification on the laboratory and industrial scale are thus on great demand. One of the numerous approaches in this field is an affinity tags system easily applicable for recombinant protein purification by affinity chromatography. The term 'affinity chromatography' was introduced in 1968 by Pedro Cuatrecasas, Meir Wilchek, and Christian B. Anfinsen (1968). Now it is the method of choice (Kabir et al., 2010). Affinity chromatography is based on specific interaction between two molecules in order to isolate the protein of interest from a pool of unwanted proteins and other contaminants. For this purpose a fusion protein is created. A short fragment of DNA can be ligated to the 5 ' or 3' - terminus of the target gene. This peptide or protein coding sequence (so called tag), which is translated in frame with protein of interest exhibits a characteristic property, strong and selective binding to the molecules immobilized on the solid matrices (Fong et al., 2010). Purification process is effective and simple. During passage of the cell extract containing the fusion protein and contaminants through an appropriate column the tagged protein is retained, while all the others migrate freely through the column (Fig. 1).

In the next step, the bound protein is eluted by a change in buffer composition /parameters (i.e. competitors, chelators, pH, ionic strength or temperature). Affinity tags are divided into three main classes according to their properties and the properties of molecules that interact with them: 1) tags, binding to small molecule ligands linked to a solid support (i.e. HIS-tag), 2) protein tags binding to a macromolecular partner immobilized on chromatography support (i.e. CBP-tag), 3) the protein-binding partner attached to the resin in an antibody which recognizes a specific peptide epitope in a recombinant protein (i.e. FLAG-tag) (Lichty et al., 2005, Arnau et al., 2006, Waugh et al., 2005). To date large number of gene fusion tags has been described, the most commonly used ones are presented in Table 6.

Tag	Comments	References
His-tag	Purification by interaction between immobilized metal ions and chelating amino acids	Valdez-Ortiz et al., 2005, Vaquero et al., 2002
FLAG	Purification based on binding the FLAG peptide to antibodies	Brodzik et al., 2009, Zhou and Li., 2005
Strep-tag II	Strong specific interaction between Streptag and strep-Tactin (streptavidin derivate) immobilised on resin	Witte et al., 2004

Table 6. Some examples of affinity tags commonly used for protein purification.

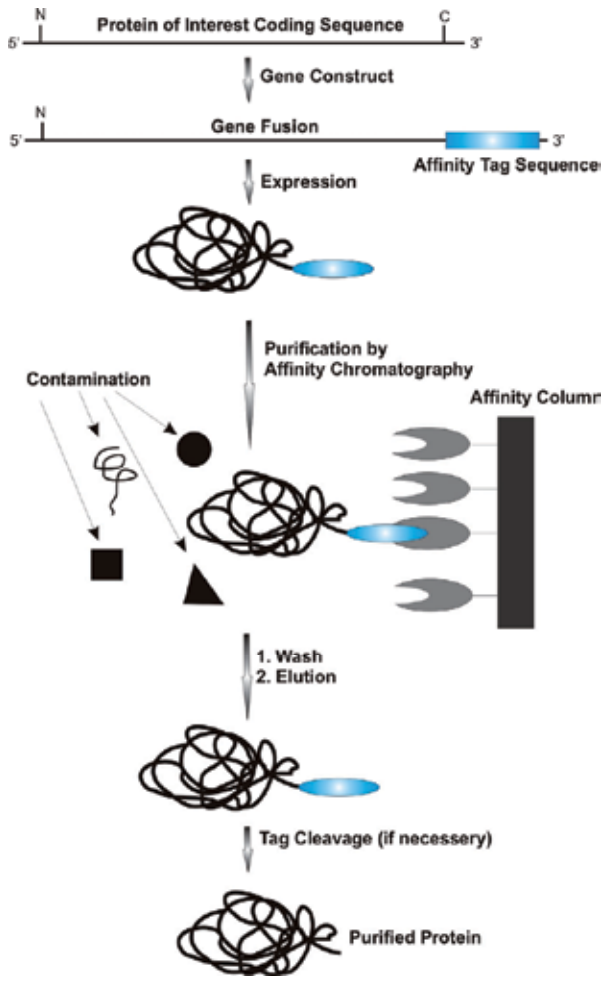

Fig. 1. Schematic representation of the recombinant protein purification process by affinity chromatography (Hearn & Acosta, 2001, modified).

4.2 Elastin-like polypeptides in recombinant protein purification

While affinity chromatography is used for purification of a broad spectrum of recombinant proteins it is not free from drawbacks. The main limitations associated with the use of this method are: 1) high cost of chromatography packing materials, 2) volume-limited sample throughput, 3) dilution of the protein product in elution buffer, 4) additional concentration step may cause loss in protein yield (Chow et al., 2008). Taking into account the above, there is a need to introduce new alternative methods for purification of recombinant proteins.

One of the possible solutions is application of non-chromatographic purification tags. Elimination of resins allows us to reduce some of the aforementioned problems.

Elastin-like polypeptides (ELP), artificial polymers containing Val-Pro-Gly-Xaa-Gly pentapeptide repeats, are an example of such tags. Such repeats occur naturally in the

hydrophobic domain of human tropoelastin (soluble precursor of elastin) and they play an important role in the process of elastin formation (Mithieux & Weiss 2005, Valiaev et al., 2008). Xaa (so called guest residue) in the ELP sequence can contain any amino acid except for proline (Meyer & Chilkoti, 1999). Occurrence of proline at these positions eliminates distinctive and very useful properties of these polymers (Trabbic-Carlson et al., 2004). Literature classification of ELP is based on the type and number of amino acids present in the guest residue positions (Meyer & Chilkoti 2004).

Elastin-like polypeptides belong to one of the three classes of thermosensitive biopolymers (Mackay and Chilkoti, 2008) whose properties are changed under the influence of moderate temperature differences. Aqueous solutions of ELP exhibit lower critical solution temperature (LCST) which causes that the above phase transition temperature (T_t) ELP pass from soluble to an insoluble form (Ge et al., 2006) in a narrow temperature range (~ 2 ° C) (Ge and Filipe, 2006). This is a reversible process called coacervation. In solutions with temperature below T_t , free polymer chains remain in a disordered soluble form. The opposite occurs in solutions with temperatures above T_t, when the polymer chains have more ordered structure (called β-helix), stabilized by hydrophobic interactions (Rodriguez-Cabello et al., 2007) that increase association of polymer chains (Serrano et al., 2007). This process is reversible. The fact that ELP –protein fusions are prone to reversible transition is of great importance (Kim et al., 2004). The process of ELP-tagged protein purification involves increasing ionic strength and/or temperature of the cell lysate to induce ELP-fusion protein aggregation (Fig. 2). Next sample centrifugation/filtration separates the ELP fusion protein from contaminants. After resolubilization of an ELP fusion, another centrifugation/filtration removes denatured and aggregated biomolecules. This process called Inverse Transition Cycling (ITC) can be repeated to achieve the required purity of the product (Floss, Schallau et al., 2010).

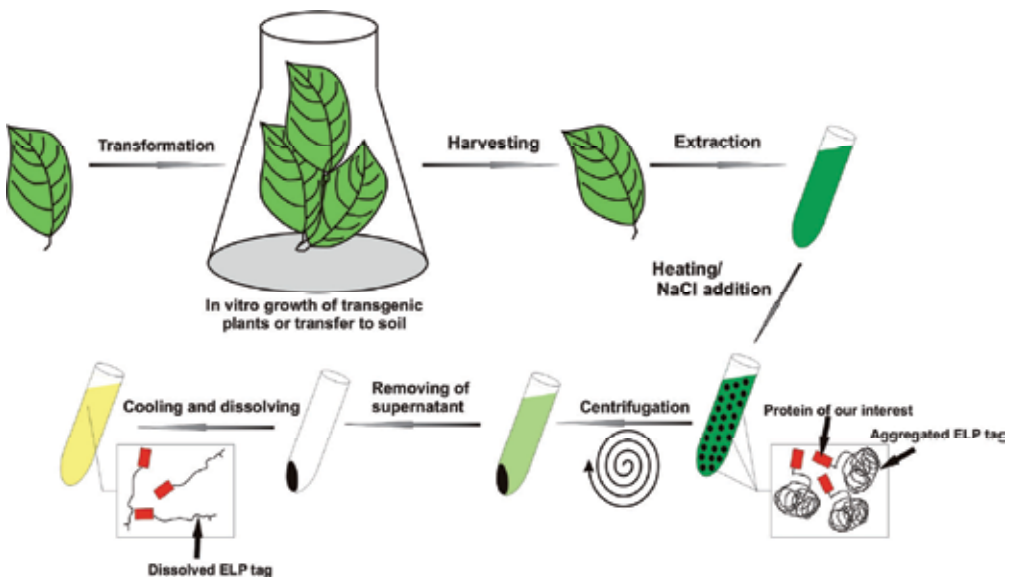

Fig. 2. Purification of ELPylated target proteins from plants using ITC (Floss et al., 2010 modified.

Purification of proteins using elastin-like polypeptides has several advantages over the traditional chromatographic methods: 1) purification of proteins with ELP tags by ITC appears to be universal for soluble recombinant proteins, 2) chromatography beads are not required, which significantly reduces the costs, 3) final concentration step is not required (Chow et al., 2008).

4.3 Application of ELP to the process of production and purification of recombinant proteins in transgenic plants

Scheller and co-workers (2004) achieved efficient and stable expression of spider's silk-ELP fusion protein in the ER of transgenic tobacco and potato. Application of ITC allowed them to obtain 80mg pure recombinant protein from 1kg tobacco leaf material. Purified biopolymer was tested as a potential component used for the cultivation of anchorage-dependent CHO-K1 cells and human chondrocytes. The most common coating substances such as collagen, fibronectin and laminin are derived from animal sources, so there is a risk of contamination of cell cultures by viruses or prions which is essentially undesirable in the case of medical applications. What is more, production of this fusion protein in plants is less costly. Lin and associates (2006) obtained active soluble glycoprotein 130 which seems to be potent drug in Crohn's disease, rheumatoid arthritis and colon cancer therapy. This work a presents creation and expression of mini-gp130-ELP. A fusion protein containing Ig-like domain and cytokine binding module of gp 130 fused to 100 repeats of ELP was expressed in tobacco leaves (ER retention). Inverse transition cycling (ITC) purification resulted in 141 μg of active mini-gp130-ELP per 1g of leaf fresh weight. Floss and co-workers (2010) demonstrated the ability of genetically engineered tobacco to produce mycobacterial antigens Ag85B and ESAT-6 as the vaccine against tuberculosis. In this work Ag85B-ELP and ESAT-6-ELP (TBAg) fusions were created, purified by inverse transition cycling and tested on animals. Production of this TBAg-ELP fusion proteins reached 4% of the tobacco leaf total soluble proteins (TSP) for the best producer plants. Further testing of the vaccine showed mycobacterium-specific immune response with no side effects in an animal model. What is more, this study also confirmed that ELP had no immunomodulating activity. Joensuu and co-workers (2009) demonstrated ELP application in production of antibodies for Foot-and-mouth disease virus (FMDV) therapy. Single chain variable antibody fragment (scFv) recognizing FMDV coat protein VP1 was expressed in transgenic tobacco plants. To recover the fusion protein in the active form the plants, ITC was performed. Finally, the authors demonstrated that scFv expressed in plants were able to bind FMDV.

It has been shown for spider silk proteins (Scheller et al., 2004), murine interleukin-4, human interleukin-10 (Patel et al., 2007) and anti-HIV type 1 antibodies (Floss et al., 2008, Floss et al., 2009) that the ELP fusion significantly enhances accumulation of recombinant proteins produced in plants. So far the mechanism of that phenomenon is not known.

5. Status of plant-derived biopharmaceuticals in clinical development

At present some non-pharmaceutical products from plants are on the market (Basaran and Rodriguez-Cerezo, 2008). Although no plant made pharmaceutical (PMP) has been commercialized as a human drug, several PMPs are at the late stage of development and some have already received regulatory approval, including a vaccine and several nutraceuticals (Table,7, 8, 9).

Antibodies	Target	Plant	Clinical trial status	Company	Source
DoxoRx	Side-effects of cancer therapy	Tobacco	Phase I	Planet Biotechnology	http://www.planet biotechnology.com
RhinoRX	Common cold	Tobacco	Phase I	Planet Biotechnology	http://www.planet biotechnology.com
IgG (ICAM1)	Common cold	Tobacco	Phase I	Planet Biotechnology	http://www.planet biotechnology.com
CaroRX	Dental caries	Tobacco	EU approved as medical advice	Planet Biotechnology,	http://www.planet biotechnology.com

Table 7. Plant derived antibodies in clinical phages of development.

Antigen or vaccine	Disease	Plant	Clinical trial status	Company	Source
Hepatitis B antigen	Hepatitis B	Lettuce	Phase I	Thomas Jefferson University	Streatfield, 2006
Hepatitis B antigen	Hepatitis B	Potato	Phase II	Arizona State University	Streatfield, 2006
Fusion proteins	Rabies	Spinach	Phase I	Thomas Jefferson University	http://www.labome.org
Heat labile toxin B subunit of *E.coli*	Diarrhea	Potato	Phase I	ProdiGene	Tacket, 2005
Capsid protein Norwalk virus	Diarrhea	Potato	Phase I	Arizona State University	Khalsa et al., 2004
Vibrio cholerae	Cholera	Potato	Phase I	Arizona State University	Tacket, 2005
HN protein of Newcastle disease virus	Newcastle disease (Poultry)	Tobacco	USDA Approved	Dow Agro Sciences	http://www.dowagro.com
Viral vaccine mixture	Diseases of horses, dogs	Tobacco	Phase I	Dow Agro Sciences	http://www.dowagro.com
Poultry vaccine	Coccidiosis infection	Canola	Phase II	Guardian Bioscence	Basaran & Rodrigez-Cerezo, 2008
Gastroenteritis virus (TGFV) capsid protein	Piglet gastroenteritis	Maize	Phase I	ProdiGene	Basaran & Rodrigez-Cerezo, 2008
H5N1 vaccine candidate	H5N1 pandemic influenza	Tobacco	Phase I	Medicago	http://www.medicago.com

Table 8. Plant derived vaccines in clinical phages of development.

Therapeutic humans protein	Disease	Plant	Clinical trial status	Company	Source
α-Galactosidase	Fabry disease	Tobacco	Phase I	Planet Biotechnology	http://www.planet biotechnology.com
Lactoferon	Hepatitis C	Duckweed	Phase II	Biolex	http://www.biolex.com
Fibrinolytic drug	Blood clot	Duckweed	Phase I	Biolex	http://www.biolex.com
Human glucocerebrosidase	Gaucher's disease	Carrot	Waiting USDA's approval	Prostalix Biotherapeutic	http.//www.prostalix.com
Insulin	Diabetes	Safflower	Phase III	SemBioSys	http.//www.sembiosysys.com
Apolipoprotein	Cardio vascular	Safflower	Phase I	SemBioSys	http.//www.sembiosysys.com

Table 9. Plant derived pharmaceuticals in clinical phages of development.

In 2006 the world's first plant made vaccine candidate for Newcastle disease in chickens, produced in a suspension cultured tobacco cell line by Dow Agro Science, was registered and approved by the US Department of Agriculture (USDA) – the final authority for veterinary vaccines. In addition, two plant made pharmaceuticals are moving through Phase II and Phase III human clinical trials. Biolex's product candidate, Locteron®, is in Phase IIb clinical testing for the treatment of chronic hepatitis CA. This company uses two genera, *Lemna* and *Spirodela*, as a platform for production of their biopharmaceuticals. The positive outcome of Phase III trials of Protalix's glucocerebrosidase (UPLYSO®) for the treatment of Gaucher's disease which is now waiting for USDA's approval is another positive example. The successful completion of Phase III trial that concerned SemBioSys insulin bioequivalent of the commercial standard represents an important landmark in the plant made pharmaceuticals scenario and, most likely, in the next few years recombinant human insulin produced in safflower will become commercially available for diabetic people.

Medicago Inc. of Canada was invited to the sixth WHO meeting about evaluation of pandemic influenza prototype vaccines in clinical trials. One of the purposes of this meeting was to make recommendations on research activities that will contribute to the development of effective pandemic vaccines. Medicago has recently reported positive results from a Phase I human clinical trial with its H5N1 avian influenza vaccine candidate (a VLP based vaccine produced with a transient expression system). The vaccine was found to be safe, well tolerated and it also induced a solid immune response. Based on these results, Medicago will process with Phase II clinical trial with the first plant made influenza vaccine (Franconi et al., 2010). These examples will pave the way to easy public acceptance of transgenic plants as new production platforms for human therapeuticals.

6. Concluding remarks

Biopharming is still a relatively new field in plant science but in the coming years it may become the premier expression system for a wide variety of new biopharmaceuticals. The use of plants as factories for the synthesis of therapeutic protein molecules will undoubtedly develop. Since the first development of a genetically modified plant in 1984, numerous comprehensive review articles have been published demonstrating the tremendous potential of plants for pharmaceutical production. As it has been clearly shown plants are no

longer considered only in terms of diet or beauty. The proteins targeted for biopharmaceutical technology form three broad categories: antibodies, vaccines, and other therapeutics. Plant bioreactors represent an attractive alternative for their synthesis requiring the lowest capital investment of all tested production systems. The events of heterologous proteins in planta production were rapidly followed with development/improvement of significant technologies (e.g. DNA delivery systems, selection methods). At present a number of promoters with tissue-specific activity or sub-cellular targeting sites that offer protein stability are known and many are still under intense study. Obviously, the construction of a transgenic plant synthesizing a functional therapeutic is a multidisciplinary process and the society of biotechnologists takes a keen interest in its success. However, over the past years various plant expression platforms have been tested and it is evident that further development and improvement are needed for more effective molecular farming. Apart from continuously increasing transgene yields efforts will need to ensure that plant-derived biopharmaceuticals would meet the same safety and efficacy standards as products of non-plant origin. There is no doubt that sooner or later the scientific limitations of molecular farming will be overcome, especially when numerous therapeutics and plant platforms are developed by many laboratories and companies. Thus, this is the regulatory requirements and public acceptance which are the greatest challenge of modern plant biotechnology. Of course, molecular farming raises less objection than technologies using genetically modified animals, but still the existing or proposed regulations remain based on public fears rather than on scientific facts.

In conclusion, "the molecular farming industry" means a natural advance in drug production technology. The dynamics of optimization and improvement of plant expression platforms illustrates its potential and tremendous scientific background. The possible success in this field will have to face the question of public acceptance. Thus, the scientists should send the clear massage to the public opinion that molecular farming is a strictly controlled technology that has strong benefits. And that probably will be more difficult than the construction of functional bioreactor itself.

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4

Molecular Breeding of Grasses by Transgenic Approaches for Biofuel Production

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1. Introduction

Since the Industrial Revolution, fossil fuels have been consumed in massive amounts. However, little fossil fuels are estimated to remain, and the number of new oil fields in the world continues to decline. Thus, the future supply of fossil fuels will be tight and will not continue to support our levels of consumption for long. In addition, rapid consumption of fossil fuels for energy production continues to elevate the atmospheric carbon dioxide concentration, which was 280 ppm before the Industrial Revolution and is 380 ppm today. The emissions, including carbon dioxide, derived from the combustion of fossil fuels act as greenhouse gases and cause the serious problem of global warming.

In this context, society must develop beyond the consumption of our limited fossil fuels. Plant biomass, which is organic matter derived from the photosynthetic fixation of atmospheric carbon dioxide, holds much promise as a future energy source. The use of plant biomass for energy is called carbon neutral, because the combustion of plant biomass only releases carbon dioxide that was originally fixed from the atmosphere by plants. Because a sustainable society based on carbon-neutral energy must be constructed to preserve the global environment, we must develop related practical technology as soon as possible.

However, recent hikes in the global price of grain, caused by various political and economic events, has made us consider shifting toward using non-grain plant biomass to improve both energy and food-supply security. Thus, research into the production of biofuel from lignocellulose, which constitutes the bodies of woody and grass plant species, is a major field of study in modern plant science.

Needless to say, this research includes biotechnological approaches. In particular, genetic engineering is expected to be a key technology in herbaceous plant breeding, because most of the bioenergy crop species are not yet domesticated well. This chapter focuses on recent progress in the molecular breeding of herbaceous plants.

2. Herbaceous perennials for dedicated lignocellulosic biofuel crops

High biomass yields are indispensable to successful cultivate energy crops that can compete with and replace fossil fuels (Karpenstein-Machan, 2001; Lewandowski et al., 2003). Considering food-supply security, net energy yield, and environmental protection,

large and fast-growing non-food grass species are among the most promising candidates for lignocellulose production. Perennial species are more attractive than annuals because they have minimal input requirements, commonly yield more aboveground biomass in the form of stems and leaves, and their well-developed root systems can serve as carbon sinks.

Name (Scientific name)[a]	Photosystem	Yield (t DM ha^{-1} yr^{-1})[b]	Location	Citation
Switchgrass (*Panicum virgatum* L.)	C4	15.9‡	U.S.A. (Oklahoma)	Aravindhakshan et al. (2010)
Sugarcane (*Saccharum* spp. hybrids)	C4	41.3[c]	Japan (South western islands)	Terajima et al. (2010)
Miscanthus species (*Miscanthus* × *giganteus*)	C4	28.7†	Central Italy (Pisa)	Angelini et al. (2009)
		12.4‡	U.S.A. (Oklahoma)	Aravindhakshan et al. (2010)
Erianthus species (*Erianthus arundinaceus* Retz.)	C4	58.4	Japan (Northern Kanto region)	Ando et al. (2011)
Giant reed (*Arundo donax* L.)	C3	37.7†	Central Italy (Pisa)	Angelini et al. (2009)

[a] Actual scientific names of experimental cultivars or lines used in the study cited are given for *Erianthus* species, *Miscanthus* species, and sugarcane.
[b] Values followed by the same obelisk were obtained by the same study.
[c] An average value calculated with the data from three different locations.

Table 1. Summary information for perennial grasses described in this review (modified and updated from Lewandowski et al., 2003)

C4 perennial grasses show promise as lignocellulosic biofuel crops because their highly efficient C4 photosynthesis often yields more biomass than C3 grasses (Jakob et al., 2009). There are many available candidate perennial grasses, which differ in their potential productivity, chemical and physical biomass properties, environmental demands and crop management requirements (Lewandowski et al., 2003). This review will focus on noteworthy C4 perennial grasses as dedicated lignocellulosic biofuel crops, including switchgrass (*Panicum virgatum* L.), *Miscanthus* species, *Erianthus* species, and sugarcane (*Saccharum* species). In addition, the high biomass C3 grass species giant reed (*Arundo donax* L.), which has a photosynthetic capacity comparable to or higher than that of C4 grasses (Angelini et al., 2009; Rossa et al., 1998), will be discussed as well. These perennial grasses are summarized in Table 1 and will be briefly described in sections 2.1 through 2.3.

2.1 Switchgrass

Switchgrass (*Panicum virgatum* L.) is an outcrossing perennial warm-season forage grass originally from the prairies of North America. In the 1990's, the United States Department of Energy sponsored a 10-year research project to evaluate and develop this grass as a dedicated herbaceous lignocellulosic energy crop because of its potential for high fuel

yields, environmental enhancement, and ability to grow well on marginal cropland without heavy fertilizing or intensive management (Bouton, 2008; McLaughlin & Kszos, 2005; Wright & Turhollow, 2010).

Switchgrasses are divided into two ecotypes: lowland and upland (Bouton, 2008; Lewandowski et al., 2003). The lowland ecotypes are tall and robust plants adapted to wetter sites; they mature later and have longer growth periods and higher biomass yields than upland ecotypes. Although somatic chromosome numbers vary in switchgrass (2n from 2x = 18 to 12x = 108) (Hopkins et al., 1996), the lowland ecotypes are predominantly tetraploid (2n = 4x = 36) (Bouton, 2008; Lewandowski et al., 2003). The upland ecotypes have shorter and fine-stemmed morphology, and are adapted to drier habitats. They are commonly octoploid (2n = 8x = 72), or occasionally tetraploid (2n = 4x = 36) (Bouton, 2008) or hexaploid (2n = 6x = 54) (Lewandowski et al., 2003). Irrespective of ecotype, switchgrasses with the same ploidy level can be intercrossed (Lewandowski et al., 2003).

2.2 *Saccharum* complex

The term "Saccharum complex" was first coined by Mukherjee (1957) and originally encompassed four closely-related interfertile genera, *Saccharum*, *Erianthus*, *Sclerostachya*, and *Narenga*. Based on species richness and the geographic distributions of endemic species, India was considered the center of maximum variation of the *Saccharum* complex (Mukherjee, 1957). Eventually, the genus *Miscanthus* was added to the *Saccharum* complex, because it was thought to be involved in the origin of *Saccharum* (Daniels et al., 1975, as cited in Alwala et al., 2006; and Amalraj & Balasundaram, 2006).

Modern sugarcane varieties are mostly derived from interspecific hybridization within the genus *Saccharum* (Amalraj & Balasundaram, 2006), and intergeneric hybridization between *Saccharum* and other genera in the *Saccharum* complex is thought to be the primary gene pool for sugarcane breeding (Cheavegatti-Gianotto et al., 2011). Thus, the *Saccharum* complex can also be considered as the primary gene pool for breeding non-domesticated *Miscanthus* and *Erianthus* as well.

In sections 2.2.1 through 2.2.3, we will address individual genera within the *Saccharum* complex. However, a comprehensive molecular breeding system with intercrossing across the complex should ultimately be undertaken in the development of novel hybrid biofuel crops.

2.2.1 Sugarcane (*Saccharum* species)

Sugarcane is a tall perennial C4 grass that is cultivated in tropical and subtropical regions of the world. Notably, this grass stores high concentrations of sucrose in the stem. Approximately, 65 to 70% of global sugar production in the form of sucrose is derived from sugarcane (FAO, 2003). The potential of sugarcane as an important energy crop was argued because of the advent of large-scale sugarcane-based ethanol production in Brazil (Tew & Cobill, 2008).

Sugarcane belongs to the genus *Saccharum*. Although six polyploid species are recognized within *Saccharum* (Table 2), modern cultivars for sugar production are mostly derived from interspecific hybridization between *S. officinarum* (2n = 8x = 80) and *S. spontaneum* (2n from 5x = 40 to 16x = 128) and are thus complex polyploids with variable chromosome numbers

(2n approx. from 100 to 120) (Henry, 2010; Piperidis et al., 2010). Of the other four species, *S. robustum*, *S. barberi*, and *S. sinense* have also provided minor contributions to the breeding of some modern sugarcane cultivars (Cheavegatti-Gianotto et al., 2011).

Species	Chromosome number (2n)	Genomic contribution to modern interspecific hybrid cultivars and elite lines (%)[a]	Note
S. barberi	From 111 to 120	-	Semi-sweet Indian cane
S. edule	From 60 to 80	-	Cultivated for edible inflorescence
S. officinarum	80	From 70 to 80	Domesticated sweet cane
S. robustum	60 or 80	-	Putative ancestor of *S. officinarum*
S. sinense	From 81 to 124	-	Semi-sweet Chinese cane
S. spontaneum	From 40 to 128	From 10 to 23	Wild cane found throughout Asia

[a] Values are the proportions of total chromosome complement reported by Piperidis et al. (2010) where the proportion of chromosomes derived from interspecific exchanges between *S. officinarum* and *S. spontaneum* are shown to be 8-13%.

Table 2. Species of *Saccharum* and their characteristics (modified from Henry, 2010)

The breeding of sugarcane as a dedicated biomass crop called "energy cane" can be categorized into three strategies with different objectives: the “sugar model”, the “sugar-and-fiber model”, and the “fiber-only model”. The fiber yield of energy cane is important because of its use for electricity generation, cellulosic ethanol production, and so forth; fiber is considered a by-product in the sugar and sugar-and-fiber models or the main product in the fiber-only model (Tew & Cobill, 2008). In the sugar model, improved sugar yield and sugar content are the main foci, so traditional sugarcane cultivars can be used. In the sugar-and-fiber and fiber-only models, Type I and Type II energy canes, respectively, would be used. Tew & Cobill (2008) defined Type I and Type II energy canes as follows:

- Type I energy cane is bred and cultivated to maximize both its sugar and fiber content.
- Type II energy cane is bred and cultivated primarily or solely for its fiber content.

Recently, Japanese breeders succeeded in developing a high-quality Type I energy cane cultivar ‘KY01-2044’ with 1.5 times the total biomass yield and 1.3 times the total sugar yield than the major Japanese sugar-producing cultivar (Asia Biomass Office, 2010; Terajima et al., 2010). The new cultivar allowed the establishment of an experimental system for simultaneous production of ethanol and sucrose from total sugar with residual fiber as a heat source. In addition to ethanol production, this system is designed to produce an amount of sucrose comparable to conventional sugar production systems.

2.2.2 *Miscanthus* species

Miscanthus is a genus of C4 perennial rhizomatous grasses widely distributed in Asia and the Pacific Islands. *Miscanthus* was thought to consist of 17 species divided into four sections (Deuter, 2000). However, recent taxonomic analyses using molecular markers revealed that the genus can be reduced to approximately 11-12 species. The species *M. sinensis* ssp. *condensatus* is sometimes recognized at specific rank as *M. condensatus* (Clifton-Brown et al., 2008) (Table 3).

Species	Chromosome number and ploidy level[a]
M. floridulus (Labill.) Warb.	2n = 2x = 38
M. intermedius (Honda) Honda	2n = 6x = 114
M. longiberbis Nakai	-
M. lutarioparius	-
M. oligostachyus Stapf.	2n = 2x = 38
M. paniculatus (B. S. Sun) Renvoize & S. L. Chen	-
M. sacchariflorus (Maxim.) Hack.	2n = 2x = 38 (in China) 2n = 4x = 76 (in Japan)
M. sinensis Anderss.	2n = 2x = 38
M. sinensis ssp. condensatus (Hackel) T. Koyama	2n = 2x = 38
M. tinctorius (Steud.) Hack.	2n = 2x = 38
M. transmorrisonensis Hayata	2n = 2x = 38
M. × *giganteus* Greef & Deuter ex Hodkinson and Renvoize (Hybrid species between *M. sacchariflorus* and *M. sinensis*)	2n = 3x = 57

[a] Major cytotypes from Deuter (2000) are shown.

Table 3. Species in the genus *Miscanthus* and their chromosome numbers

The basic chromosome number of *Miscanthus* species is 19, and polyploids and aneuploids are observed (Deuter, 2000). Of the species in Table 3, *M. sacchariflorus*, *M. sinensis*, *M. sinensis* ssp. *condensatus*, *M. floridulus*, and *M.* × *giganteus* are of interest for biomass production (Deuter, 2000). In particular, the triploid hybrid species *M.* × *giganteus* shows superior characteristics, such as high biomass yield, and is thus thought to be the most practical *Miscanthus* species for bioenergy production, especially in Europe and North America (Lewandowski et al., 2000; Pyter et al., 2007). The existence of this promising hybrid will further encourage interspecific hybridization within *Miscanthus,* because the genus has a lot of genetic diversity within and between species. In addition, the frost tolerance, growth at low temperature, and robustness against pests and diseases of *Miscanthus* make it a potential gene pool for developing widely-adaptable stress-tolerant cultivars of sugarcane through intergeneric hybridization (Clifton-Brown et al., 2008).

2.2.3 *Erianthus* species

Erianthus is a tall C4 perennial rhizomatous grass. The genus *Erianthus* was erected by André Michaux in *Flora Boreali-Americana* in 1803 for the New World species *E. saccharoides* Michaux (Tagane et al., 2011). Old World species have distinct morphology from New World species (Grassl, 1972, as cited in Tagane et al., 2011) and are characterized by the

presence of a distinctive luteolin, di-C-glycoside, that is not present in the New World species (Williams et al., 1974). The genus is widely distributed in the Americas (New World species) and in the Mediterranean, India, China, South East Asia, and New Guinea (Old World species) (Amalraj & Balasundaram, 2006).

The seven Old World species comprise the section *Ripidium* (Table 4). The basic chromosome number of these *Erianthus* species is x = 10, the same as *Saccharum officinarum*. Polyploids are also observed, as in other genera in the *Saccharum* complex.

Species	Chromosome number[a]
E. arundinaceus (Retz.) Jesw.	2n = 30, 40, 60
E. bengalense (Retz.)	2n = 20, 30, 40, 60
E. elephantinus Hook. f.	2n = 20
E. hostii Griseb.	2n = 20
E. kanashiroi Ohwi	2n = 60
E. procerus (Roxb.) Raizada	2n = 40
E. ravennae (L.)	2n = 20

[a] Chromosome numbers shown by Amalraj & Balasundaram (2006) are shown.

Table 4. Species in the genus *Erianthus* sect. *Ripidium* and their chromosome numbers

The Old World species are increasingly used in sugarcane breeding because of their high biomass yields, drought tolerance, and resistance to pests and diseases. Two species in particular, *E. arundinaceus* and *E. procerus,* are considered most useful due to their disease resistance, ratooning ability, vigor, and environmental stress tolerance (Tagane et al., 2011). Recently, Ando et al. (2011) reported the wintering ability and high biomass yields of the *E. arundinaceus* clone JW630 grown experimentally in Nasushiobara, Japan, where the monthly mean minimum air temperatures from December to March are below freezing, indicating its potential as breeding stock for cold-tolerant sugarcane and as a dedicated lignocellulosic biofuel crop (Table 1).

2.3 Giant reed

Giant reed (*Arundo donax* L.) is a rhizomatous C3 perennial grass that evolved in Asia but is also considered a native to the Mediterranean region (Lewandowski et al., 2003). The grass is used to make reeds for woodwind musical instruments, in construction, and as a source of cellulose for rayon manufacture and paper pulp production (Perdue, 1958).

Recently, giant reed has gained attention as a source of lignocellulose for bioenergy production because of its high yield and strong pest resistance (Lewandowski et al., 2003). Studies of the giant reed in Central Italy demonstrated biomass production equivalent to or higher than *Miscanthus* × *giganteus* (Angelini et al., 2009) (Table 1). Because giant reed exhibits heavy metal tolerance, it could be used simultaneously for both phytoremediation and bioenergy production (Papazoglou, 2007; Papazoglou et al., 2005).

Giant reed is highly polyploid, with a possible base chromosome number x = 12 [(2n = 10x = 120 - 8 or 2n = 9x = 108 + 4) (Gorenflot et al., 1972) or (2n = 110) (Lewandowski et al., 2003)]. Although isozyme and DNA analyses have revealed genetic diversity in giant reed (Khudamrongsawat et al., 2004; Lewandowski et al., 2003), the plant is thought to produce

no viable seed, owing to aberrant division of the megaspore mother cell (Bhanwra et al., 1982). This behavior suggests that conventional breeding through sexual hybridization cannot be performed.

3. Plant regeneration systems

In most case, effective plant regeneration systems with *in vitro* cell cultures are required to establish reliable transformation systems in plants. In this section, we summarize the regeneration systems of switchgrass, sugarcane, *Miscanthus*, *Erianthus*, and giant reed.

3.1 Switchgrass regeneration systems

Biotechnology research on the molecular breeding of switchgrass began in 1992 at the University of Tennessee with the support of Oak Ridge National Laboratory (Vogel & Jung, 2001). This work yielded the first reported plant regeneration system through embryogenesis and organogenesis (Denchev & Conger, 1994). In the experiments, mature caryopses and young leaf segments were used as explants for callus induction. Then, the influences of the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-trichloropyridine-2-carboxylic acid (picloram) in combination with 6-benzyladenine (BA) were examined on the plant regeneration system. The combination of 2,4-D and BA led to best results in experiments with mature caryopses (Denchev & Conger, 1995). Alexandrova et al. (1996a) increased the efficiency of switchgrass regeneration by using immature inflorescences obtained from node cultures in aseptic conditions; one immature inflorescence can produce hundreds of spikelets of the same genotype in a single Petri dish that can be easily used as explants for callus induction. Most recently, a new medium, LP9, for the production, maintenance, and regeneration of switchgrass callus was reported (Burris et al., 2009). Callus produced on LP9 can be easily propagated, maintains its regenerability for at least six months, and is adaptable to *Agrobacterium*-mediated transformation (Burris et al., 2009).

Embryogenic suspension cultures initiated from embryogenic callus were also established in switchgrass (Gupta & Conger, 1999), and osmotic pre-treatment with 0.3 M each of sorbitol and mannitol was effective for plant regeneration from suspension cultures (Odjakova & Conger, 1999). Another type of culture system that is highly attractive for gene transfer experiments was also established: multiple shoot clumps were induced from intact seedlings with various combinations of 2,4-D and 1-phenyl-3-(1,2,3-thidiazol-5-yl) urea (TDZ) (Gupta & Conger, 1998).

Most of the research mentioned above used the lowland cultivar 'Alamo'. However, because switchgrass is outcrossing and self-infertile, genetic variation exists not only among cultivars but also within a cultivar, indicating that the establishment of a totipotent tissue culture is not highly dependent on the cultivar itself but rather on screening highly regenerable genotypes as in other outcrossing plants, like ryegrasses (Takahashi et al., 2004). Therefore, Odjakova & Conger (1999) and Burris et al. (2009) used specific single genotypes Alamo 2702 and Alamo 2, respectively, in their experiments, and Alexandrova et al. (1996b) developed a micropropagation procedure with node culture for the multiplication of such selected genotypes. Interestingly, the selected line HR8, having high somatic embryogenic capacity, was recently bred using recurrent tissue culture selection to allow easy induction of embryogenic callus from mature seeds (Xu et al., 2011).

3.2 Sugarcane regeneration systems

The first sugarcane regeneration system via callus culture was reported in 1964 (Nickell, 1964, as cited in Lakshmanan, 2006) and was followed by numerous reports resulting from a great deal of research activity. These early reports have been well-reviewed elsewhere (Lakshmanan, 2006; Suprasanna et al., 2008a). Although many papers on plant regeneration from sugarcane calli are still being published, they generally vary only in reporting specific culture conditions, which must be optimized for each cultivar and genotype because of the outcrossing nature of sugarcane. Most callus cultures are supplemented with the auxin 2,4-D, but sometimes other synthetic auxins, such as picloram and 3,6-dichloro-2-methoxybenzoic acid (dicamba) are used, with or without the cytokinins BA, TDZ, or kinetin. Research on sugarcane regeneration systems may be stalled. To spark innovation, here we update the previous reviews (Lakshmanan, 2006; Suprasanna et al., 2008a) by describing innovative reports published since 2007.

In addition to plant hormones, exogenous amino acids may positively or negatively influence somatic embryogenesis in plants. In sugarcane tissue culture, the addition of arginine to culture media was found to significantly induce somatic embryogenesis and to promote plant regeneration (Nieves et al., 2008). Similarly, glycine, arginine, and cysteine positively affected somatic embryogenesis and subsequent plant regeneration; 0.75 mM glycine was the most effective treatment studied (Asad et al., 2009).

The moisture status of *in vitro* cultures influences plant regeneration in sugarcane. Partial desiccation has been reported to enhance plant regeneration from calli (Garcia et al., 2007; Kaur & Gosal, 2009). A similar phenomenon was observed even in irradiated embryogenic cultures (Suprasanna et al., 2008b).

A fundamental but important aspect of sugarcane tissue culture is the selection of the initial explants. In most previous reports, explants were obtained from field-grown plants. However, Garcia et al. (2007) recommended using sugarcane plants grown *in vitro* because they provided a year-round source of physiologically uniform explants and could be prevented from releasing large amounts of phenolic compounds, which can hamper tissue culture (Garcia et al., 2007). Basnayake et al. (2011) recommended using explants obtained from donor plants with good water supplies, especially when working with genotypes that are recalcitrant to tissue culture due to phenolic compounds.

3.3 *Miscanthus* regeneration systems

To the best of our knowledge, the first preliminary regeneration experiments in *Miscanthus* were conducted with immature inflorescences, mature leaves, immature leaves, nodal segments, internodal segments, meristematic regions, and ovules as explants, which were cultured on media containing a wide range of auxin types (Gawel et al., 1987, as cited in Gawel et al., 1990). In these experiments, immature inflorescences were the only explants that produced calli, and only media containing 2,4-D or picloram with no cytokinin produced a regeneration response (Gawel et al., 1987, as cited in Gawel et al., 1990). The research group thus focused on immature inflorescences as explants and published that 9.0 μM of 2,4-D was effective for callus induction and subsequent plant regeneration in *M. sinensis* cultivars 'Gracillimus', 'Variegatus', and 'Zebrinus' (Gawel et al., 1990). In these experiments, callus induction and plant regeneration were not distinguished from one

another; shoot formation occurred during successive culture on the same culture medium as callus induction (Gawel et al., 1990).

Many researchers have focused on the high-biomass species *M.* × *giganteus*. The auxins 2,4-D or 2,4,5-trichlorophenoxyacetic acid in combination with the cytokinin 6-furfurylaminopurine were best for inducing regenerable embryogenic callus from immature inflorescences (Lewandowski & Kahnt, 1992). Although immature inflorescences emit fewer browning substances than shoot tips or leaf primordia (Lewandowski & Kahnt, 1993), browning substances from the explants were detrimental to tissue culture (Lewandowski & Kahnt, 1992). Ascorbic acid, cysteine, and watering the explants were not effective treatments, but liquid culture gave better results because the browning substances did not accumulate around the explants or calli (Lewandowski & Kahnt, 1992).

The effects of different explants, such as shoot apices, leaves and root sections of *in vitro*-propagated plants and leaf and immature inflorescence sections from greenhouse-grown plants, were examined in *M.* × *giganteus*. Shoot apices had the highest percentage of embryogenic callus formation, while immature inflorescence-derived callus had the highest regenerability (Holme & Petersen, 1996). The growth conditions of donor plants influence tissue culture (Creemers-Molenaar et al., 1988), as has been observed in *M.* × *giganteus* (Holme & Petersen, 1996). Leaf explants from *in vitro*-propagated shoots and from greenhouse-grown plants showed differences in embryogenic callus formation; the best results were obtained from leaves of *in vitro*-propagated shoots and older leaves of greenhouse-grown plants (Holme & Petersen, 1996). However, supplying BA to the callus induction medium led to different results: a higher percentage of regenerable shoot-forming callus was formed on shoot apices compared with leaf sections of *in vitro*-grown shoots when 0.4 μM BA was supplied (Petersen, 1997). Also, small immature inflorescences of *M.* × *giganteus*, between 2.5 and 8 mm, were more suitable for embryogenic callus formation than explants from shorter or longer inflorescences, shoot apices or leaf explants, indicating that the size and type of explant influence culture responses (Petersen et al., 1999). Furthermore, different carbon sources, and their sterilization methods, influenced *M.* × *giganteus* tissue culture. Significant differences were reported for carbon sources and their sterilization methods in tissue cultures of various explant-derived calli. Leaf explants were more affected by the carbon sources than were shoot apices or immature inflorescences, and both callus proliferation and plant regeneration were generally improved by the use of filter-sterilized carbon sources (Petersen et al., 1999).

As mentioned above, severe browning is a major problem in *Miscanthus* tissue culture, but supplying proline to callus induction and suspension cultures effectively prevented browning in *M.* × *giganteus* (Holme et al., 1997). Proline is thought to inhibit polyphenol oxidase, which causes enzymatic browning of cultured tissues (Öztürk & Demir, 2002). The addition of proline to callus induction media increased embryogenic callus formation on shoot apices and leaf explants. However, results varied with proline concentration and the basal salts in the medium. Specifically, 12.5 to 50 mM proline in callus induction media with Murashige & Skoog (MS) salts (Murashige & Skoog, 1962) increased embryogenic callus formation more than media with N6 salts (Chu et al., 1975). The inhibitory effects of proline on tissue browning in *Miscanthus* were confirmed by another group (Głowacka et al., 2010). Conversely, adding honey instead of sucrose to callus induction media inhibited browning of cultured immature inflorescences, and a combination of the honey and banana pulp was best for inducing regenerable callus in *M.* × *giganteus* (Płażek & Dubert, 2009, 2010).

Media component (mg L^{-1} final conc.)	Media		
	HB[a]	MS	N6
Macroelements			
KNO_3	2000	1900	2830
NH_4NO_3	1500	1650	
$(NH_4)_2SO_4$			463
$CaCl_2 \cdot 2H_2O$	200	440	166
$MgSO_4 \cdot 7H_2O$	300	370	185
KH_2PO_4	300	170	400
Microelements			
Na_2-EDTA	37.25	37.3	37.25
$FeSO_4 \cdot 7H_2O$	27.85	27.8	27.85
$MnSO_4 \cdot 4H_2O$	11.25	22.3	4.4
$ZnSO_4 \cdot 7H_2O$	4.3	8.6	1.5
$CuSO_4 \cdot 5H_2O$	0.0125	0.025	
$CoCl_2 \cdot 6H_2O$	0.0125	0.025	
H_3BO_3	3.1	6.2	1.6
KI	0.415	0.83	0.8
Vitamins			
Inositol	100	100	
Nicotinic acid	0.5	0.5	0.5
Pyridoxine HCl	0.5	0.5	0.5
Thiamine HCl	0.4	0.1	1
Another organic			
Glycine	2	2	2

[a] Composition presented in Sun et al. (1999).

Table 5. Composition of HB, MS, and N6 basal media

Recently, studies involving tissue cultures of *M. sinensis* have been reported. Głowacka & Jeżowski (2009a, 2009b) reported culturing anthers of *M. sinensis* and subsequently demonstrated the effects of inflorescence developmental stage on callus induction and plant regeneration (Głowacka et al., 2010). Mature seeds can also serve as explants for tissue culture in *M. sinensis*. Mature seeds of 18 accessions from various sites in Japan were subjected to callus induction, and a combination of a relatively high 2,4-D concentration (5 mg L^{-1}) and a relatively low BA concentration (0.01 mg L^{-1}) efficiently induced regenerable calli that could be used to produce transgenic *Miscanthus* plants (Wang et al., 2011). Interestingly, there was a correlation between the average annual air temperature at accession collecting sites and the frequency of embryogenic callus induction; seeds collected from warmer regions formed a higher percentage of embryogenic calli (Wang et al., 2011). Most recently, Zhang et al. (2011) germinated seeds collected in China and compared explants from the epicotyls, young leaves, and radicles. The epicotyl was best for embryogenic callus formation and plant regeneration from the callus. In the experiments, Holley & Baker (HB) medium (Holley & Baker, 1963, as cited in Zhang et al., 2011) was better for callus induction than other common basal media, such as MS medium, N6 medium (Chu et al., 1975), or half strength of the MS medium. Zhang et al. surmised that HB medium would work well for callus induction in other grass species, because the medium had been effective for callus culture of wheat (Sun et al., 1999). The

components of HB medium will be of interest to many researchers, so we compare HB, MS, and N6 media in Table 5. We were unable to obtain the original publication (Holley & Baker, 1963, as reported in Zhang et al., 2011), so we give the composition of HB basal medium presented in Sun et al. (1999).

3.4 *Erianthus* regeneration systems

To date, only one published report concerns the plant regeneration system of *Erianthus* species. Callus induction and plant regeneration from calli was achieved in *E. elephantinus* (Jalaja & Sreenivasan, 1999). Calli were induced from expanding leaves, leaf sheaths, and immature inflorescences as explants on MS basal medium containing 2 mg L^{-1} 2,4-D. As in *Miscanthus* species, tissue browning occurred, especially in explants from leaf sheaths (Jalaja & Sreenivasan, 1999). Unfortunately, detailed data on the frequencies of callus induction and plan regeneration from the callus were not reported because the study focused mainly on somaclonal variation, such as morphology, pollen fertility, and chromosome aberrations, of the regenerants (Jalaja & Sreenivasan, 1999).

3.4.1 Protocol for plant regeneration system of *Erianthus arundinaceus*

We have recently succeeded in establishing a plant regeneration system in *E. arundinaceus*. Here, we present the simplified protocol. All culture media used were based on MS medium containing 3% (w v^{-1}) sucrose, adjusted to pH 5.8, and solidified with 0.25% (w v^{-1}) Gelrite (Wako, Osaka, Japan). Components of the culture media are shown in Table 6.

Media	Components[a]
Plant maintenance medium	0.3% (w v^{-1}) activated charcoal 3%(w v^{-1}) sucrose
Callus induction medium	5 mg L^{-1} 2,4-D 25 mM L-proline 750 mg^{-1} $MgCl_2 \cdot 6H_2O$ 3%(w v^{-1}) sucrose
Multiple shoot formation medium	1 mg L^{-1} BA 3 mg L^{-1} NAA 3%(w v^{-1}) sucrose
Shoot elongation medium	0.3 mg L^{-1} GA 3%(w v^{-1}) sucrose

[a] Additives added to MS basal medium are shown for each medium.

Table 6. Culture media used in our plant regeneration system for *Erianthus arundinaceus*

- Establishment of *in vitro*-grown plants

We obtained *in vitro*-grown plants of the *E. arundinaceus* clone JW630 (Ando et al., 2011). Shoot tips, each containing an apical meristem, of greenhouse-grown plants (approx. 3 cm long) were used as explant sources. After removing the mature outer leaves, the shoot tips were submerged in 70% ethanol for 1 min, surface-sterilized in 10% (v v^{-1}) sodium hypochlorite solution (1% available chlorine) for 20 min, rinsed twice in sterile distilled

water under aseptic conditions, aseptically stripped of more outer leaves, and cultured on plant maintenance medium under long-day conditions [16 h light (80 μmol m^{-2} s^{-1})/8 h dark; 28°C]. The explants were subcultured every two weeks until rooting.

- Callus induction from axillary buds of *in vitro*-grown plants

Axillary buds at the proximal end of each leaf were isolated from *in vitro*-grown plants under a stereomicroscope. The isolated buds were placed on callus induction medium, and cultured under continuous fluorescent light (40 μmol m^{-2} s^{-1}) at 25°C. They were subcultured every two weeks.

- Plant regeneration from calli

For plant regeneration, calli were transferred to the plant maintenance medium and cultured under long-day conditions [16 h light (80 μmol m^{-2} s^{-1})/8 h dark; 28°C].

- Results and tips

As with other C4 plants, such as switchgrass, sugarcane, and *Miscanthus*, explant browning was severe at first. Rooting of the explants took about two months, but once rooted, the *in vitro* plants grew vigorously on the plant maintenance medium (Figure 1A). Calli were easily induced from axillary buds of *in vitro*-grown plants (Figure 1B), and plant regeneration was observed from the calli (Figure 1C). However, calli induced from explants from field-grown plants were rare due to severe bacterial contamination and tissue browning, even when we used apical meristems, immature inflorescences, and young leaf rolls (data not shown). The *in vitro*-grown plants produced multiple shoot clumps when cultured on multiple shoot formation medium (Figure 2A), and the newly formed short shoots maintained their morphological states and could elongate to normal morphology after being transferred to shoot elongation medium containing gibberellic acid (GA) (Figure 2B).

Fig. 1. Plant regeneration system for *Erianthus arundinaceus*. An *in vitro*-grown plant cultured for three months (A), a callus induced from an axially bud of the *in vitro*-grown plants (B), and plant regeneration from calli (C).

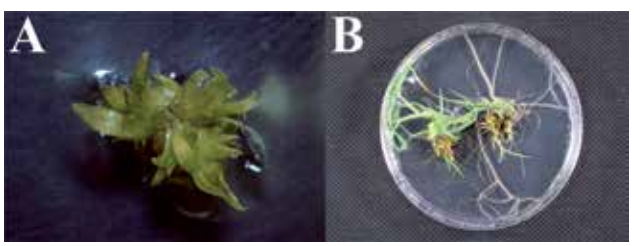

Fig. 2. Multiple shoot formation in *Erianthus arundinaceus*. A multiple shoot clump derived from an *in vitro*-grown plant (A), and elongated shoots from the multiple shoot clumps (B).

3.5 Giant reed regeneration system

To the best of our knowledge, only one scientific article on the regeneration of giant reed has been published so far (Takahashi et al., 2010). In the system, *in vitro* propagation was first optimized for the year-round production of explants (Figure 3).

Fig. 3. An *in vitro*-grown giant reed plant. The photo was taken two months after subculture (adapted from Takahashi et al., 2010).

Calli were induced from axillary buds isolated from the *in vitro*-grown plants. Several combinations of 2,4-D and BA were used for callus induction. They influenced both callus induction frequency and later plant regeneration on hormone-free media because of possible carryover effects of plant hormones in the original callus induction media. Media supplemented with 3 mg L^{-1} 2,4-D appeared to stabilize callus formation and subsequent shoot formation on hormone-free medium (Figure 4).

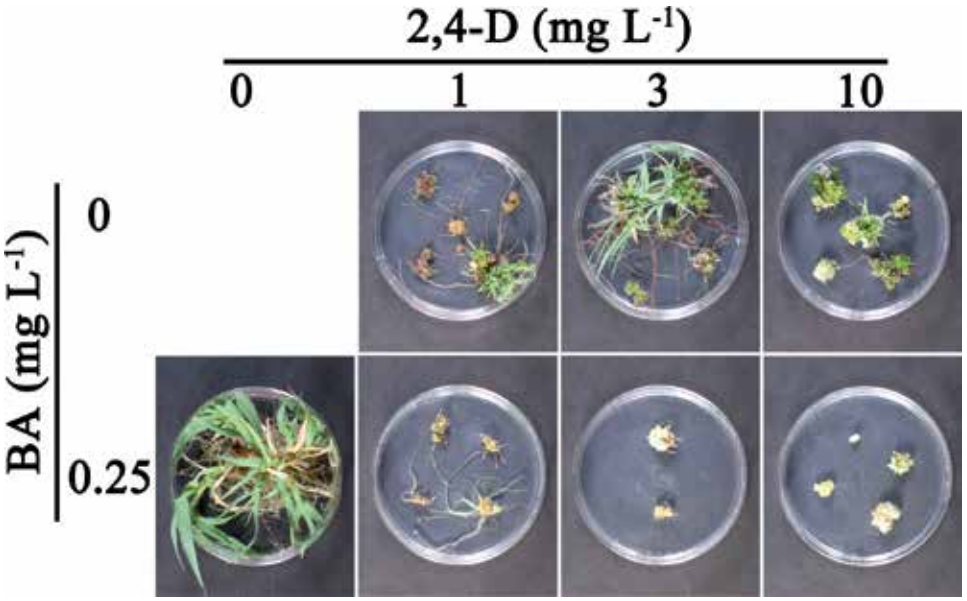

Fig. 4. Effect of original callus induction media on subsequent shoot formation on hormone-free media in giant reed. The concentrations of 2,4-D and BA are those in original callus induction media (modified and adapted from Takahashi et al., 2010).

In total, 11 genotypes were treated under optimized conditions, and data suggest genotypic effects in tissue culture response, although the giant reed is thought to propagate asexually because its seeds are non-viable. Ex-callus induction frequencies and shoot formation frequencies ranged from 82.9 to 100% and from 0 to 37.5%, respectively.

4. Transformation systems

We summarize here several earlier reports on transformation systems in switchgrass, sugarcane, *Miscanthus*, and giant reed. We know of no such reports for *Erianthus*.

4.1 Switchgrass transformation systems

There are some reports on the establishment of transformation systems in switchgrass. Gene transfer methods and names of transgenes used so far are shown in Table 7.

Transgenes[a]	Gene transfer methods	Reference
sgfp, [*bar*]	Gene gun	Richards et al. (2001)
uidA, [*bar*]	*Agrobacterium*	Somleva et al. (2002)
pporRFP, [*hph*]	*Agrobacterium*	Burris et al. (2009)
uidA, *GUSPlus*, [*hph*]	*Agrobacterium*	Xi et al. (2009)
sgfp, [*hph*]	*Agrobacterium*	Li & Qu (2011)
sgfp, *GUSPlus*, [*bar*][b], [*hph*]	*Agrobacterium*	Xu et al. (2011)
uidA, [*bar*], [*hph*], [*nph*]	*Agrobacterium*	Song et al. (2011)

[a] Selectable marker genes in brackets.
[b] Not used as a selectable marker.

Table 7. A list of transgenes and gene transfer methods in used in reported switchgrass transformation systems

The first transgenic switchgrass plants were produced by the gene gun method (Richards et al., 2001). Tungsten particles coated with a plasmid harboring the *sgfp* and *bar* genes were introduced into immature inflorescence-derived embryogenic calli. In total, 2,430 calli were subjected to the gene transfer, and 97 plants eventually showed tolerance, conferred by the *bar* gene, to 0.1% Basta herbicide (Richards et al., 2001). Basta tolerance was also observed in T_1 transgenic progeny resulting from crosses between transgenic and nontransgenic control plants, indicating inheritance of the *bar* gene (Richards et al., 2001).

All subsequent reports on switchgrass transformation have used *Agrobacterium*-mediated transformation (Table 7). The first such report involved sophisticated experiments that examined the effects of various explants of different genotypes for callus induction and of various concentrations of acetosyringone during inoculation and cocultivation on gene transfer efficiency (Somleva et al., 2002). The gene transformation efficiencies ranged from 0 to 97.3%, but the efficiencies were clearly depended on genotype and kind of explant. Improvements in basal media should lead to efficient transformation in switchgrass. Recently, switchgrass Type II calli were induced from newly-developed LP9 medium (see section 3.1) and were subjected to the *Agrobacterium*-mediated transformation with a final transformation efficiency of 4.4% (Burris et al., 2009).

Because *Agrobacterium* is the causal agent of crown gall, interactions between *Agrobacterium* and plant cells during gene transfer often trigger an undesired plant defense mechanism, called hypersensitive cell death, that leads to lower gene transfer efficiency. Thus, highly compatible *Agrobacterium* strains must be selected for plant transformation. Indeed, different strains of *Agrobacterium*-strain varied in their transient transgene expression efficiencies (Chen et al., 2010) and in stable transformation rates in switchgrass (Song et al., 2011; Xi et al., 2009). For stable transformation of switchgrass, the most noteworthy *Agrobacterium* strain so far has been EHA105; two different research groups found it to be more effective than other strains, such as AGL1, GV3101, and LBA4404 (Song et al., 2011; Xi et al., 2009).

Li & Qu (2011) recently developed the most high-through-put system to date, with a stable transformation efficiency of over 90%. Although most previous transformation systems employed the cultivar 'Alamo', the authors a found higher transformation efficiency with the cultivar 'Performer'. Their modifications included infection under vacuum, co-cultivation in desiccation conditions, resting between co-cultivation and selection, and L-proline supplementation in the callus culture and selection media (Li & Qu, 2011). Interestingly, this system also employed *Agrobacterium* strain EHA105, suggesting that it may be highly compatible with 'Performer'. Switchgrass transformation using a EHA105 with the switchgrass selected line HR8, which has high somatic embryogenic capacity (Xu et al., 2011) (see section 3.1), may be a promising avenue for future research.

Transgene inheritance and phenotypic expression in progeny derived from *Agrobacterium*-mediated transformation were observed in some of these reports (Li & Qu, 2011; Somleva et al., 2002; Xi et al., 2009).

4.2 Sugarcane transformation systems

Numerous reports on transformation systems in sugarcane have been previously reviewed (Hotta et al., 2010; Lakshmanan et al., 2005; Suprasanna et al., 2008a). Here, we summarize two key studies published in 2011.

Sophisticated adjustment of culture conditions for several cultivars have resulted in transformation systems widely-adaptable to different genotypes. Basnayake et al. (2011) investigated the effects of 2,4-D levels during callus proliferation, geneticin concentrations during selection, and/or light intensity during regeneration in 16 Australian sugarcane cultivars destined for microprojectile-mediated transformation. This study will be a useful guide for the rapid optimization of key tissue culture variables for efficient genetic transformation of other sugarcane cultivars.

Microprojectile-mediated gene transfer is the most common gene transfer method in sugarcane (Hotta et al., 2010), but the method often causes complex transgene integration that may result in gene silencing. However, minimal expression cassettes lacking vector backbone sequences have been reported to support simple transgene integration in plants. Most recently, a linear minimal expression cassette for the *npt* gene was introduced into embryogenic callus by microprojectile-mediated gene transfer of different amounts of the expression cassette DNA (Kim et al., 2011). Genomic DNA from transgenic sugarcane plants displayed two to 13 *npt* hybridization signals on Southern blots, and the authors observed a trend toward reduced transgene integration complexity and reduced transgene expression levels when lower amounts of the minimal expression cassette were used per shot. This

suggests that backbone free minimal expression cassettes might be efficiently integrated and expressed in sugarcane and other plant species.

4.3 *Miscanthus* transformation systems

The first transgenic *Miscanthus* plants produced via particle bombardment were reported in *Miscanthus sacchariflorus* (Zili et al., 2004, as cited in Jakob et al., 2009), although we were unable to obtain the original reference and cannot report the details. Later, transgenic *M. sinensis* plants were produced by particle bombardment methods (Wang et al., 2011); four transgenic plants containing a foreign *hph* gene with or without the *gfp* were recovered from 120 bombarded calli, and foreign gene expression was confirmed by reverse transcription-PCR analyses. So far, there are no other reports on *Miscanthus* transformation.

4.4 Giant reed transformation systems

An optimized particle bombardment protocol for gene transfer with embryogenic calli was recently reported in giant reed (Dhir et al., 2010). In the study, embryogenic calli were induced from inflorescence segments collected from field-grown mature plants, and several physical parameters, such as helium pressure, bombardment distance to target tissue, and vacuum pressure, together with other factors such as gold microparticle size, DNA concentration, and the number of bombardments, were examined with transient expression of beta-glucuronidase (GUS) and green fluorescent protein (GFP) genes (Dhir et al., 2010). Unfortunately, however, no transgenic plants were obtained due to the lack of regeneration potential of the tissue culture system (Dhir et al., 2010).

We also tried to produce transgenic giant reed plants by using particle bombardment-mediated gene transfer in combination with the plant regeneration system described above (see section 3.5). We obtained more than 100 hygromycin-resistant calli from ca. 5,000 bombarded calli (Figure 5A). Transformation of these calli with foreign *hph* was confirmed by PCR analyses (data not shown). However, we could not obtain transgenic plants from the resistant calli, because the calli had lost their shoot formation potential and produced only adventitious roots on regeneration medium (Figure 5B). Thus, improvements in these tissue culture systems are needed to produce transgenic giant reed plants.

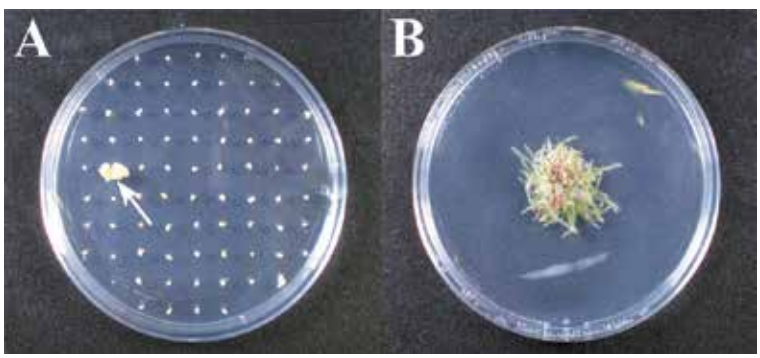

Fig. 5. Transgenic calli of giant reed. A hygromycin-resistant callus propagated during selective culture with the antibiotic hygromycin (arrow) (A), and adventitious root formation from the calli on plant regeneration medium (B).

5. Transgenic plants with improved traits

Here, we summarize reports of transgenic switchgrass and sugarcane plants having distinctive improved traits that will be of significance for biofuel production. In switchgrass, we know of only four such reports, as shown in Table 8. However, in sugarcane, several reports exist on the metabolic engineering of value-added sugarcane via carbohydrate biosynthesis and increased sucrose accumulation; the target genes and the resulting transgenic plants have been discussed in previous reviews (Hotta et al., 2010; Lakshmanan et al., 2005; Suprasanna et al., 2008a; Waclawovsky et al., 2010). Thus, for transgenic sugarcane, we focus here on reports published from 2007 to 2011 (Table 8). No studies have yet been reported for improved traits in *Miscanthus*, *Erianthus*, or giant reed.

Transgenes[a]	Gene transfer methods	Reference
Switchgrass		
phaA, *phaB*, *phaC*, [*bar*]	*Agrobacterium*	Somleva et al. (2008)
PvCAD[b] [*hph*]	*Agrobacterium*	Fu et al. (2011b)
PviCAD2[b] [*hph*]	*Agrobacterium*	Saathoff et al. (2011)
COMT[b] [*hph*]	*Agrobacterium*	Fu et al. (2011a)
Sugarcane		
phaA, *phaB*, *phaC*, [*npt*]	Gene gun	Petrasovits et al. (2007)
mds6pdh, *zmglk*, [*npt*]	Gene gun	Chong et al. (2007)
HIS-*CaneCPI-1*, [*npt*]	Gene gun	Ribeiro et al. (2008)
avidin[c], [*adhA*]	Gene gun	Jackson et al. (2010)
mutB, [*npt*]	Gene gun	Hamerli & Birch (2011)

[a] Selectable marker genes in brackets.
[b] Partial inverted repeat sequences for RNAi technology.
[c] Several signal peptides were fused for subcellular localization.

Table 8. A list of transgenic plants with improved traits

5.1 Transgenic switchgrass plants

A pioneering study of transgenic switchgrass involved the successful production of value-added transgenic plants that could synthesize polyhydroxybutyrate (PHB), a biodegradable alternative to standard consumer plastic (Somleva et al., 2008). Primary transgenic plants containing up to 3.72% dry weight of PHB in leaf tissues and 1.23% dry weight of PHB in whole tillers were obtained. Polymer accumulation was also analyzed in the T1 generation. This achievement resulted from the successful expression of a functional multigene pathway involving complex metabolic engineering (Somleva et al., 2008).

In the first half of 2011, three reports were published on the downregulation of lignin biosynthesis genes using RNAi technology to improve ethanol production from lignocellulosic biomass (Fu et al., 2011a; Fu et al., 2011b; Saathoff et al., 2011). The first two reports were published at almost the same time and both targeted the gene encoding cinnamyl alcohol dehydrogenase (CAD), a key enzyme for catalyzing the last step of lignin

monomer biosynthesis. The resulting transgenic plants showed significantly fewer transcripts of the target gene, reduced CAD activity, lower lignin content (Fu et al., 2011b; Saathoff et al., 2011), and altered lignin composition (Fu et al., 2011b). Furthermore, these modified lignin biosynthesizers had improved sugar release from cell walls with or without chemical pre-treatment (Fu et al., 2011b; Saathoff et al., 2011). In another study, the expression of the lignin biosynthesis-related *O*-methyltransferase gene was similarly downregulated, and the resultant transgenic plants had reduced lignin content, digestibility by cellulase, and up to 38% more efficient ethanol yield using conventional biomass fermentation processes (Fu et al., 2011a).

5.2 Transgenic sugarcane plants

PHB production is also targeted in sugarcane molecular breeding. The same gene set and the same strategy used with transgenic switchgrass (Somleva et al., 2008) were also employed for producing transgenic sugarcane (Petrasovits et al., 2007). In the study, PHB accumulated in leaves of transgenic plants to a maximum of 1.88% of dry weight without obvious deleterious effects. The PHB concentration in culm internodes of the transgenic plants was much lower (0.0033%) than in leaves (Petrasovits et al., 2007).

The same research group also examined sugar manipulation in sugarcane by engineering a new carbon sink for the six-carbon sugar alcohol sorbitol; sorbitol has intrinsic value as a non-caloric sweetener and is also used to manufacture ascorbic acid (Chong et al., 2007). Transgenic sugarcane plants expressing the *mds6pdh* gene accumulated the sorbitol. The average amounts of sorbitol detected in the most productive line were 120 mg g^{-1} dry weight (equivalent to 61% of the soluble sugars) in the leaf lamina and 10 mg g^{-1} dry weight in the stalk pith, but the accumulation caused evident necrosis in expanding leaves and reduced growth (Chong et al., 2007). More recently, another group focused on production of the sucrose isomer trehalulose (Hamerli & Birch, 2011). The transgenic sugarcane plants accumulated the trehalulose up to 600 mM in juice from mature nodes. Contrary to the case of the sorbitol-accumulating sugarcane, the trehalulose-accumulating transgenic plants were vigorous, and trehalulose production in selected lines was retained over multiple vegetative generations under glasshouse and field conditions (Hamerli & Birch, 2011).

A high value-added dedicated lignocellulosic biofuel crop could also be developed by accumulating heterologous functional proteins that could be used for therapeutic, industrial, or other purposes. This concept is adaptable to the production of cystatin, a natural inhibitor of cysteine proteinases, that can be used to protect against insect attacks (Ribeiro et al., 2008). A transformed sugarcane plant expressing high levels of the His-tagged cystatin gene $_{HIS}$*CaneCPI-1* was reported to be useful for production and purification of functional $_{HIS}$CaneCPI-1 protein (Ribeiro et al., 2008). The $_{HIS}$CaneCPI-1 protein purified through affinity chromatography in a nickel column was able to inhibit the catalytic activity of midgut cysteine proteinase purified from the sugarcane weevil *Sphenophorus levis* and human cathepsin L in nanomolar amounts, indicating that this system can be used for the production of functional recombinant proteins (Ribeiro et al., 2008). The accumulated recombinant proteins may disturb cell metabolism. If so, subcellular targeting of the recombinant proteins would be necessary to optimize protein yield and avoid detrimental effects of the accumulated protein. Subcellular targeting peptides are thus considered a useful tool for compartmentalization of recombinant proteins. To find ideal subcellular

targeting for recombinant protein accumulation in sugar cane, Jackson et al. (2010) used the glycoprotein avidin, which is potentially toxic to cells, as a test protein fused with several subcellular targeting peptides. Accumulation of avidin was directed to the apoplast, endoplasmic reticulum, and lytic and delta type vacuoles. The study identified the delta type vacuole as a promising target, but the efficiency may depend on tissue type. If the protein is resistant or can be protected by proteolytic attack, the lytic vacuole would be a preferable target (Jackson et al., 2010).

6. Conclusions

We described recent progress in the identification of candidate herbaceous perennials for dedicated lignocellulosic biofuel crops. The biotechnological approaches for molecular breeding of these plants as dedicated biofuel crops are still in immature stages. Biotic and abiotic stress tolerance and herbicide resistance are the minimum required traits. Other traits and related technologies will be important and will make large impacts in the molecular breeding of biofuel crop; these include low-fertilizer needs, sophisticated manipulation technology of secondary cell wall biosynthesis, high-efficiency photosynthesis for high productivity, promoted and synchronized flowering for hybridization, accelerated generation and, especially, organellar transformation for the effective accumulation of high-value chemicals and proteins. These traits and candidate target genes are reviewed in other works (Jakob et al., 2009; Vega-Sánchez & Ronald, 2010; Vogel & Jung, 2001).

7. Acknowledgments

This work was supported by the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (C) (23580027).

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5

Bioactive Beads-Mediated Transformation of Rice with Large DNA Fragments Containing *Aegilops tauschii* Genes, with Special Reference to Bead-Production Methodology

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1. Introduction

Plant transformation is a technique that allows us to transfer genes from one species to another in order to introduce new characteristics into the recipient. The plant transformation technique has become widely adopted as a method both to understand plant physiology and to improve plant characteristics. There are now many established gene-transfer methods, both direct and indirect, for the stable introduction of novel genes into plant species. Examples include *Agrobacterium*-mediated transformation, particle bombardment (biolistic), and protoplast electroporation (Klein and Fitzpatrick-McElligott, 1993; Tzfira and Citovsky, 2006). Recently, a direct gene-transfer method called bioactive beads (BABs)-mediated transformation has been developed (Sone et al., 2002; Wada et al., 2011a, 2011b). This method involves immobilization of DNA molecules on alginate beads and their transfer to plant cells with the assistance of a polyethylene glycol (PEG) solution. Alginate, a hydrophilic polysaccharide that solidifies in the presence of Ca^{2+} ions, is utilized as a barrier membrane to produce calcium-alginate beads for immobilizing high-density DNA molecules. The original procedure for bead production was described in Sone et al. (2002) (Fig. 1). Firstly, a plasmid DNA solution was mixed with a $CaCl_2$ solution. Isoamyl alcohol was then added to a 1.5 ml micro-tube containing an aqueous phase 1% sodium alginate solution to form a water/oil mixture that was emulsified by sonication using an ultrasonic disrupter (UR-20P; Tomy Seiko, Tokyo, Japan) for 10 s. Bioactive beads (BABs) encapsulating DNA molecules were generated after immediately adding a $CaCl_2$ solution containing plasmid DNA into the emulsified solution. The DNA-immobilizing BABs were utilized in combination with PEG solution for protoplast transformation.

This method was successfully used for transformation of tobacco BY-2 protoplasts with a ten-fold higher transformation efficiency than the conventional PEG transformation method that is using naked plasmid DNA (Sone et al., 2002). The BABs transformation method has a wide applicability beyond tobacco BY-2 protoplasts to many organisms, both in plant cells, e.g., eggplant (*Solanum integrifolium*, Liu et al., 2004), tobacco (*Nicotiana tabaccum* SR-1, Liu et

al., 2004) (Fig. 2), carrot (*Daucus carota*, Liu et al., 2004), rice (*Oryza sativa*, Wada et al., 2009) and in human cells, e.g., lymphocyte cell line K562 cells and human carcinoma HeLa cells (Higashi et al., 2004).

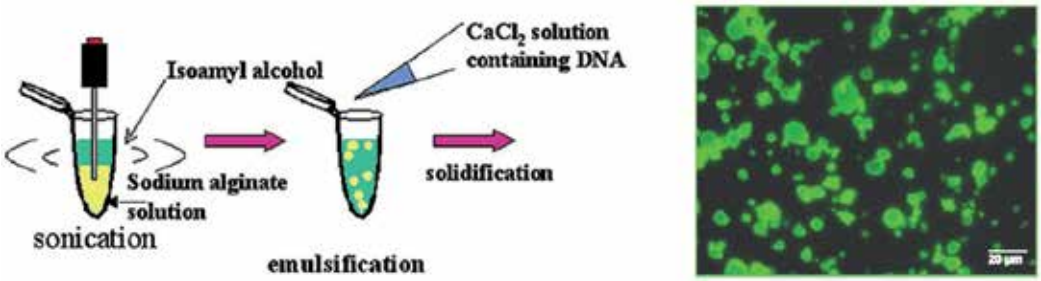

Fig. 1. Schematic diagram shows the preparation of bioactive beads (BABs) immobilizing DNA molecules

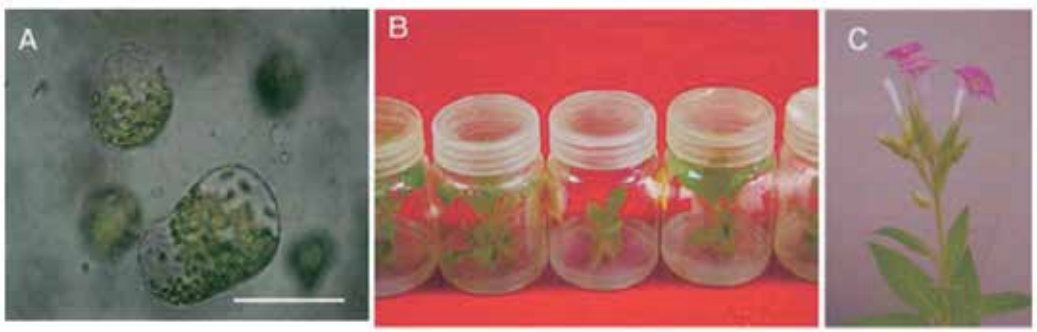

Fig. 2. Transgenic tobacco SR-1. A Cell division of protoplast 5 days after co-transformation. B Transgenic tobacco SR-1 regenerated plants 4 months after being transformed. C Flowering transgenic tobacco SR-1, 5.5 months after transformation. (With kind permission from Springer Science+Business Media: Journal of Plant Research, Obtaining transgenic plants using the bio-active beads method, 117, 2004b, 95-99, Liu, H.; Kawabe, A.; Matsunaga, S.; Murakawa, T.; Mizukami, A.; Yanagisawa, M.; Nagamori, E.; Harashima, S.; Kobayashi, A & Fukui, K., Figure 2)

Mizukami and associates (2003) have introduced yeast artificial chromosomes (YACs) into yeast spheroplasts using this method, and revealed the higher physical stability of 468 kb of YAC DNA embedded in and/or on the bioactive beads in solution compared to naked chromosome DNA molecules. The authors also checked whether YAC DNA molecules immobilized on bioactive beads would be intact even after vortex treatment. The result showed that naked YAC DNA molecules was degraded in the solution, resulting in no visible band after electrophoresis, but the band was clearly observed in the case of the YAC DNA molecules immobilized on the beads. This shows that BABs can stabilize large DNA fragments in solution. Moreover, yeast chromosomal DNA with YAC DNAs (128 kb, 256 kb and 468 kb) was embedded in and/or on the beads and transferred into recipient yeast cells lacking those YAC DNAs. Pulse field gel electrophoresis clearly showed that YAC DNAs up to 468 kb in size were successfully introduced into recipient

cells by PEG treatment with bioactive beads. This utility, coupled with the fact that the method does not require any sophisticated equipment and is easy to practice, clearly suggests the bioactive beads method as an alternative transformation method, especially for large DNA molecules.

Despite the advantages of BAB transformation mentioned above, there is insufficient information regarding the ideal production conditions, such as the shape and size of BABs, or the concentration of DNA that is suitable for the most efficient BABs transformation. Thus, the production conditions for BABs should be optimized. Here, improvement of the BAB production system enabling uniform size and shape will be reported. Using this system, various sizes of beads immobilizing the pUC18-sGFP construct could be produced. Its applicability to plant transformation has already been examined by using tobacco BY-2 protoplasts.

As described above, the BAB method has the ability to transfer large DNA fragments into yeast spheroplasts and into the protoplasts derived from tobacco BY-2 cells suspension. Moreover, recently our group has successfully produced transgenic rice with large DNA inserts containing *Aegilops tauschii* hardness genes by using this method. The detailed transformation procedures and characterization of transgenic plants will be described in this chapter.

2. Improvement of efficacy of a BAB transformation method

The capturing of DNA fragments by BABs can prevent their physical damage during transformation processes. As a result, the BAB transformation method provides a feasible large DNA fragment transfer method into many organisms (Mizukami et al., 2003; Wada et al., 2009). However, the mechanism for DNA transfer is still obscure and experimental conditions are not yet optimized, resulting in low DNA transfer efficiency (Liu et al., 2004). During preparation of BABs by the original sonication method, $CaCl_2$-containing DNA molecules were added into an alginate emulsion solution, and the beads were then collected by centrifugation and used for transformation (Sone et al. 2002). It was, however, found that large amounts of DNA remained in the $CaCl_2$ solution, resulting in a poor efficiency, both in DNA immobilization and transformation. Therefore, a more efficient BAB production system with higher DNA immobilization and DNA transformation efficiencies is desired.

A new system for bead production was developed using an in-house device called a bead-maker. The bead-maker has 2 major components; an automated micro-syringe, and a vibrator (Fig. 3). The automated micro-syringe was assembled by placing a syringe (100 μl gastight syringe, Hamilton, Nevada, USA) on a micro-syringe pump (MSP-RT, AS ONE) so that the flow rate of the solution could be accurately controlled. The vibrator consists of a loudspeaker (FR-8, 4Ω, Visaton, Germany), attached to a moveable rod fixed to a wooden board and connected to a sine wave sound generator (AG-203D Kenwood, Tokyo, Japan). A capillary tube (30 μm ϕ, GL Sciences, Tokyo, Japan) from the syringe was connected to the moveable rod so it could be vibrated simultaneously when the rod was vibrated. The frequency and amplitude of the sine wave sound generator are selectable. As a result, the

vibration of the moveable rod as well as the capillary tube linking the micro-syringe and the vibrator are controllable.

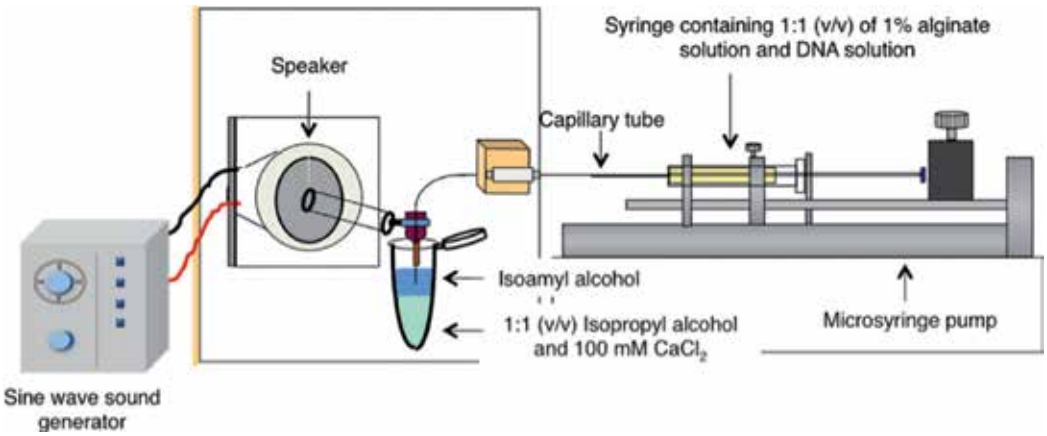

Fig. 3. Schematic diagram of a bead-maker

To test the effectiveness of this new system for plasmid DNA immobilization, bead production using 1% alginate containing 0.5 μg/μl of pUC18-sGFP was carried out. First, a DNA-containing alginate solution was prepared by mixing 100 μl of sodium alginate solution (1% w/v) with 100 μl of pUC18-sGFP (0.5 μg/μl), and then the freshly-prepared solution was slowly loaded into the micro-syringe. The syringe was connected to the capillary tube and placed on the micro-syringe pump. The solidifying solution was then prepared by firstly adding 750 μl of a mixture of 0.1 M $CaCl_2$ and isopropyl alcohol (1:1) in a 1.5 ml micro-centrifuge tube, followed by adding 750 μl of isoamyl alcohol and placing the tube in a plastic rack as shown in Fig. 3. As the bead-maker was working, the DNA-containing alginate solution was pumped out at a steady flow rate. Simultaneously, the sine wave sound generator produced sound waves at the speaker resulting in the vibration of the moveable rod connecting to the speaker. Consequently, vibration of the capillary tube linked to the moveable rod dropped alginate-DNA solution into the solidifying solution. The isoamyl alcohol kept the droplets spherical and the mixture of $CaCl_2$ and the isopropyl alcohol solidified the alginate-DNA droplets. The spherical beads were collected and washed at least twice in 0.1 M $CaCl_2$ solution by centrifugation (5000 *g* for 5 min) and re-suspended in a sufficient volume of 100 mM $CaCl_2$ solution. To verify the efficiency of DNA immobilization, the beads were stained by a DNA-staining dye, YOYO-1, and were observed under a fluorescence microscope (Zeiss). Images were captured using a CCD (charge-coupled device) camera (Fig. 4a-d). Observation of bead shape under the microscope revealed them to be spherical. Contrastingly, the shapes of beads made using the original system, with normal sonication, were irregular; many were not spherical. Qualitatively, the green fluorescence intensity emitted from DNA-immobilized beads, which correlates with the amount of DNA immobilized by the BABs, was measured using image analysis software, Image J (http://rsbweb.nih.gov/ij/), to further compare the efficiency of DNA immobilization of these two systems. The intensity of green fluorescence emitted from beads made using the new system was obviously

higher than that of the beads made by sonication. To quantify this result, the green fluorescence intensities from more than 50 beads made using both the new system and the sonication system were measured (Fig. 4e). The mean intensity from the improved beads was much higher than that from beads made by sonication, indicating that the amount of DNA immobilized by the improved beads was higher than that by beads made by the original sonication system.

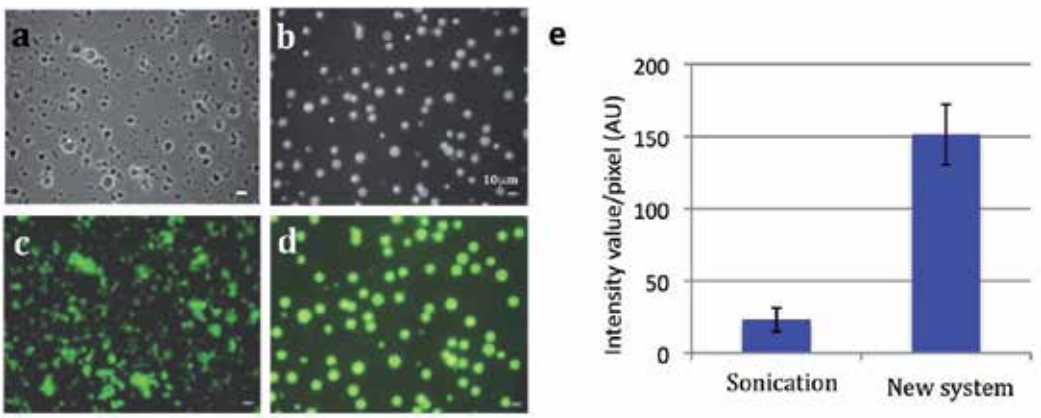

Fig. 4. Improvement of BAB production was achieved using the new BABs production system. BABs made by the new system were spherical, size-controllable and more highly efficient at DNA immobilization compared to BABs made by the original sonication system. Phase contrast (a-b) and fluorescent images (c-d) of plasmid DNA-immobilized beads made using the sonication system (a and c), and using the new system (b-d). Bars = 10 μm. Mean intensity of green fluorescent intensity from plasmid-DNA immobilized beads stained by YOYO-1 (e). Error bars: ±1 s.d.

We further investigated whether the bead size influenced transformation efficiency. As described above, solution flow rate and the frequency and amplitude of the sine wave were adjustable. Various combinations of these parameters were tested to obtain uniformly sized BABs. Investigation of the effect of solution flow rate, vibration frequency, and vibration amplitude on the size of beads indicated that a solution flow rate at 0.4 μl/min was the most suitable for producing beads of a uniform size, compared to other flow rates tested at 0.2, 0.8, 2 and 5 μl/min. However, the bead size was controllable by changing the vibration frequency and amplitude. The vibration amplitude had a direct effect on bead sizes: smaller beads are produced with a higher amplitude. The frequency of the sine wave affected the bead size through the strength of the capillary tube vibration; frequencies causing strong vibration produced smaller beads while weak vibration produces larger beads.

Three different sizes of BABs immobilizing the same amounts of pUC18-sGFP were selected (Fig. 5) and used for transformation into tobacco BY-2 protoplasts in combination with PEG treatment (Fig. 6). Transient assays of GFP-expressing protoplasts were carried out 24 h and 48 h after transformation (Fig. 6c). The results showed a negative correlation between bead size and transformation efficiency, that is, as the size of beads decreased, the transformation

efficiency increased. Moreover, with the new system, a *ca.* 10% transformation efficiency was achieved (Fig. 6 (⑥)). One to six μm diameter beads provided higher transformation efficiencies than beads made by sonication method. Thus it is likely that immobilization of DNA molecules on beads made by the new system is more efficient than that made by sonication system. Furthermore, it was also found that transformation efficiency obtained from one to six μm beads was higher than that obtained from seven to twelve μm beads. Two explanations are possible to reveal out why smaller beads have better transformation efficiency. One is that DNA is introduced into plant cells through physical uptake of bioactive beads. Smaller beads should be more easily incorporated into plant protoplasts. The other is because a higher number of smaller beads means a larger total surface area for the same volume of alginate solution being used. This means that a higher number or a larger total surface area of smaller beads might adhere to the protoplast membrane than when using larger beads, consequently enhancing the interaction between DNA-immobilized beads and protoplasts, and ultimately increasing DNA transfer between beads and protoplasts. From these results, it is concluded that, aside from DNA concentration, bead size optimization is also an important factor in achieving high transformation efficiency. This new system developed for BABs production with higher transformation efficiency should facilitate more applicability of BABs transformation and even enable multiple gene delivery into plant cells.

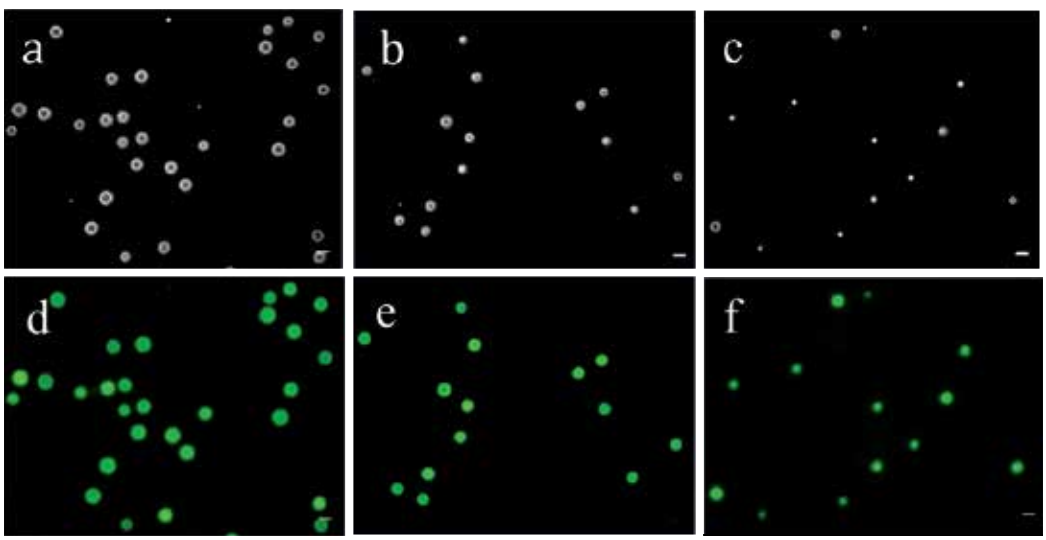

Fig. 5. Phase contrast (a-c) and fluorescence image (d-f) of BABs made under optimized conditions. BABs of 7-12 μm diameter made with 0.4 μl/min solution flow rate, frequency of 250 Hz, and amplitude set to 7 (a,d). BABs of 5-8 μm diameter made with 0.4 μl/min solution flow rate, frequency of 270 Hz, and amplitude set to 10 (b,e). BABs of 1-6 μm diameter made with 0.4 μl/min solution flow rate, frequency of 250 Hz, and amplitude set at 10 (c,f). Bars = 10 μm

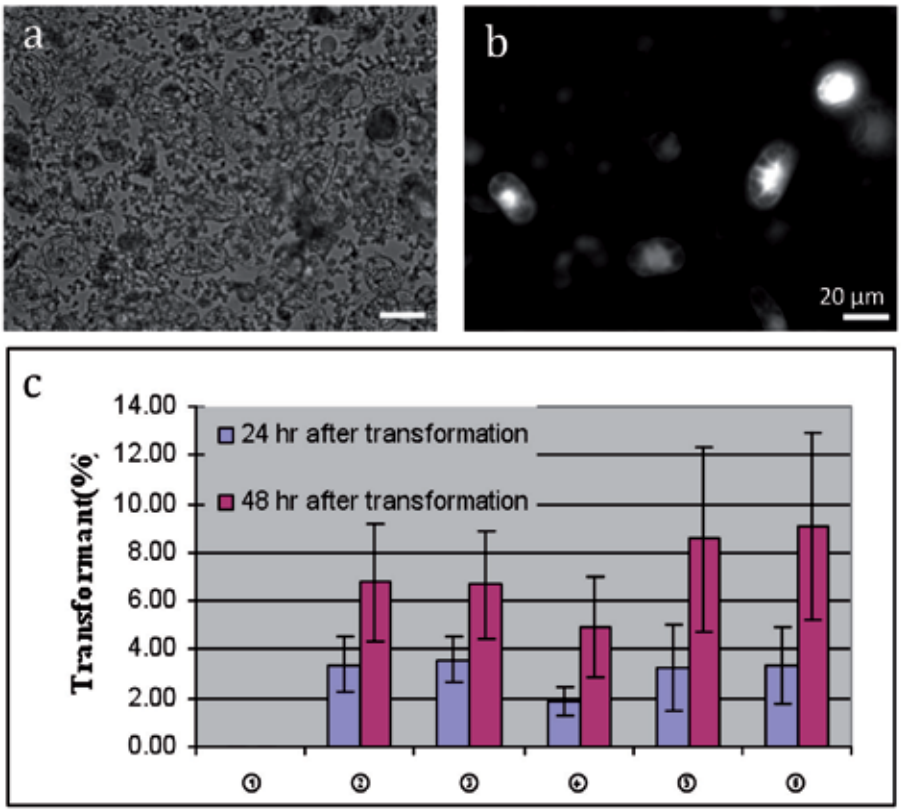

Fig. 6. Transformation of tobacco BY-2 protoplasts using BABs immobilizing pUC18-sGFP. Phase contrast image of tobacco BY-2 protoplast transformed with the improved BABs (a), tobacco BY-2 protoplasts expressing GFP protein (b). Bars = 20 μm. Transformation efficiency after 24 h and 48 h of transformation: ①, negative control (distilled water); ②, 50μg naked pUC18-sGFP; ③, sonicated beads; ④, beads made using the new system, size: 7-12 μm (68%); ⑤, beads made using the new system, size: 5-8 μm (62%), 2-3 μm (16%); ⑥, beads made using new system, size: 1-6 μm (70%). Error bars: ±1 s.d.

3. Large DNA transfer into plants using the bioactive beads method

3.1 Transformation of rice with large DNA molecules using BAB method

Transformation of large DNA fragments is a promising approach to extend the reach of plant genetic engineering. Until now, plant genetic engineering has been performed using single or small numbers of genes, resulting in successful production of genetically engineered plants such as herbicide- and insect-resistant plants (Gonsalves, 1998; Khan et al., 2009; Song et al., 2003b; Tai et al., 1999; Wang et al., 2005). To produce transgenic plants with more variety of phenotypes, however, multiple gene transfer will be required (Dafny-Yellin et al., 2007; Daniell et al., 2002; Halpin, 2005; Naqvi et al., 2010). Even single traits are often the result of expression of multiple genes. For example, a single metabolic pathway may be related to several genes. Thus if we want to manipulate the metabolic pathway, it will be best achieved by manipulating several genes simultaneously. Introducing these

genes at same time is preferable because: (1) introduction of multiple genes through crossing of different transgenic plants is time-consuming and laborious, and (2) co-transformation of different kinds of plasmid DNAs needs preparation of many constructs, which also takes time and needs different kinds of marker genes depending on the number of plasmids to be introduced. Therefore, transformation with large DNAs including multiple genes is promising to enhance the efficiency of transformation. In addition, large DNA transfer will enable the regulatory regions of transgenes to be transferred with the genes of interest. This will allow introduced genes to be expressed at the physiological level.

Although this approach is expected to have such advantages, it is still difficult to transfer large DNA fragments into plants. This difficulty is due to the lack of a reliable method of introducing large DNA fragments into plants. For general plant transformation, *Agrobacterium*-mediated transformation and particle bombardment are the methods normally used (Bhalla, 2006; Rakoczy-Trojanowska, 2002). However, these methods are difficult to apply to large DNA transfer into plants because of the instability of large DNAs in *Agrobacterium* cells and during the bombardment process. Song and associates (2003a) and Nakano and co-workers (2005) have recently reported that DNA fragments larger than 100 kb are unstable in *Agrobacterium* cells, resulting in deletion and/or rearrangements in *Agrobacterium* cells before their transformation into plants. Their data suggest that *Agrobacterium*-mediated transformation is not suitable for large DNA transfer. In particle bombardment, DNAs coated on metal particles generally suffer from physical damage during bombardment, resulting in fragmentation of large DNAs. However, some laboratories, have reported success in introducing DNA fragments over 100 kb in size by particle bombardment (Van Eck et al., 1995; Mullen et al., 1998; Phan et al., 2007). This is a promising improvement, but successful reports of large DNA transfers using this method are still limited. In addition, particle bombardment needs the equipment. Thus, an easy and inexpensive method needs to be developed for large DNA transfer. As described above, our group has recently developed such a new transformation method, namely the BAB method (Higashi et al., 2004; Liu et al., 2004a; Liu et al., 2004b; Mizukami et al., 2003; Sone et al., 2002; Wada et al., 2011a, 2011b). This method is easy and inexpensive. The method has been applied to yeast, mammalian cells (HeLa, K562 cells), and plant cells (tobacco BY-II, tobacco SR-I, carrot, egg plant, rice). Transformation efficiency is about 10 times higher than PEG treatment without bioactive beads. An important characteristic of the BAB method is its transformation capability with large DNA fragments. As mentioned above, 468 kb of YAC DNA was effectively transferred into yeast cells. This result clearly suggests that the BAB method is a suitable for large DNA transfer.

To examine the applicability of the BAB method to plant transformation with large DNA fragment, 124 kb of YAC DNA was introduced into cultured tobacco BY-2 cells (Liu et al., 2004b). The transient expression of introduced genes was detected in the transgenic suspension cells. To investigate this in more detail, the bioactive beads method was applied to the transformation of rice with *ca*. 100 kb BAC DNA (Wada et al., 2009). Rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) was used as the plant material and BAC DNA including hardness genes from *Agilops tauschii* was introduced using the bioactive beads method. The hardness genes consist of three genes (*puroindoline a, puroindoline b, GSP-1* gene) and are scattered in a *ca*. 100 kb region on the short arm of chromosome 5D in *Aegilops tauschi* (Turnbull et al., 2003). The BAC DNA encompasses the hardness locus and also contains the regulatory regions of each gene (Fig. 7). Because of this, it was expected that the expression of each gene would be shown at the regular physiological level.

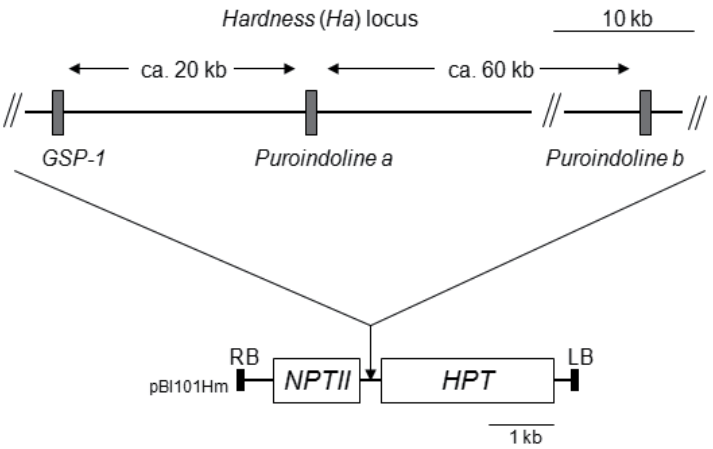

Fig. 7. Schematic diagram of the construct, used in this transformation, pBI BAC10-60. (With kind permission from Springer Science+Business Media: Plant Cell Reports, Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes, 28, 2009, 750-68, Wada, N.; Kajiyama, S.; Akiyama, Y.; Kawakami, S.; No, D.; Uchiyama, S.; Otani, M.; Shimada, T.; Nose, N.; Suzuki, G.; Mukai, Y.; Fukui, K.; Figure 1)

As a result, nine transgenic plants were obtained and analyzed. The PCR analyses showed that each gene was integrated into the rice genome (Table 1). Some transgenic plants contained most transgenes, but transgenic plants with all the transgenes could not be obtained. This indicates that rearrangement of introduced DNA molecules occurred during transformation.

	Lines	*NPTII* gene	*Pinb* gene	*Pina* gene	*GSP-1* gene	*HPT* gene
Non-transgenic plant		–	–	–	–	–
Transgenic plants with pBI BAC10-60	9-1-1	–	+	–	+	+
	9-1-2	–	+	–	+	+
	9-1-3	+	+	–	+	+
	9-1-4	+	+	–	+	+
	9-1-6	–	+	–	+	+
	9-1-7	+	+	–	+	+
	9-1-8	+	+	–	+	+
	9-1-9	+	+	–	+	+
	9-1-10	–	–	–	+	–

Table 1. Profiles of T_0 transgenic plants as determined by PCR analysis. +: gene detected, – : gene not detected. (With kind permission from Springer Science+Business Media: Plant Cell Reports, Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes, 28, 2009, 750-68, Wada, N., Kajiyama, S., Akiyama, Y., Kawakami, S., No, D., Uchiyama, S., Otani, M., Shimada, T., Nose, N., Suzuki, G., Mukai, Y., Fukui, K.; Table 3)

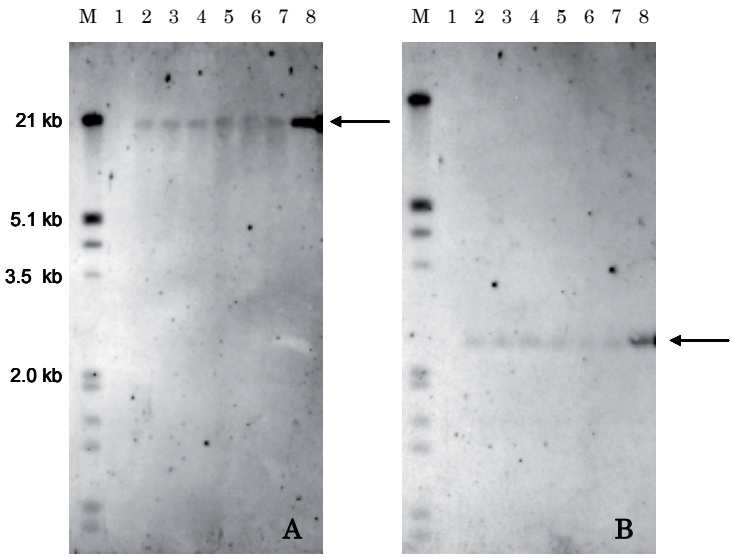

Fig. 8. Genomic Southern blot analysis of T_0 transgenic plants. Total DNA of rice plants was digested with *Hin*dIII and probed with (A) the *HPT* gene and (B) *Pinb* gene. Lane 1 : control plant, Lane 2 : transgenic plant 9-1-3, Lanes 3 to 7 : transgenic plants 9-1-6 to 9-1-10, Lane 8 : pBI BAC DNA digested with *Hin*dIII. The amount of BAC DNA corresponds to two copies insertion of the transgene in rice genome. Arrows indicate the locations of the observed bands (These figures, from Wada et al. (2011), are reproduced with permission from John Wiley and Sons, Inc.)

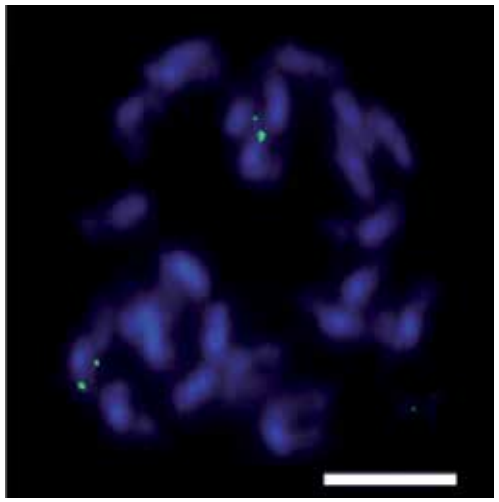

Fig. 9. FISH analysis of the kernels of the T_1 transgenic plant 9-1-6-3. The two paired green signals indicate the integration sites of pBI BAC 10-60. Bar = 10 μm. (With kind permission from Springer Science+Business Media: Plant Cell Reports, Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes, 28, 2009, 750-68, Wada, N.; Kajiyama, S.; Akiyama, Y.; Kawakami, S.; No, D.; Uchiyama, S.; Otani, M.; Shimada, T.; Nose, N.; Suzuki, G.; Mukai, Y.; Fukui, K.; Figure 4)

The copy numbers of *puroindoline b* (*Pinb*) and *HPT* genes were checked by genomic Southern blot analysis. The results showed that these two regions were integrated into the rice genome as a single copy of intact fragment (Fig. 8). T_0 plants showed sterility: 7 out of 9 transgenic plants did not produce any seeds. Two transgenic plants were partially fertile and produced some seeds. They recovered their fertility in successive generations. Segregation tests of the T_1 generation showed that transgenes were inherited into the next generation in Mendelian mode. Homozygous plants were also obtained. FISH analysis revealed that the transgenes were integrated into the telomeric region of a pair of rice chromosomes in the homozygous plants (Fig. 9). The expression of the introduced gene, *Pinb*, was also confirmed at the mRNA and protein levels. Thus, the promoter region of *Pinb* was functional even in rice cells. These results indicate that the BAB method can introduce multiple genes into plants and produce stable transgenic plants that can pass introduced transgenes on to successive generations.

To examine if the introduced gene is functional in transgenic rice, a phenotypic analysis was performed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Wada et al., 2010). The results indicated that the endosperm structure changed in the transgenic rice to a more loosely packed structure (Fig. 10). The hardness genes are known to affect the softness of wheat endosperms by giving the loosely packed endosperm structure. Thus, the EM observations clearly indicated that the introduced hardness gene functioned in similar manner as in wheat endosperm. Analysis of physico-chemical properties of the rice flour also indicated that the transgenic rice endosperm had the phenotype of soft textured seed. The results suggest that the PINB protein localized at the surface of the starch compounds, resulting in preventing the adhesion of each starch compound and changing some physico-chemical properties, such as flour particle size, and pasting properties. These results indicate that the hardness locus introduced was functional in transgenic rice. This suggests that introduction of a genomic locus that controls a trait could be a good strategy for adding desirable traits to plants.

The results obtained indicated that the BAB method is a promising method for plant transformation with large DNA fragments. Co-transformation experiments with two or three kinds of BAC DNAs simultaneously were also successful (data not shown). Co-transformation can increase the number of genes that can be introduced simultaneously. However, some aspects of the BAB method could be improved. First, the intactness of introduced DNA fragments should be examined. In our experiments, the deletion of some transgenes was observed. The rearrangement of introduced DNA fragments might also occur in other regions that were not checked. How often and to what extent such rearrangements occur with the BAB compared to conventional methods needs to be investigated to fully establish that the BAB method has advantages over other conventional methods for transformation with large DNA fragments. Second, further improvement in the transformation efficiency of the BAB method should be achieved. We have succeeded in immobilizing proteins on BABs (data not shown). The immobilization of large DNAs with proteins, such as VirE2, might target the introduced DNA into the host genome more efficiently because VirE2 is known to target T-DNA into nuclei and protect the T-DNA during *Agrobacterium*-mediated transformation (Gelvin, 2003; Gopalakrishna et al., 2003). Despite these points that could be improved, our results clearly indicate that the bioactive beads method could be an alternative way of producing stable transgenic plants with multiple transgenes.

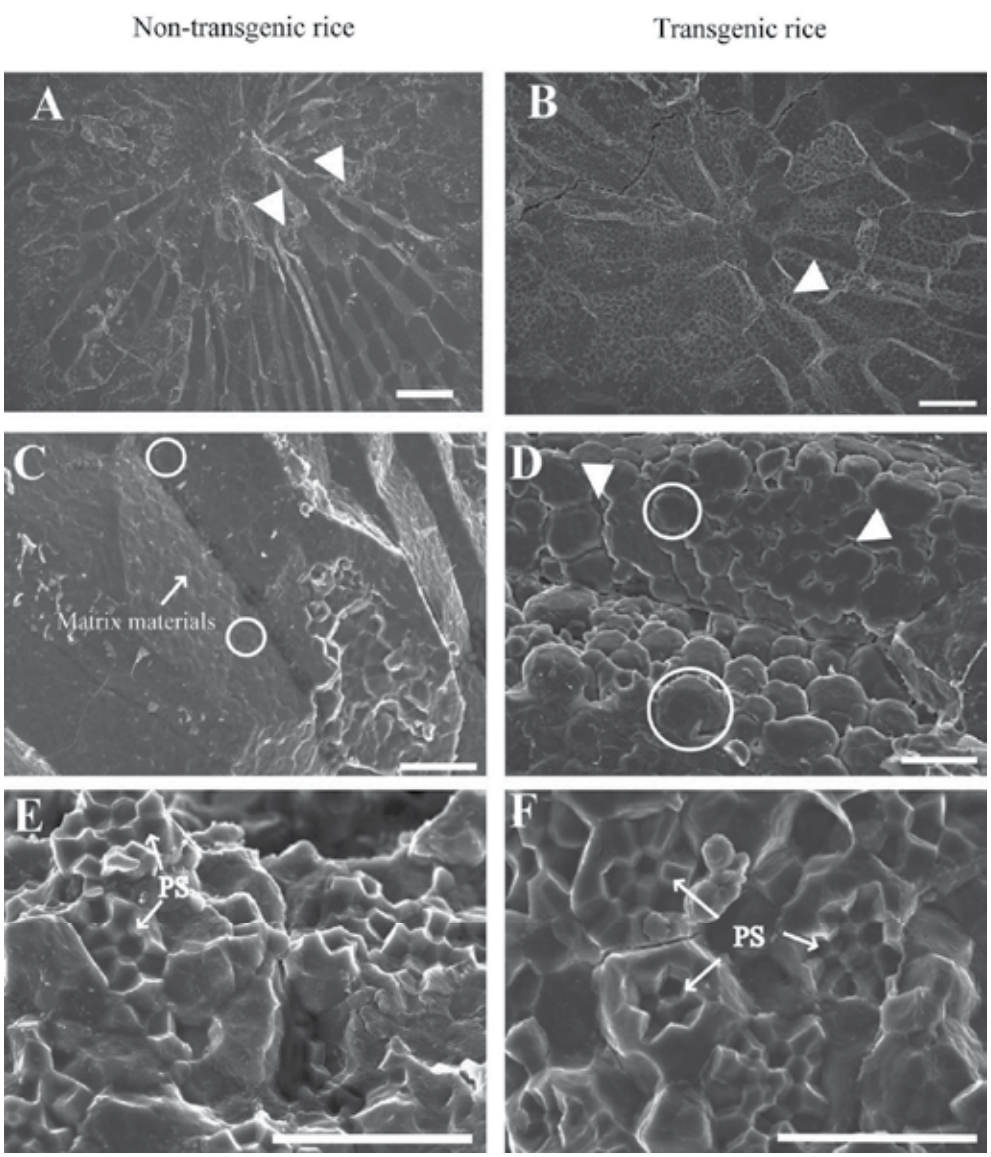

Fig. 10. SEM observation of rice endosperm cells. (A,B) Low magnification view of transversely fractured surface of milled rice of (A) non-transgenic rice and (B) transgenic rice. Arrowheads indicate intracellularly cleaved site. Bars: 100 μm. (C,D) Higher magnification view of intercellularly cleaved site of (C) non-transgenic rice and (D) transgenic rice. Bars: 20 μm. (C) Compound starch granules (circles) embedded within matrix material in non-transgenic rice. Intracellularly cleaved sites can also be seen. (D) Starch compound granules (circles) surrounded by airspaces (arrowheads). (E,F) Higher magnification view of intracellularly cleaved site of (E) non-transgenic rice and (F) transgenic rice. Partially split compound starch granules (PS) exposing individual starch granules with sharp angles and edges can be seen. Bars: 20 μm (Reproduced from Wada et al. (2010) with permission of Elsevier Science.)

In mammalian cells, chromosome engineering systems including artificial chromosomes, have been developed (Basu & Willard, 2005; Ikeno et al., 1998; Kazuki et al., 2011; Oshimura & Katoh, 2008). An artificial chromosome has a capacity to carry chromosomal fragments, with virtually no size limitation to the transgenes that can be transferred (Kuroiwa et al., 2000). In addition, microcell-mediated transfer (MMCT) has made it possible to introduce genes that cannot be transferred by conventional transfection. For example, a chromosomal region including the dystrophin gene (2.4 Mb) has been introduced into a mouse genome using human artificial chromosomes (HACs) and MMCT (Hoshiya et al., 2009). HACs can be engineered by recombination technology in cells that are the most suitable for each step (e.g. chicken DT40 cells for homologous recombination, hamster CHO cells for site-specific recombination, Kazuki et al. 2010). However, there is no such system for plants. Recently three reports have been published on the production of plant artificial chromosomes (Carlson et al., 2007; Yu et al., 2007; Ananiev et al., 2009). However, there is still no report of utilizing them for plant transformation with large DNA fragments. Thus, a plant transformation system using large DNA fragments has yet to be developed. The BAB method can be used to introduce large DNA fragments into a plant genome as a part of plant transformation system with large DNA fragments.

3.2 Further utility of bioactive beads

BABs can be not only applied to DNA transformation, but also to the immobilization of proteins (Zhou et al., 2009). BSA (Bovine serum albumin) protein was successfully entrapped by BABs and its interaction with FITC-labeled anti BSA was clearly observed. Moreover, the authors improved the efficacy of protein immobilization in BABs by treating the alginate solution with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (NHSS) to cross-link the desired protein (BSA) to the alginate carboxyl groups prior to solidification. It was found that cross-linking beads provided high protein-retention ability for up to 2 weeks after immobilization. Such improved protein-immobilizing beads with high retention capacity might have the potential to be an alternative choice for detecting antigen-antibody interactions.

BABs have also been successfully used in the immobilization of a single yeast cell displaying hydrolyzing enzymes to capture fluorescent molecules released after enzymatic reaction (Zhou et al., 2009). The retention of fluorescent products by yeast-encapsulating BABs enabled active and non-active cells to be differentiated by sorting in a flow cytometer. Using such a developed system, a library screening for novel enzymatic activities on the surface of yeast cells should be possible.

4. Conclusion

An alternative transformation method, BABs-mediated transformation, has been developed by applying a drug delivery system (DDS) in which highly concentrated DNA molecules are entrapped by small autonomously degradable alginate beads and transferred into plant cells in combination with polyethylene glycol (PEG) treatment. This transformation method is easy to perform, is applicable to a range of organisms, allows large-sized DNAs to be delivered, and facilitates the transportation of multiple genes of up to 468 kb size into yeast spheroplasts. Moreover, our latest results on transformation of BAC DNA containing *A. tauschii* hardness genes into rice protoplasts, along with an improvement in the efficiency of

DNA immobilization by BABs have verified that this method is capable of producing transgenic rice that carry large DNA fragments and can facilitate the production of useful transgenic plants by introduction of multiple genes simultaneously with high efficiency. Further development of the BAB method will contribute to the development of more flexible plant genetic engineering methodologies using large DNA fragments. This will open up new possibilities for plant genetic engineering and make it possible to produce a number of useful transgenic plants in the near future.

5. Acknowledgements

This work is supported by a fund from the Handai Frontier Research Center's Strategic Research Base supported by the Special Coordination Fund for Promoting Science and Technology of the Government of Japan to K. F. This research was partially supported by a Grant-in-Aid for Scientific Research (B) (No. 19380194) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We would also like to express special thanks to the Global COE (Center of Excellence) Program, "Global Education and Research Center for Bio-Environmental Chemistry", of Osaka University. N.K. would like to thank the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) and the International Graduate Program for Frontier Biotechnology, Graduate School of Engineering, Osaka University for fellowship support. N.W. was supported by a Research Fellowship from the Japanese Society for the Promotion of Science for Young Scientists.

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6

Genetic Transformation of Immature Sorghum Inflorescence via Microprojectile Bombardment

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1. Introduction

Sorghum bicolor is one of the most important cereals in the world after rice, maize, wheat and barley. In 2010, more than 60 million tons were produced from approximately 50 million ha around the world. It is an important crop in the arid and semi-arid regions of the world and it is primarily used in Brazil as a supply for an increasing livestock market. However, the Brazilian sorghum productivity is low (1,500 to 2,500 kg/ha) and extremely variable along the years, typical of a culture sowed in marginal climate conditions and mainly without the use of high input technologies. Conventional breeding programs have already done a great deal of research to increase the sorghum productivity, though in some fields, the gains obtained by these programs are reaching stationary levels due to the lack of genetic variability (Nwanze *et al.*, 1995). Alternatively, recombinant DNA technology and the generation of transgenic plants can increment conventional breeding programs through the amplification of the gene pool that can be used to improve sorghum environmental fitness and nutritional qualities.

However, unlike others Poaceae, sorghum transformation has been a challenge mainly due to recalcitrance in tissue culture and long periods of selection required for the recovery and regeneration of putative transgenic plants (Casas *et al.*, 1993, Zhao *et al.*, 2000; Jeoung *et al.*, 2002; Howe *et al.*, 2005). Since the earliest 90's, laboratories around the world have generated improvements in sorghum regeneration and transformation that are ensuing in more consistent protocols. Transgenic sorghum plants have been generated via biolistic (Casas *et al.*, 1993; Casas *et al.*, 1997; Zhu *et al.*, 1998; Able *et al.*, 2001; Emani *et al.*, 2002; Jeoung *et al.*, 2002; Devi and Sticklen, 2003; Tadesse *et al.*, 2003; Girijashankar *et al.*, 2005) or *Agrobacterium* mediated transformation (Zhao *et al.*, 2000; Carvalho *et al.*, 2004; Gao *et al.*, 2005; Howe *et al.*, 2006; Van Nguyen *et al.*, 2007; Nguyen et al., 2007; Gurel et al., 2009; Lu et al. 2009; Mall et al., 2011).

Even though the efficiency of sorghum transformation using the microprojectile bombardment had been improved, by all this studies, since the initial experiments (from 0,3 to 1,3%), it is still low if compared with the efficiency of sorghum transformation mediated by *Agrobacterium tumefaciens* from 2.1% – 4.5% (Zhao et al., 2000; Gao et al., 2005; Howe et

*Corresponding Author

al., 2006). So, there is still need for more improvements in the microprojectile bombardment, once this technique can be used for genotypes and explants not susceptible to the transformation mediated by *Agrobacterium tumefaciens*.

2. Genetic transformation of *Sorghum bicolor*

2.1 *In vitro* regeneration of transgenic sorghum cells

Sorghum tissue culture is reported to be highly recalcitrant mainly because the release of toxic phenolics compounds in culture media, lack of regeneration in long term *in vitro* cultures, and high degree of genotype dependence. Consequently, cell transformation followed by plant regeneration remains extremely complicated in sorghum transgenic technology. So, the establishment of sorghum regeneration systems from somatic cells constitutes a prerequisite of extreme importance within the process of transgenic sorghum plants production.

The regeneration of sorghum *in vitro* has been achieved from a variety of tissues, such as immature embryos (Bhat and Kuruvinashetti 1995; Bai et al. 1995; Elkonin and Pakhomova 1996), immature inflorescences (Kaeppler and Pedersen 1997), young leaves (Han et al. 1997) and shoot tips (Nahadi and de-Wet 1995; Patil and Kuruvinashetti 1998; Shyamala and Devi, 2003). Immature zygotic embryos and inflorescences are the explants with higher embryogenic competence and frequently used to regenerate various cultivars of sorghum. Gupta and co-workers (2006) compared the regeneration in tissue culture of immature embryos and immature inflorescences from five genotypes of *S. sudanense* (SDSL 98984, SDSL 98988, SDSL 981125, SDSL 981142 and SDSL 981144) and three genotypes of *S. bicolor* (2219B, GD 68727 and 981013). They indicated that the regeneration potential of immature inflorescences was much superior to that of immature embryos and their performance was almost equivalent across the genotypes tested. The superiority of immature inflorescences can be due to its higher proportion of meristematic tissues (floral meristems, rachis, rachillae, and primordial of various floral organs) in comparison to immature embryos (mainly scutellum) according to authors.

However, the main explants used in the transformation of sorghum are immature zygotic embryos between 1.5 and 2.0 mm in length (Casas et al, 1993; Emani et al, 2002; Jeoung et al, 2002; Gao et al, 2005, Howe et al, 2006; Gurel et al, 2009). One of the constrains in working with immature embryos is the intensive labor to generate large quantities of explants to be used in the transformation procedures. In this sense, immature inflorescences are easier to isolate, show very good regeneration rates in tissue culture and, morphogenetic competence over a wider size range (1–5 cm) than immature embryos. Besides, it is faster to grow donor plants for the production of immature inflorescence than for immature embryo (Cai and Butler, 1990; Kaepler and Pedersen, 1997; Jogeswar et al 2007; Brandão et al. 2007).

Even though outstanding studies aiming to identify sorghum genotypes able to produce high quality callus from immature inflorescence have been conducted, the efficiency to produce transgenic sorghum plants using this type of explant is still very low.

2.2 Genetic transformation of *Sorghum bicolor* via microprojectile bombardment

The bombardment of plant cells with the DNA of interest is a direct method of transformation designed (Taylor and Fauquet, 2002) in the late 80's to manipulate the

genome of plants recalcitrant to transformation via *Agrobacterium*, including the cereals (Klein et al 1987; Taylor and Fauquet, 2002). In the transformation via particle bombardment, microprojectiles of metal physically covered with the gene of interest are launched toward the target cells, using equipment known as "gene gun" (Sandford et al. 1987). The velocity of these particles is fast enough (1500 km/h) to penetrate the cell wall of a target tissue and does not cause cell death. The precipitated DNA on the microprojectiles is released progressively into the cell after the blast, and integrated into the genome (Taylor and Fauquet, 2002). The acceleration of microprojectiles is obtained by a high voltage electrical discharge or compressed gas (helium). The metal particles used must be non-toxic, non-reactive, and lower than the diameter of the target cell. The most commonly used are gold or tungsten. Several physical parameters correlated with the biolistic equipment such as pressure, distance of the macro and micro-carrier flight, and vacuum, must be optimized for successful transformation. In addition to these parameters, the biology of the plant material and the gene of interest (GOI) should also be studied in preliminary experiments (Sandford et al., 1993). Since the 90's, the biolistic was used to transform a wide variety of plants, including sorghum.

Some advantages of the microprojectile bombardment are related to its efficiency in the transformation of monocots, the use of simple vectors, easier to handle, as well as the possibility of inserting more than one GOI into cells efficiently (Chen et al. 1998; Wu et al. 2002). Although considered a very efficient method in cereals, one drawbacks of this technique is the occurrence of multiple copies of the GOI in the transgenic plant and complex integration patterns (Wang and Frame, 2004).

The biolistic has proved to be an efficient method for introducing new features in sorghum and a few transformation protocols are already available (Casas et al. 1993; Casas et al. 1997; Zhu et al. 1998; Able et al. 2001; Devi and Sticklen, 2002 ; Emani et al. 2002; Tadesse et al., 2003; Girijashankar et al., 2005). The optimization of physical and biological parameters was the subject of most of the work published about sorghum transformation via bombardment. The pioneer work was done by Casas and collaborators between 1993 and 1997. Initially, using anthocyanin (*R* and *C1* genes) and β-glucuronidase (*uidA* gene) as reporter genes, they established an ideal pressure and microprojectile flying distance to transform immature sorghum embryos via bombardment. In this work it was also shown that the bialaphos was suitable to select transgenic cells of sorghum. Major problems in the protocol were the low efficiency (0,3%) and long time to select transgenic callus (7 months). In the next work (1997), the group tried to improve the transformation efficiency by using immature sorghum inflorescence, an explant with higher morphogenetic potential compared to immature embryos. At this time a greater number of transgenic plants were obtained but the overall efficiency of the process was low and the selection protocol generated many escapes.

Optimization of physical and biological parameters to produce transgenic sorghum plants was also the purpose of the work by Able et al. (2001) and Tadesse et al. (2003). Able and co-workers (2001) analyzed the transient expression of the reporter genes GUS and GFP over different physical bombardment parameters to identify the best conditions to generate transgenic sorghum plants using the particle inflow gun (PIG). Three transgenics events were confirmed by molecular analyses. Tadesse and associates (2003) also used reporter

genes to test different acceleration pressures, target distances, gap widths and macroprojectile travel distances to bombard immature and mature embryos, shoot tips and embryogenic calli. The strength of four different promoters (*ubi1*, *act1D*, *adh1* and *CaMV35S*) was also analyzed in transient assays. The optimization of the transformation conditions generated a protocol with an efficiency of 0.5 to 1.3% of transgenic sorghum production from shoots tips and immature embryos, respectively.

Currently few sorghum events expressing genes with agronomical interest were developed by the laboratories working with transformation of this specie via microprojectile bombardment. The gene *chi II*, encoding rice chitinase under the control of the constitutive CaMV35S promoter, has been transferred to sorghum for resistance to stalk rot (*Fusarium thapsinum*) by Zhu et al. (1998). Chitinases are proteins produced by plants as defense against pathogen attack; they function by degrading the fungal cell wall. Their work was done with calli developed from immature zygotic embryos as target tissue for microprojectile bombardment and, six independent events that were bialaphos-resistant and containing the chitinase gene were reported.

Another chitinase, the gene *ECH2*, and the *bar* gene were used to produce disease and herbicide resistant transgenic plants, respectively. Devi and Sticklen (2003) transformed shoots clumps, originated from mature sorghum seeds cultivated in presence of N^6-benzyladenine (BA), via microjectile bombardment with these genes. Shoot clumps were used with the purpose to develop a transformation protocol using an explant easier to be obtained throughout the year. Only five different events were generated, but the proficiency of sorghum shoot meristems for regeneration and transformation was demonstrated.

The *cry1Ac* gene from *Bacillus thuringiensis* under the control of the wound-inducible promoter from the maize protease inhibitor gene (*mpiC1*) was inserted in the genome of three independent transgenic sorghum events. Shoot apices were bombarded and subcultured in a MS medium supplemented with benzylaminopurine (BAP) and naphthalene acetic acid (NAA). Leaf damage by the spotted stem borer (*Chilo partellus*) was reduced up to 60% in the sorghum transgenic plants generated (Girijashankar et al., 2005).

To withstand toxic aluminum concentrations present in acidic soils, sorghum was genetically modified to express the *ALMT1* (Sasaki et al. 2004) gene from wheat (Brandão, 2007). The *ALMT1* gene codes a malate transporter Al^{+3} activated protein that is highly expressed in wheat root apices of aluminum tolerant cultivars. Transgenic sorghum plants grown in hydroponic culture under stress of Al^{+3}, showed a higher level of aluminum tolerance when compared with isogenic non-transgenic control plants.

3. Genetic transformation of immature sorghum inflorescence

Here, we report improvements made in the transformation process via microprojectile bombardment that enable us to obtain a protocol where putative transgenic plants can be produced with an efficiency ranging from 1.01 to 3.33% using immature inflorescences of sorghum.

3.1 Material and methods

3.1.1 Plant material and explants preparation

Seeds from nine *Sorghum bicolor* (Moench L.) accessions were obtained from the Embrapa Maize and Sorghum National Research Center – Brazil. Shoots were collected at different developmental stages (3 to 5 cm in length), from field plants prior to the appearance of the flag leaf (Figure 1). The outermost leaf blades were removed and shoots rinsed with 70% ethanol and sterile distilled water. After that, immature inflorescences were dissected, chopped in approximately 5 mm long segments and cultivated on callus induction medium (CIM) as described by Tadesse et al. (2003), with minor modifications [MS salts (Murashige and Skoog, 1962), 1 mg.L^{-1} thiamine HCl, 7.5 mg.L^{-1} glycine, 100 mg.L^{-1} DL-asparagine, 100 mg.L^{-1} myo-inosital, 0.2 mg.L^{-1} kinetin, 2.5 mg.L^{-1} 2,4-D and 30 g.L^{-1} sucrose]. Medium pH was adjusted to 5.8 with 1 N potassium hydroxide prior to autoclaving.Cultures were incubated at 25^{o}C in dark for three to four weeks.

For the biolistic experiments 30 calli pieces of approximately 3 mm diameter (Figure 2B) were uniformly distributed within a 35 mm diameter circle of 60 x 15 mm Petri dishes containing CIM media in which a higher osmotic value was achieved by the addition of 12% sucrose.

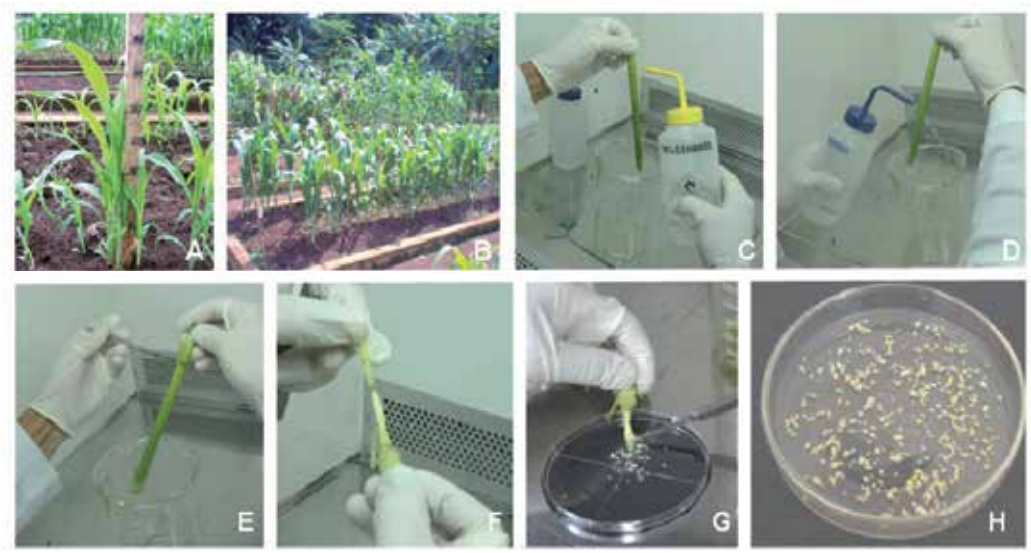

Fig. 1. Harvest and cultivation of immature sorghum inflorescence. (A and B) Sorghum plant used to isolate immature inflorescence; (C) Rinsing of sorghum shoots, without the outermost leaves, with 70% ethanol; (D) Rinsing of shoots with sterile distilled water; (E and F) Dissection of immature inflorescence; (G) Cutting the immature inflorescence in 5mm pieces; (H) Culturing inflorescence pieces.

3.1.2 Plasmid constructs

The genetic cassettes p35S::*C1* and p35S::*Bperu* (Goff *et al.* 1990), used in the transient transformation experiments, were kindly provided by Dr. Vicki Chandler from the Department of Plant Science, University of Arizona, Tucson, Arizona. These plasmids

contain the CaMV35S promoter, directing the expression of *B-peru* (1.9Kb) and *C1* (1.1 Kb) cDNAs, maize *Adh1* intron and nopaline synthase terminator. For the stable transformation experiments, the plasmid pCAMBIA3301 (Cambia, Canberra, Australia) which contains β-glucuronidase (GUS) reporter gene (Jefferson et al., 1987) and the *bar* gene (De Block et al., 1987) that encodes phosphinothricin acetyltransferase (PAT), both driven by the CaMV35S promoter was used.

3.1.3 Particle bombardment

Embryogenic calli were bombarded with tungsten microprojectiles using a biolistic particle helium acceleration device (Biomics – Brasília / Brazil). For the transient experiments 3 μL of each plasmid (stock 1 μg/μL), p35S::*C1* and p35S::*Bperu*, were co-precipitated with tungsten particles, while for the stable transformation 10 μL pCAMBIA3301 plasmid (stock 1 μg/μL) were used. To precipitate DNA onto the microparticles, plasmid DNA were mixed with 50μL (60 $mg.mL^{-1}$) tungsten particle M10 (Sylvania, GTE Chemicals/ Towanda – USA) under low agitation. Next, 50μL of 2.5M $CaCl_2$ and 20μL of 0.1M spermidine were sequentially added and homogenized. The mixture was kept for three minutes under low agitation and for an additional three minutes without agitation. Particles coated with DNA were centrifuged for five seconds and the supernatant was removed. DNA-coated particles were washed carefully once with 70% ethanol, twice with 100% ethanol, and suspended in 100% ethanol. Eight microliters of DNA-coated particle were deposited at the center of sterile macro-carries membrane (Ficael, São Paulo, SP).

Treatments	Osmotic Media (hours)	Helium Pressure (psi)	Microcarrier Flying Distance (cm)
1	0	1 000	3
2	0	1 000	6
3	0	1 000	9
4	4	1 000	3
5	4	1 000	6
6	4	1 000	9
7	24	1 000	3
8	24	1 000	6
9	24	1 000	9
10	0	1 200	3
11	0	1 200	6
12	0	1 200	9
13	4	1 200	3
14	4	1 200	6
15	4	1 200	9
16	24	1 200	3
17	24	1 200	6
18	24	1 200	9

Table 1. Conditions tested in transient transformation experiments.

Eighteen treatments (Table 1) were designed to test the permanence of explants on osmoticum prior to bombardment (0, 4 and 24 hs), pressure of the accelerating helium pulse (1000 and 1200 psi), and microprojectile flying distance (3, 6 and 9 cm), in transient sorghum transformation. The distance between the high pressure chamber and the macro-carrier membrane (8 mm), the distance between the macro-carrier membrane and the retention screen (17 mm) and the vacuum pressure (27 mmHg) were maintained constant. For each treatment three plates containing 30 calli pieces were bombarded once.

3.1.4 Expression analysis

A*nthocyanin:* For the anthocyanin expression studies, bombarded calli were incubated at 27°C for 2 d in the darkness. The number of anthocyanin spots was scored under a stereoscope (Zeiss Stemi SV11, Germany).

GUS: GUS expression was detected after explants were incubated at 37°C for 20 h in a solution containing 1 mM 5-bromo-4-chloro-3-indolyl β-D glucuronide (X-Gluc, Sigma Chem. Co., São Paulo, Brazil), 50 mM phosphate buffer pH 6.8, 20% methanol, 1% Triton X-100 (Rueb and Hensgens, 1998). Chlorophyll was extracted from leaf tissue in 70% ethanol for 30-60 min.

PAT: Seeds from four independent transgenic T_0 events tested positive by PCR and PAT activity were propagated, and the T_1 plants screened for PAT and GUS enzyme activity inheritance. From each T_0 line 50 seeds were propagated in greenhouse. Seedlings with four leaves were sprayed with 0.6% aqueous solution of the commercial herbicide Finale® (Bayer, São Paulo, Brazil) to confirm expression of the *bar* gene. Control non-transgenic plants or segregating seedlings showed symptoms in 2–5 d and died within 2 weeks. Leaf material from PPT-resistant plants was GUS stained as described above. Chi-square goodness of fit was used to test the significance of observed versus expected ratios.

3.1.5 Selection procedures

Explants were cultured on solid CIM media at 25°C in the dark for one week and transferred to selective SE media (modified CIM supplemented with 0.5 $mg.L^{-1}$ kinetin and without DL-asparagine) containing 15 $\mu L.L^{-1}$ of the herbicide Finale® (3 $mg.L^{-1}$ 4-hidroxi(methyl) phosphynol-DL-homoalanine ammonium salt) for one week. After that, the explants were transferred to a media containing 30 and 45 $\mu L.L^{-1}$ herbicide every week. Growing calli were cultured for one more week in a SE media supplemented with 45 $\mu L.L^{-1}$ herbicide, and subsequently transferred to a callus maturation media RM [MS salts and vitamins (Murashige and Skoog, 1962), 60 $g.L^{-1}$ sucrose, 100 mg. L^{-1} myo-inositol, 0.2 $mg.L^{-1}$ NAA, 3 g/L phytagel, pH 5.8) supplemented with 30 $\mu L.L^{-1}$ herbicide and cultured in the dark at 25°C for somatic embryo maturation. Approximately 2 to 4 weeks later, mature somatic embryos showing a white and opaque coloration were transferred to Magenta boxes (Sigma, São Paulo, Brazil) containing germination media composed by MS media without plant growth regulators, supplemented with 15 $\mu L.L^{-1}$ herbicide and placed in a lighted (16 h / 60 $\mu mol\ m^{-2}\ s^{-1}$) growth room. Germinated plantlets (4-6 cm) were cultured in soil, for the first week under a plastic lid, in a greenhouse.

3.1.6 Plant DNA extraction, polymerase chain reaction (PCR) and Southern blot hybridization analysis

Total genomic DNA was isolated from leaf tissue of primary transformants using a CTAB protocol described by Saghai-Maroof *et al.* (1984). The presence of *bar* and *uid*A genes were detected, initially, by the polymerase chain reaction (PCR). The 407 bp coding region of *bar* gene was amplified using primers (AGA AAC CAC GTC ATG CC and TGC ACC ATC GTC AAC CAC). The 406 bp coding region of *uid*A gene was amplified using the primers (TCG TGC TGC GTT TCG ATG and GCA TCA CGC AGT TCA ACG). Each 25 uL amplification reactions containing 50 ng of template DNA, 5 µM each primer, 500 µM dNTP mixture, 2.5 µl TAQ DNA polymerase reaction buffer and 1 unit Taq DNA polymerase (Invitrogen, São Paulo, Brazil) were carried out using a thermal cycler (Eppendorff Mastercycler, Hamburg, Germany) under the following conditions: 94ºC for 5 min; 30 cycles 94ºC for 60 s; 55ºC for 30s, and a final extension at 72ºC for 10 min. The amplified products were separated by electrophoresis on a 1.2% agarose gel and visualized with ethidium bromide.

For Southern blot analysis 10 µg of total genomic DNA from each T_0 plant were completely digested with *Xho*I at 37ºC overnight. Digested DNA fragments were separated by electrophoresis in 0.8% agarose gel, and then transferred to a Hybond-N^+ nylon membrane (Amersham, São Paulo, Brazil) according to Sambrook *et al.* (1989). The blot was hybridized with a P^{32} labeled *bar* gene coding region. Negative control samples consisted of non-transgenic genomic DNA. After overnight hybridization at 65°C, the membranes were washed in 2X SSC, 0.1% SDS at room temperature and in 1X SSC, 0.1% SDS at 65°C and exposed to Kodak™ XAR-5 film at 75ºC for 3 d.

3.1.7 Statistical data analysis

The data obtained from the transient experiment was collected in Microsoft Excel (Version 5).The experimental design was based on randomized blocks, factorial 2x3x3 (2 pressure of the accelerating helium pulse, 3 time of explants on osmoticum and 3 microprojectile flying distance), with triplicates, totalizing 54 experimental units. The data were subjected to ANOVA and means compared by Tukey test ($p< 0.05$), using the statistical program SISVAR 4.0 (Ferreira, 2000).

4. Results

4.1 Explant preparation and selection of transformed calli *in vitro*

Nine *Sorghum bicolor* accessions were selected and screened for the quality of callus produced four weeks after cultivation of immature inflorescence on callus induction media (CIM). Embryogenic calli were produced at different efficiency levels by all of the accessions (data not shown). For this study, the genotype CMSXS102B, which gave the highest percentage of immature inflorescence sections producing embryogenic callus (85%), was used.

Embryogenic calli from immature inflorescence of sorghum were bombarded three to four weeks after cultivation in CIM media (Fig. 2*B*), with the plasmids containing the *B-peru* and *C1* or *bar* and GUS genes for transient and stable transformation experiments, respectively. Within this cultivation period, explants started to expand and enlarge at the cut edges.

Initially, the bombarded embryogenic calli had a compact appearance and then they developed into more friable structures in the next weeks of cultivation. After bombardment, calli were transferred back to CIM media without selection pressure for one week, and then moved to selection medium.

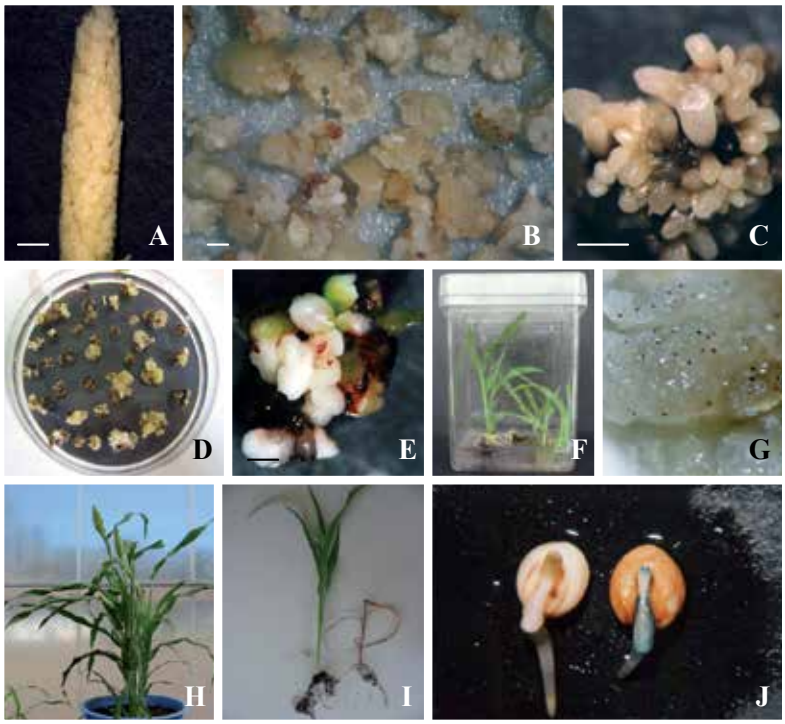

Fig. 2. Genetic transformation of calli from immature sorghum inflorescence. (A) Immature sorghum inflorescence; (B) Calli used for the particle bombardment; (C) Embryogenic callus induced from immature inflorescence; (D) Herbicide resistant calli after 6 weeks particle bombardment; (E) Maturation of calli after selection; (F) Regeneration of herbicide resistant callus, (G) Transient expression of anthocyanin; (H) Putative transgenic *Sorghum* plants in greenhouse; (I) Transformed (left) and wild type (right) sorghum plants weeks after application of herbicide Finale®; (J) GUS expression of wild type (left) and transgenic (right) germinated seeds. *Bar* = 1mm.

Growth of bombarded callus was slightly inhibited and some of them turned brown on selection medium supplemented with 3.0 mg.L^{-1} of PPT, compared to non-bombarded ones. After one week of cultivation the concentration of PPT on selection medium was increased to 6 mg.L^{-1} and most of the bombarded calli turned black. In order to reduce escapes within chimerical clusters, surviving bombarded clumps were carefully divided and cultured, at one week intervals, onto selection medium supplemented with 9 mg.L-1 PPT, during four weeks. At this herbicide concentration complete inhibition of non-transgenic calli growth was observed, most calli turned dark, necrotic and died (Fig. 2*D*). After six weeks of selection, surviving calli were transferred onto maturation media supplemented with 6

mg.L^{-1} PPT. On this medium, as soon as the yellowish calli become white and opaque, between 2 to 4 weeks of cultivation, they were transferred onto germination medium (Figure 2E). A concentration of 3 mg.L^{-1} PPT was used for the differentiation and germination of mature calli that occurs around 20 d of cultivation. Control non-bombarded explants did not survive on selection medium containing 6 or 9 mg.L^{-1} PPT. This selection procedure has been used successfully in different transformation experiments with calli derived from immature inflorescence of sorghum. The overall time for selection and regeneration of putative transgenic plants using this protocol is around 16 weeks.

4.2 Transient and stable transformation

The optimization of DNA delivery parameters was initially performed by using the transient expression of maize *R* and *C1* transcriptional activators. All bombardments were carried out with plasmidial DNA from the same stock, and the number of anthocyanin spots ranged from 3 to 348 depending on the particle bombardment conditions used (Fig. 2G).

Statistical analyses of the transient anthocyanin expression (Table 2) identified interactions among the different factors studied. Embryogenic calli submitted to 1000 psi of helium accelerating pressure, cultivated during 4 h in a higher osmotic medium and positioned at 3 cm from the micro-carrier launch platform (Treatment 4) presented a larger number of cells expressing anthocyanin than when the explants were positioned at 6 or 9 cm (Treatment 5 and 6). Without the pre-cultivation of explants in an osmotic medium, there were no differences among the positions (3, 6 or 9 cm) of explants (Treatments 1 to 3) and the overall amount of anthocyanin spots was lower. At 1200 psi acceleration pressure, treatments 10 to 18, the best results obtained were when calli were cultivated in osmotic medium for 24 h and positioned at 3 or 6 cm from the micro-carrier launch platform (Treatments 16 and 17).

	TARGET DISTANCE		
Time in osmotic medium	**Pressure 1000 psi**		
	3 cm	6 cm	9 cm
Without pre-treatment	(1) 30,283 aA	(2) 47,246 aA	(3) 37,013 aA
4 hours	(4) 91,356 bB	(5) 52,786 aAB	(6) 31,170 aA
24 hours	(7) 74,930 aA	(8) 79,240 aA	(9) 42,813 aA
Time in osmotic medium		**Pressure 1200 psi**	
Without pre-treatment	(10) 43,183 aA	(11) 41,686 aA	(12) 44,626 aA
4 hours	(13) 44,736 aA	(14) 74, 263 aA	(15) 44,370 aA
24 hours	(16) 88,576 bB	(17) 59,086 aB	(18) 18,343 aA

Note. Means followed by the same small letter in the vertical and capital letter in the horizontal are not significantly different at 5% level according to Tukey's multiple range test. Numbers in parentheses represent the different treatments.

Table 2. Mean number of anthocyanin spots induced transiently by p35S::*C1* and p35S::*Bperu* constructs in embryogenic callus of the sorghum.

The highest number of cells expressing the anthocyanin genes was obtained with embryogenic calli cultivated in osmotic medium during 4 h before the bombardment, positioned at 3 cm distant from the microcarrier release platform and using 1000 psi

accelerating pressure. Therefore, these conditions were used in six independent experiments to test stable transformation of embryogenic calli obtained from immature sorghum inflorescences, with a cassette containing the *bar* and *iud*A genes. The transformation efficiency for these sets of experiments ranged from 1.01% to 3.33 % (Table 3).

Experiment number	Number of transgenic events	Number of calli bombarded	% Efficiency
RLB3962008	1	93	1.07
RLB5420703	3	120	2.5
RLB5430703	2	60	3.33
RLB5222202	2	180	1.11
RLB020106	3	296	1.01
RLB120705	2	150	1.33

Table 3. Biolistic transformation of calli from immature sorghum inflorescence

4.3 Evaluation of transgenic material

To estimate the transgene copy number and the inheritance of the *bar* gene, T_1 progenies were tested for their tolerance to the herbicide Finale ®. Germinated T_1 transgenic and control seedlings at the stage of five leaves were sprayed with herbicide and scored for damage 7 and 14 d after the application. Transgenic seedlings segregated for the presence of the *bar* gene, there were plants with and without tissue damage, while all control non-transgenic plants presented necrosis and died (Fig. 2I).

Transgenic Lines Number	Herbicide-resistant plants	Herbicide-sensitive plants	Segregation ratio	Chi-square
1 (RLB3962008)	28	16	3:1	$\chi^2 = 3,03$; P>0,05
2 (RLB5222202)	07	01	3:1	$\chi^2 = 0,67$; P>0,05
3 (RLB5430703)	34	12	3:1	$\chi^2 = 0,029$; P>0,05
4 (RLB5420703)	37	8	3:1	$\chi^2 = 1,25$; P>0,05
5 (RLB2109056A)	23	25	1:1	$\chi^2 = 0,83$; P>0,05
6 (RLB2109053B)	28	20	1:1	$\chi^2 = 1,33$; P>0,05
Wild-type plant (CMSXS102B)	0	50	ND[a]	ND[a]

[a] – ND, Not determined

Table 4. Independent transgenic T_1 generation plants analyzed for the inheritance and segregation of herbicide resistance

Segregation data obtained from six T_1 progenies sprayed with herbicide is presented in Table 4. Among the progenies of self -pollinated T_0 transgenic plant lines, Chi-square tests showed a Mendelian segregation ratio of 3:1 in four lines. This ratio indicated that the *bar* gene was inserted in a single locus, efficiently inherited and transcribed in T_1 progeny plants. Two lines (Lines 5 and 6) showed a 1:1 ratio, suggesting semi-dominance.

Leaves of all T_0 transgenic events tested negative for the β-glucuronidase expression. However, GUS expression could be detected in germinated seeds (T_1) of event RLB5420703 (Fig. 2J).

The presence of *uid*A and *bar* genes in genomic DNA of all independent T_0 lines was confirmed by PCR analysis of genomic DNA. The results revealed the presence of 406 bp band of *uid*A and 407bp band of *bar* genes in all of the plants tested (Fig. 3A and B).

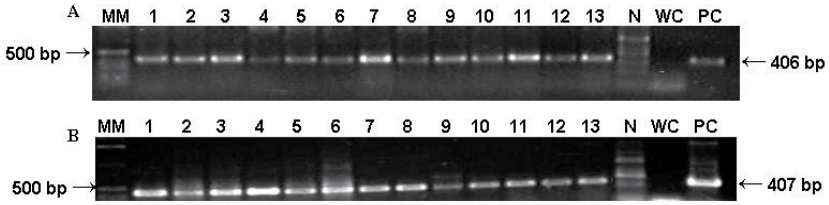

Fig. 3. PCR analysis of genomic DNA extracted from transgenic primary generation (T_0) of *Sorghum bicolor*. (A) PCR amplification of a *uid*A gene, showing the 406 bp fragment (lanes 1-13); (B) PCR amplification of *bar* gene, showing a 407 bp fragment (lanes 1-13). M: molecular weight marker, N: control wild type sorghum plants; WC: water control; PC: plasmid positive control.

The stable integration of the *bar* gene in the transgenic events were analyzed by Southern blotting of genomic DNA digested with *Xho*I, which cuts outside the *bar* coding sequence releasing a 564 bp fragment. No hybridization signal was present in the digested DNA from the untransformed plants (Fig. 4).

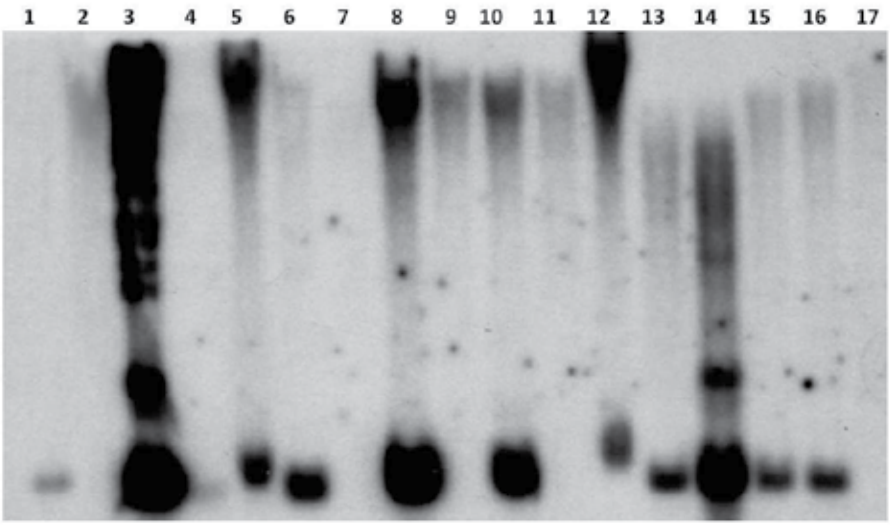

Fig. 4. Southern blot analysis of genomic DNA from transgenic *Sorghum bicolor* plants (cultivar CMSXS 102B). Genomic DNA was digested with *xho*I and hybridized with a radiolabelled 520 pb *bar* fragment. Lane 1: plasmid positive control; lanes 2-16: transformed plants ; lane 17: wild type *S. bicolor* total DNA.

5. Discussion

Immature inflorescence proved to be an excellent organ to increase considerably the quantity of tissue competent of embryogenic callus production. A large number of high quality calli is relatively easier and faster to produce from immature inflorescence.

Transient expression of anthocyanin allows us to detect in a rapid and precise manner the most efficient combination of biolistics parameters that rendered a higher transient expression. The frequency of transient activity expression as an indicator of stable transformation efficiency has already been used, successfully, by Christiansen *et al.* (2005) to optimize the transformation conditions of *Brachypodium distachyon*. They observed that treatments with a higher number of GUS spots where the ones that produced a larger number of stable transgenic events.

An important step in the transformation via biolistics is the wound suffered by the explant during the microparticle entry into the cell. Usually to minimize this type of problem and to increase the capacity for somatic embryogenesis and plant regeneration, the target cells are plasmolised by an osmotic treatment (Vain *et al.* 1993). In this study all treatments where sorghum explants were incubated in an osmotic media a few hours before bombardment produced a higher number of anthocyanin spots, confirming that plasmolysis of cells can reduce damage and increase the efficiency of bombardments.

Acceleration pressure and microcarrier flying distance are parameters that influence the ability to deliver DNA into various explants. Analyzing the transient expression of anthocyanin, it was observed that a helium gas pressure of 1000 psi combined with a distance of 3 cm rendered the higher number of anthocyanin spots. This combination of biolistic physical parameters when tested in stable transformation experiments showed an efficiency of up to 3.33% of transgenic sorghum events production. Even though, biolistic parameters should be optimized for each equipment and explant used, other authors found optimal bombardment conditions similar to our results. Casas *et al.* (1993) and Tadesse *et al.* (2003) were able to generate transgenic sorghum plants via biolistic using a macro-carrier flying distance of 6 cm and a pressure of 1100 psi with an efficiency ranging from 0,3% to 1,3%.

We introduced the *uidA* and *bar* genes under the control of the CaMV35S promoter into sorghum CMSXS102B genome. The transformed plants were determined by a combination of PCR and Southern blot analysis, together with assays demonstrating functional gene product activity, PAT and GUS. Histochemical GUS activity was absent in leaves of the T_0 plants, but could be detected in the T_1 germinating seeds of one of the events investigated in this study. The absence of GUS expression in transgenic sorghum has been reported by several investigators (Emani *et al.*, 2002; Carvalho *et al.*, 2004; Girijashankar *et al.*, 2005; Van Nguyen *et al.*, 2007). Factors such as methylation based silencing (Emani *et al.*, 2002), regulatory sequences present in the genetic cassette (Tadesse *et al.*, 2003; Carvalho *et al.*, 2004) and phenolic compounds typically present in the sorghum tissue culture (Carvalho *et al.*, 2004) might have contributed for the absence of GUS expression.

The analysis of PPT-resistance showed that the trait was expressed by all of the transgenic events recovered, probably because of the herbicide selection pressure. In addition, it was inherited by the T_1 progenies with a typical Mendelian segregation pattern in four out of six transgenic lines studied. Two lines showed a 1:1 segregation ratio; this type of transgene segregation had already been reported in wheat and maize (Cheng *et al.*, 1997; Ishida *et al.*

1996). This abnormal segregation pattern might be partially caused by gene silence or non-detectable gene expression in the transgenic plants (Cheng *et al*., 1997; Vaucheret *et al*., 1998).

We report a transformation methodology for calli derived from immature inflorescence of sorghum, via biolistics; these transformation conditions are already being used at Embrapa Maize and Sorghum to introduce genes of agronomical interest into the sorghum genome.

6. Acknowledgements

We thank the McKnight Foundation, Fapemig "Fundação de Apoio a Pesquisa do Estado de Minas Gerais" and CNPq "Conselho Nacional de Desenvolvimento Científico e Tecnológico"-Brazil for financial support. We also acknowledge Dr. Vicki Chandler from the Department of Plant Science, University of Arizona, Tucson, Arizona /USA for the donation of genetic cassettes p35S::*C1* and p35S::*Bperu.* This publication has been funded by "Fundo MP2 Embrapa / Monsanto".

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7

Phytoremediation of Bis-Phenol A via Secretory Fungal Peroxidases Produced by Transgenic Plants

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1. Introduction

The fungal lignin-degrading enzymes lignin peroxidase (LiP, E.C. 1.11.1.14), Mn-dependent peroxidase (MnP, E.C. 1.11.1.13), and phenol oxidase (laccase) (Lac, E.C. 1.10.3.2) can degrade or polymerize organic pollutants such as polychlorophenols, polycyclic aromatic hydrocarbons, and chlorinated hydrocarbons (Fernando and Aust, 1994; Hammel, 1989; Hirano et al., 2000; Levin et al., 2003; Lin et al., 1990; Lovley et al., 1994; Mohn and Tiedje, 1992; Reddyy et al., 1998). However, to maintain such fungal lignin-degrading enzymes at adequate levels for degradation or detoxification (bioremediation), appropriate additions of both microorganisms and nutrients are essential over long periods of time. Recently, phytoremediation technology has gained attention for its potential as an ecological remediation tool of contaminated soil and water, as plants can grow autotrophically. Establishment of effective phytoremediation technology is a suitable strategy for the long-term remediation of contaminated areas. Phytoremediation includes some processes based on the plant functions as follows; phytostabilization, which is accumulation of pollutants in the rhizosphere by absorption on the root surface, precipitation, and complexation of pollutants; rhizodegradation, which is degradation of pollutants by interaction with rhizosphere microorganisms; phytoaccumulation (phytoextraction), which is uptake and accumulation of pollutants by plants; phytodegradation (phytotransformation), which is uptake and degradation of pollutants by plants; and phytovolatilization, which is uptake and volatilization of pollutants by transpiration from contaminated area. To widely apply the benefit of phytoremediation, improvement and reinforcement of the abilities for uptake, accumulation and degradation of pollutants using genetic engineering are one of the important development subjects.

There have been many reports of phytoremediation using transgenic plants. For example, glutathione S transferase and cytochrome P450 expression showed high resistance to pesticides (Gullner et al., 2001; Doty et al., 2000), the overexpression of bacterial mercury

reductase showed high resistance to organic mercury (Bizilly et al., 2003) and effective volatilization of ionic mercury (Haque et al., 2010), pentaerythritol tetranitrate reductase-expressing plants were able to degrade glycerol trinitrate and 2,4,6-trinitrotoluene (French et al., 1999), introduction of bacterial genes involved in polychlorinated biphenyl (PCB) degradation in plants showed removal of PCB from a contaminated area (Novakova et al., 2009), the bacterial arsenite S-adenosylmethyltransferase expression induced arsenic methylation and volatilization (Xiang-Yan et al., 2011), the expression of gamma-glutamylcysteine synthetase and the genes involved in phytochelatin synthesis in plant showed more resistance and accumulation of cadmium (Zhu et al, 1999, Wawrzyński et al, 2006), and the yeast metallothionein expressing tobacco showed effective copper uptake (Thomas et al, 2003).

Recently, attempts are carried out to enhance the environmental remediation in contaminated area by using appropriate genetically modified plants with usage of fungal peroxidases. This chapter mainly focused on the removal of bis-phenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane), which is one of the major chemicals used in plastics and resins and is well known to disrupt endocrine systems in humans and other animals, from contaminated areas with usage of transgenic technology. Although many organisms can degrade and metabolize BPA, which can lead to a reduction of the estrogenicity and toxicity of BPA (Kang et al., 2006), lignin-degrading basidiomycete fungi are particularly powerful degraders of organic pollutants including BPA. These fungi produce oxidative enzymes, such as LiP, Lac, and MnP, which can degrade and polymerize BPA both *in vivo* and *in vitro*. Therefore, an overview of our recent results regarding the phytoremediation of BPA with fungal peroxidase-expressing transgenic plants by lignin peroxidase (LiP), laccase (Lac), and manganese peroxidase (MnP) were presented together with the other potential uses of these transgenic plants in this chapter.

2. LiP-expressing transgenic tobacco

cDNA (Accession no. AB158478.1) encoding LiP from the reverse transcription (RT) products of total RNA prepared from mycelia of *Trametes versicolor* IFO1030 was isolated. The cloned cDNA was ligated into binary vector pBI121 (Brasileiro et al., 1991) with double cauliflower mosaic virus (CaMV) 35S promoter sequence (Figure 1), and was introduced into the genome of the tobacco (*Nicotiana tabacum* Samsun NN) by the leaf-disk method via *Agrobacterium tumefacience* LBA4404 (Liang et al., 1989).

Integration of the cDNA into the genome of tobacco was confirmed by polymerase chain reaction (PCR) upon 10 independent transgenic lines. Two of the lines showed growth inhibition and thus were excluded from further analysis. Western blot analysis with root extracts of transgenic tobaccos and antiserum raised against LiP protein were performed to confirm the production of LiP protein in roots of transgenic lines. . To prepare the antiserum against LiP of *T. versicolor* IFO1030, we synthesized one peptide, whose sequence was ^{240}CNGTTFPGTGDNQG^{254}E, and conjugated it with keyhole limpet hemocyanin (KLH). The resultant peptide-KLH conjugant was injected into a 10-wk-old rabbit. After four injections, antiserum was collected and used for Western blot analysis. The expected signal was observed in the cell-free extracts of roots from LiP transgenic tobaccos (Figure 2).

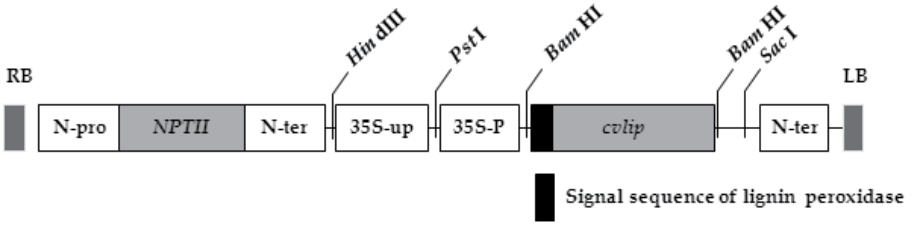

Fig. 1. Gene construct of T-DNA region of Ti plasmid.

RB, Right border of T-DNA; N-pro, promoter region of nopaline synthase gene; *NPTII*, neomycin phosphotransferase gene; N-ter, terminator region of nopaline synthase gene; 35S-up, upstream region of cauliflower mosaic virus (CaMV) 35S promoter sequence; 35S-P, CaMV 35S promoter sequence; *cvlip*, cDNA encoding LiP of *T. versicolor* IFO1030 plus signal sequence; LB, left border of T-DNA.

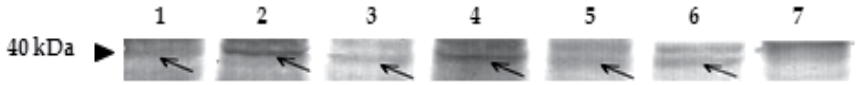

Fig. 2. Western blot analysis of LiP in cell-free extracts of roots of LiP transgenic lines.

Lanes; 1, LiP transgenic line (FLP)-1; 2, FLP-2; 3, FLP-3; 4, FLP-4; 5, FLP-5; 6, FLP-8; 7, control plant.

To test the ability of BPA removal by LiP-expressing transgenic plants, we transferred two-month-old transgenic lines on MS medium (Murashige and Skoog, 1962) to fresh MS liquid medium containing 3 g/L of glucose and 100 µg/L of kanamycin. After one week of incubation at 25°C, BPA was added to the medium at the final concentration of 100 µM and the medium was hydroponically incubated for another week. The six LiP-expressing transgenic lines showed 2- to 4-fold higher BPA removal ability than that of control plants during aqueous cultivation (Figure 3). LiP is a well-known enzyme that carries out direct and indirect oxidation of a number of environmental pollutants. Our confirmation that transgenic plants could express LiP in their roots and remove BPA will help us to establish improved methods for phytoremediation of contaminated environments.

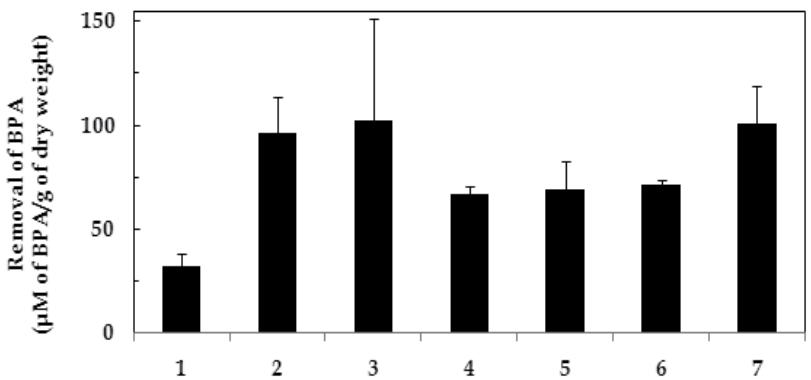

Fig. 3. Removal of BPA by LiP-expressing transgenic lines.

The levels of BPA were analyzed by HPLC (λ=278 nm). The values shown are the average of results from three independent experiments. Lanes; 1, control; 2, FLP-1; 3, FLP-2; 4, FLP-3; 5, FLP-4; 6, FLP-5; 7, FLP-8. Error bars on the graph indicate standard deviations (*N*=3).

3. Lac-expressing transgenic tobacco

Lac is a member of the multicopper oxidase family found in a wide range of organisms such as animals, plants, bacteria, and fungi. The reduction of oxygen to water is accompanied by the oxidation of substrate by laccase.

cDNA encoding Lac (Accession no. D13372.1) from the reverse transcription products of total RNA prepared from mycelia of *T. versicolor* IFO1030 was cloned. The cDNA under the control of double CaMV 35S promoter was introduced into the genome of *N. tabacum* Samsun NN by the leaf-disk method via *A. tumefacience* LBA4404 (Figure 4).

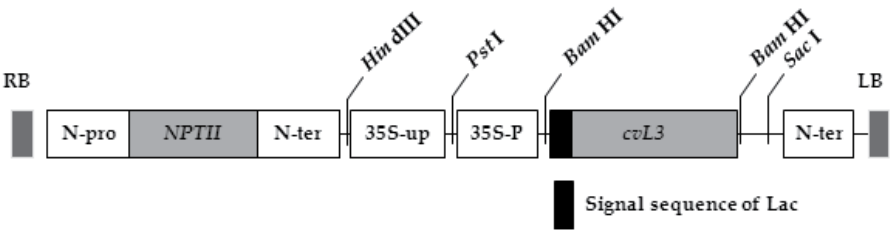

Fig. 4. Gene construct of T-DNA region of Ti plasmid.

cvL3, cDNA encoding Lac of *T. versicolor* IFO1030 plus signal sequence. Other abbreviations are listed in Figure 1.

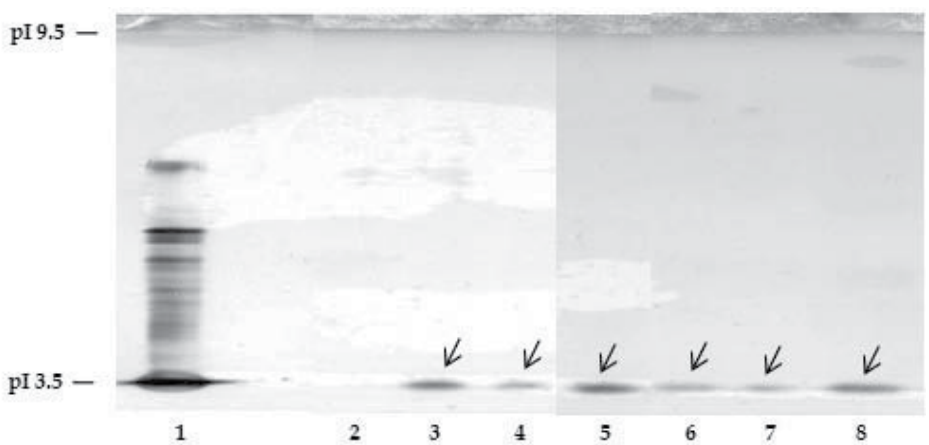

Fig. 5. Active staining of secreted Lac from the roots of transgenic lines.

Concentrated 60 μg of crude extracellular protein was analyzed by IEF and active staining using 4-chloro-1-naphthol. Lanes, 1, Concentrated aqueous cultivation medium of *T. versicolor* IFO1030; 2, control; 3, Lac transgenic line (FL)-4; 4, FL-5; 5, FL-9; 6, FL-20; 7, FL-22; 8, FL-23.

Two-month-old transgenic lines, which were incubated on MS medium, were transferred to fresh MS liquid medium and subjected to further incubation. After two weeks, to confirm the expression of Lac protein and secretion from the roots of each transgenic line into the rhizosphere, we concentrated the aqueous culture medium and analyzed it by iso-electric focusing electrophoresis (IEF) and active staining using 4-chloro-1-naphtol (Figure 5). Six independent transgenic lines apparently secreted active Lac protein into their rhizosphere, and we tested four of those to determine their ability to remove BPA. As described above, four independent transgenic lines were cultivated hydroponically. After one week of incubation, BPA was added to the medium at the final concentration of 100 μM and hydroponic incubation was done for another week. The ability to remove BPA of these Lac-expressing transgenic tobaccos was more than 5-fold that of the control line during hydroponic cultivation (Figure 6).

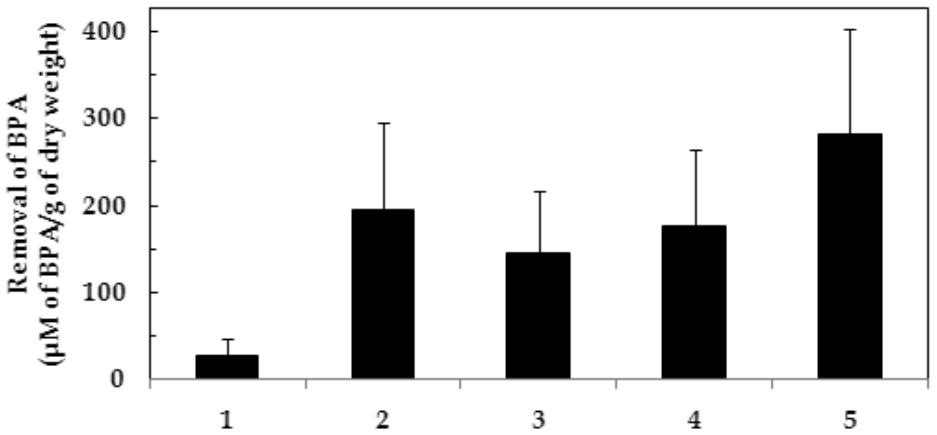

Fig. 6. BPA removal ability of Lac transgenic lines.

The levels of BPA were analyzed by HPLC (λ=278 nm). The results shown are the average of three independent experiments. Lanes; 1, control; 2, FL-4; 3, FL-9; 4, FL-20; 5, FL-22. Error bars on the graph indicate standard deviations (*N*=3).

All of these Lac-expressing transgenic tobaccos were somewhat shorter than control plants at the flowering stage, and most of the transgenic anthers failed to dehisce after blooming, while the anthers of control plants were normally dehiscent (Figure 7). In addition, the nondehiscent anthers were brown in contrast to the greenish control lines. Brown pigmentation and rough epidermis were observed on the surface of transgenic anthers. Greater Lac activity was detected in the cell-free extracts of transgenic anthers than in the controls; however, there was no correlation with lignin contents in transgenic anthers (Figure 8). Histochemical analysis of anther tissues revealed apparent deformation of the stomium in transgenic plants (Figure 9). Beals reported that the stomium in anther tissue plays a crucial role in the dehiscence of anthers in tobacco (Beals, 1997), indicating that such deformation of stomium observed in the transgenic anther tissue might affect the appearance of the nondehiscent phenotype. The expression of Lac could promote the efficient removal of BPA, but it also influences some aspects of flower development.

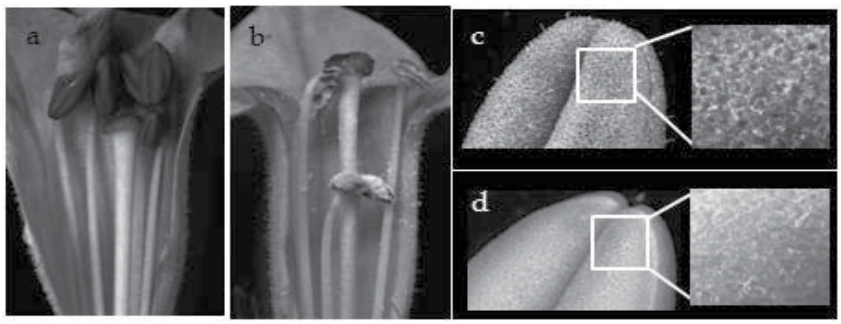

Fig. 7. Phenotypes of anthers of Lac transgenic lines.

Transgenic and control tobaccos were cultivated at 24°C. a, Transgenic flower with nondehiscent anthers. b, Control flower with normal anthers. c, Stereomicroscopic view of a transgenic anther. d, Stereomicroscopic view of a normal anther.

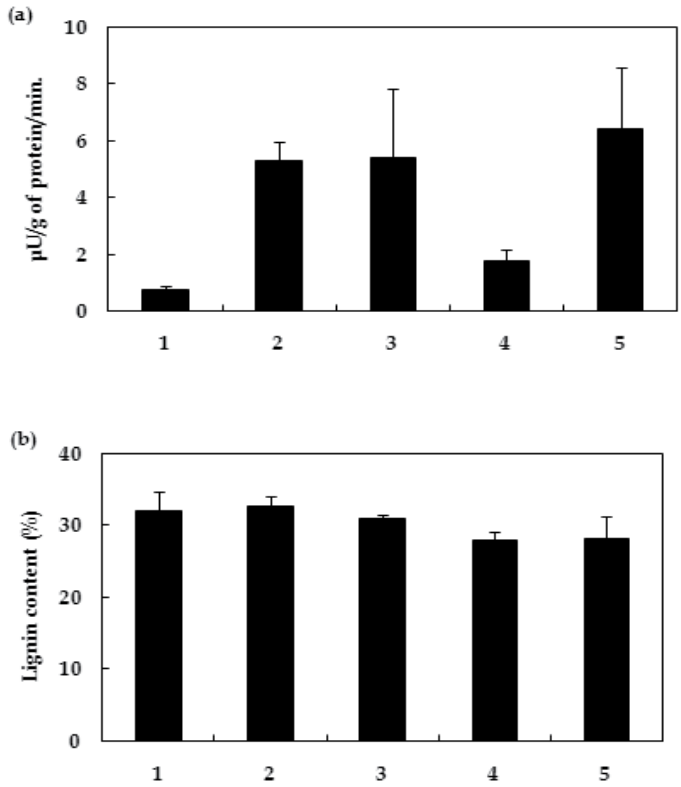

Fig. 8. Laccase activity and lignin content in anther tissues.

Transgenic and control tobaccos were cultivated at 24°C. a, Laccase activity. Cell-free extracts were prepared from both transgenic and control anthers before they dehisced. Laccase activity was calculated using the extinction coefficient (6400 $M^{-1}cm^{-1}$) of oxidized guaiacol (λ=436nm), and activity was expressed as definitive units (1 unit = 1 mol guaiacol

oxidized per min) (Eggert et al, 1996). b, Lignin content. Lignin was quantified by the Klason method. The results shown are the average of three independent experiments. Error bars on the graph indicate standard deviations (*N*=3). Lanes; 1, control; 2, FL4; 3, FL9; 4, FL20; 5, FL22

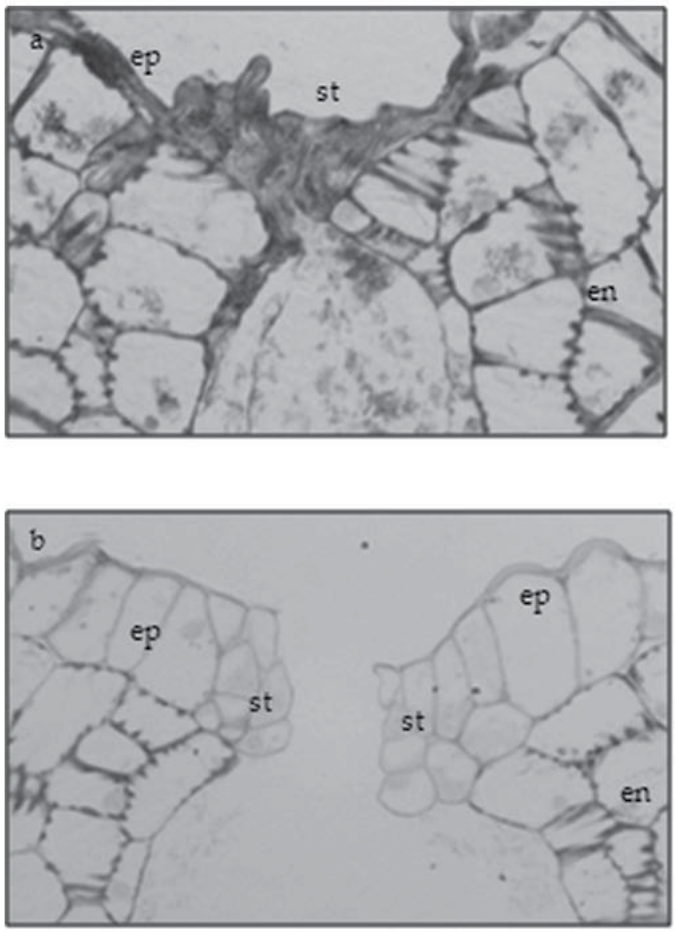

Fig. 9. Histochemical analysis of anther tissues.

Safranin-stained thin sections of a mature anther from a transgenic (a) and a control plant (b). ep, epidermis; st, stomium; en, endothecium cell.

4. MnP-expressing transgenic hybrid aspen

MnP is a heme peroxidase that can oxidize phenolic compounds in the presence of Mn (II) and hydrogen peroxide. Mn (II) is oxidized to Mn (III) by MnP; the resultant Mn (III) makes a chelating compound with an organic acid, and then organic compounds such as BPA are oxidized by the chelating compound. Previously, we isolated a cDNA (Accession no. AR429405) encoding MnP from *T. versicolor* and introduced it into the genome of *N. tabacum* Samsun NN. The transgenic tobacco could express MnP and produce Mn (III) as a result of Mn (II) oxidation in the rhizosphere during hydroponic cultivation (Iimura et al., 2002).

Moreover, isolated cDNA was also introduced into the genome of hybrid aspen Y63 (*Populus seiboldii* x *Populus gradientata*) under the control of double CaMV 35S promoter (Figure 10), as described previously (Kajita et al., 2004). Integration of the T-DNA into the genome of each transgenic line was confirmed by PCR. Although the expression of cDNA encoding MnP was confirmed by RT-PCR in six independent transgenic lines, MnP activity was detected in four of the six lines (Figure 11). The BPA-removing activities of the four MnP-expressing transgenic hybrid aspens were more than twice that of the control lines (Figure 12). Interestingly, the expression of the MnP gene showed no phenotypical differences between the MnP-expressing and control plants, unlike the expressions of the LiP and Lac genes. The lack of negative effects of MnP expression on growth and development will be advantageous when it is used in phytoremediation. Our results showed that the transgenic plants could express MnP in their roots and contribute to the effective removal of BPA from a hydroponic medium.

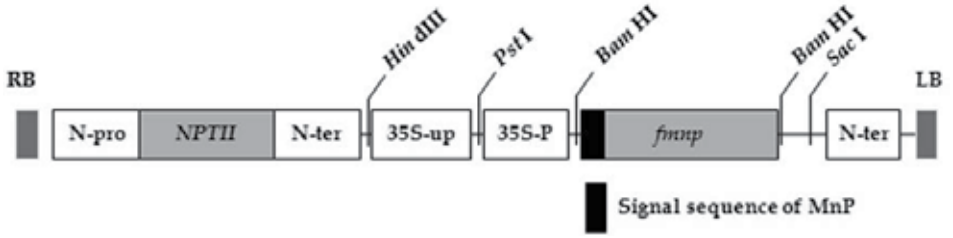

Fig. 10. Gene construct of T-DNA region of Ti plasmid.

fmnp, cDNA encoding MnP of *T. versicolor* IFO1030 plus signal sequence. Other abbreviations are listed in Figure 1.

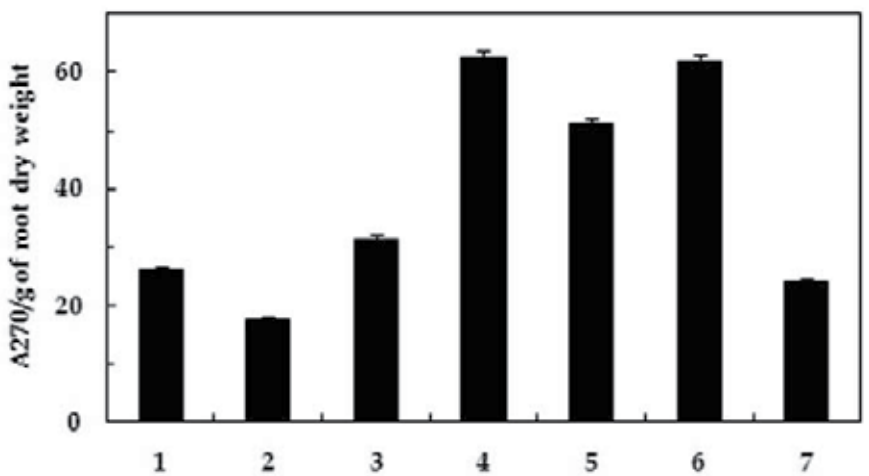

Fig. 11. MnP activity in root exudates of transgenic lines. Undamaged root tissues were dipped in 50 mM malonate buffer (pH 4.5) containing 1 mM manganese sulfate. After incubation for 24 hrs at 37°C, the absorbance of supernatant was measured at 270 nm. Lanes; 1, control plant; 2, MnP transgenic line (FM)-1; 3, FM-2; 4, FM-3; 5, FM-4; 6, FM-7; 7, FM-8. Error bars on the graph indicate standard deviations (*N*=3).

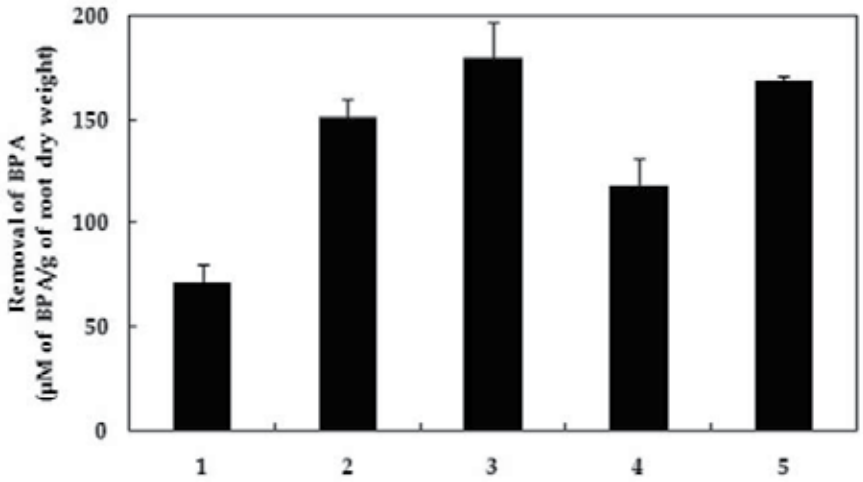

Fig. 12. BPA removal ability of MnP transgenic lines.

The levels of BPA were analyzed by HPLC (λ=278 nm). The values shown are the average of results from three independent experiments. Lanes; 1, control; 2, FM-2; 3, FM-3; 4, FM-4; 5, FM-7. Error bars on the graph indicate standard deviations (*N*=3).

As described above, fungal peroxidase (LiP, Lac, and MnP)-expressing transgenic plants showed effective BPA removal ability, but no reaction products of BPA conversions by these fungal peroxidase-expressing transgenic plants were detected under our analytical conditions. The enzymatic reaction of fungal peroxidases is non-specific and free radical-based, so it is difficult to detect the reaction products. BPA might be degraded or polymerized, as reported in some previous studies of lignolytic enzymes (Hirano et al., 2000; Fukuda et al., 2001; Tsutsumi et al., 2001; Uchida et al., 2001). The increase of BPA removal efficiency by the fungal peroxidase expression in plants would contribute to the development of remediation systems for the cleanup of contaminated areas.

5. Conclusions

Plants can metabolize BPA. Cultured cells of plants were able to glucosylate BPA (Nakajima et al., 2002; Hamada et al., 2002), and, in seedlings, BPA was absorbed from roots and translocated to leaves after glucosylation (Nakajima et al., 2002). In addition, some glycosylated forms of BPA showed less estrogenic activity than that of non-glycosylated BPA (Morohoshi et al., 2003), and oxidative enzymes in plants such as peroxidases stimulated the degradation and polymerization of BPA (Sakuyama et al., 2003). Although the ability of plants to detoxify might be useful for remediation of soil and water contaminated with BPA, the expressions of fungal peroxidases in plants by genetic engineering, as reviewed above, reinforces their ability with respect to the detoxification of BPA. Furthermore it is worth noting that the MnP- and Lac-expressing transgenic plants could remove pentachlorophenol effectively from contaminated areas during hydroponic cultivation (Iimura et al., 2002; Sonoki et al., 2005). Plants could secrete Lac and generate Mn (III) in the rhizosphere, and then the Lac and Mn (III) might be able to affect hydrophobic substrates, such as pentachlorophenol, which is difficult for plant roots to absorb.

Plants producing fungal secretory peroxidases would provide us useful tools for the remediation of areas contaminated with environmental pollutants. Further studies on the effective expression and the secretion of introduced enzymes and the application with other substrates will play an important role in the development of phytoremediation technology.

6. Acknowledgement

This work was supported in part by Grant-in-Aid for Scientific Research A (no. 21248037) from Japan Society for the Promotion of Science (JSPS) and Hirosaki University Grant for Exploratory Research by Young Scientists.

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8

Biological Activity of *Rehmannia glutinosa* Transformed with Resveratrol Synthase Genes

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1. Introduction

The rate of increase of food crop production has decreased due different factors such as global climate change, alteration in use of land, pests, disease, salinity and drought. Food production was said to be inadequate for the increasing world population (WHO, 1996a). Therefore, it is essential to increase food production and distribution in order to meet its demand and free from hunger. Furthermore, transgenic plants can enhance yields, harvesting of crops, reduce dependency on chemical insecticides. Production of transgenic plants can address the global problems such as climate change, deficiency of food and nutrition. Development of transgenic plant involves manipulation or transfer of genes from other organisms which may improve yield, quality, herbicides, and pest or diseases resistant or environmental conditions, increased agricultural productivity and better quality foods. Modification of genetic constitute of plant by inserting transgene enhances nutritional composition of the foods and improve human health and minimizes the use of pesticides and insecticides.

A number of transgenic crop plants has been produced from a variety of crop plants to date with enhanced agronomic characteristics, for example, transgenic tomatoes with improved shelf-life, transgenic fruits and vegetables with delayed ripening time and increased length of storage. Moreover, pest and disease resistance crops have been produced, viz., papaya-ringspot-virus-resistant papaya (Gonsalves 1998), insect resistance cotton, transgenic rice plants that are resistant to rice yellow mottle virus (RYMV) (Pinto et al 1999), improved nutritional contents in the transgenic rice which exhibits an increased production of beta-carotene as a precursor to vitamin A (Ye et al 2000). In addition, technology of transgenic plant production can be used to produce vaccines and bioactive compounds in plants. For example, expression of anti-cancer antibody in rice resulted in production of vaccines against infectious disease from potato (Thanavala et al

1995). *Rehmannia glutinosa* is perennial medicinal plant belongs to the family *Scrophulariaceae*. Its root has long been used in Korea for medicinal purposes such as hemantic, robustness, cardiotonic drug, diabetes treatment, antifebriel and detoxification purposes (Choi et al., 1995). Roots of *R. glutinosa* are usually infected with various pathogens during storage and these infections cause great damage to the roots and impede the intensive farming of the crop (Lim et al 2003).

Resveratrol is found in a limited number of unrelated plants and possess antifungal activity and induction in response to pathogen infection. Resveratrol is well known for its potent antioxidant activity and health-promoting effects, cardioprotection (Ignatowicz and Baer-Dubowska 2001) and reduction of cancer risk have also been observed (Jang et al. 1997; Cal et al. 2003).It can also exert neuroprotective effects by increasing heme oxygenase activity in the brain (Zhuang et al. 2003). The expression of RS transcripts has been associated with an increasedresistance to various fungal pathogens in transgenic tobacco (Hain et al. 1993) and tomato (Thomzik et al. 1997).

The purpose of this report is to better understand the role of transgene to improve the nutrition value of important crops and to evaluate the biological activity of secondary metabolic substances such as resveratrol, SOD, phenolic compounds in *Rehmannia glutinosa* under environmental stress.

2. Transformation of *R. glutinosa* with RS gene

The peanut RS genomic DNA sequence, AhRS3 (GenBank Accession number, AF227963) a polypeptide of 389 amino acid residues, was cloned into the Xba I/Cla I sites of binary expression vector pGA643 under the CaMV35S promoter. This produced a recombinant AhRS3 expression plasmid, pMG-AhRS3. *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pMG-AhRS3, which contains the neomycin phosphotransferase gene (npt II) directed by the nos promoter as a selectable marker, was used. A single colony of this strain was grown for 24 h at 28 ± 1 ^{o}C with shaking (150 rpm) in 20 ml of liquid Luria–Bertani (LB) medium (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract, pH 7) containing 100 mg/l kanamycin. The cells were centrifuged for 10 min at 7,000 x *g* at 4 ^{o}C and resuspended in liquid inoculation medium (MS medium with 20 g/l of sucrose) to obtain a final OD_{600} = 1.0 for use in plant infection. The surface-sterilized leaf explants were cultured for 2–3 days on MS medium containing 1 mg/l 6-benzylamino-purine (BAP), 2 mg/l thidiazuron (TDZ), 0.2 mg/l naphthalene acetic acid (NAA), Murashige and Skoog (MS) vitamin, 3% sucrose, and 0.8% agar (pH 5.2). Pre-treated explants were dipped into the Agrobacterium suspension in liquid inoculation medium for 10–15 min, blotted dry on sterile filter paper and incubated in a shoot induction medium (MS medium containing 2 mg/l BAP, 1 mg/l TDZ, 0.2 mg/l NAA, MS vitamin, 3% sucrose, and 0.8% agar at pH 5.2.) in the dark at 23±1 ^{o}C. After co-cultivation for 2 days, the explants were transferred to shoot induction medium containing 50 mg/l kanamycin and 200 mg/l timentin (mixture of ticarcillin disodium and clavulanate potassium) and were transferred to fresh selection medium every 2 weeks. Putative transgenic shoots were regenerated 6–8 weeks after the first sub-culture and were incubated in a growth chamber with a 16 h at 23±1 ^{o}C for 30 days. Putative transgenic

plantlets were then transferred to pots containing autoclaved vermiculite and were grown in the glasshouse.

Transgene-positive T_0 lines were selected by PCR screening. The lines containing RS gene and *npt* II transgene sequences were chosen for the evaluation of biological activities. Transformation of transgene into the plant genome was confirmed by Southern blot analysis.

2.1 Scavenging of DPPH radicals and Inhibition of lipid peroxidation of transgenic *R. glutinosa*

Free radical-scavenging activity was evaluated using trolox as standard antioxidants. The radical-scavenging activity was measured using the stable radical 1,1-diphenyl–2-picrylhydrazyl (DPPH) as previously described (Xing et al., 1996). Various concentrations of the extracts were added to 4 ml 0.004% methanol solution of DPPH. The mixture was shaken and left for 30 min at room temperature in the dark, and the absorbance was measured with a spectrophotometer at 517 nm. The radical-scavenging activity was expressed as a percentage of the absorption of DPPH in the presence and in the absence of the compound. Calculated IC_{50} values indicate the concentration of sample required to scavenge 50% of the DPPH radical. DPPH activity was calculated as

DPPH activity (%) = $(A_{blank} - A_{sample})/A_{blank} \times 100$,

where, A_{blank} is the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound.

Inhibition of lipid peroxidation was determined by measuring thiobarbituric acid-reactive substance production (Buege and Aust 1978).

2.1.1 Measurement of photosynthesis rate

Stomatal conductance (g_s), net CO_2 assimilation rate (A), and the ratio of internal CO_2 concentration (C_i) were measured according to the method described by Ivan et al. (2001) using a portable photosynthesis system (LCA-4; Analytical Development Co., Hoddesdon, Herts, UK) at an air temperature 28 ± 1 °C. For this measurement, ten fully expanded young leaves were selected from the transgenic lines and control plants at the maximum tillering stage. All measurements were performed three times on sunny days between 1000 and 1400 hours on the surface of leaves from August to September under a saturating photosynthetic photo flux density (PPFD) of 1,500 µmol/m/s. Measurements were made at the center of the leaf surface immediately after the CO_2 concentration decrease was stable. Each leaf was allowed to stabilize for 4–6 min before measuring the g_s, C_i, and A.

2.1.2 HPLC analysis

The HPLC analysis was applied using the modified method of Banwart et al. 1985. The mobile phase consisted of solvent A and B. Solvent A contained 98% water and 2% glacial acetic acid in 0.018M ammonium acetate. Solvent B was 70% solvent A and 30% organic solution, the latter being composed of 82% methanol, 16% n-butanol and 2% glacial acetic acid in 0.018M ammonium acetate. Following injection of 20µL of the sample, the flow

rate of the mobile phases was maintained at 1mL min−1. A linear HPLC gradient was employed. The HPLC system consisted of a Young-Lin M930 liquid chromatograph pump and an M720 detector (Young-Lin Instruments Co., Ltd). The column for quantitative analysis was a YMC-Pack ODS-AM-303 (250×4.6mm I.D.), and the UV absorption was measured at 280 nm.

2.1.3 SOD activity

SOD activity of root *R. glutinosa* was measured by the nitro blue tetrazolium (NBT) reduction method (Beyer and Fridovich, 1987). Test tubes containing reaction solution with 3mL of assay buffer, 60 _L of crude enzyme and 30_L of riboflavin were illuminated for 7 min in an aluminium foil lined box containing two 20-W Slyvania Groiux Fluorescent lamps at 25 ◦C. After reaction, the absorbance of the blank solution and reaction solution was measured with a spectrophotometer (Hitachi Ltd., Tokyo, Japan) at 560 nm. SOD activities were calculated as a following equation:

SOD activity (%) = (1 − *A/B*) × 100

A: absorbance of sample; *B*: absorbance of blank.

2.1.4 Paper disc diffusion assay

Bacterial pathogens and fungal strains were grown in liquid medium (micrococcus, nutrient, and YM media) for 20 h to a final concentration of 10^6–10^7 CFU/ml. Aliquots of 0.1 ml of the test microorganisms were spread over the surface of agar plates. Sterilized filter-paper discs (Whatman No. 1, 6 mm) were saturated with 50 µl of the methanol extract at 10,000 ppm and left to dry in a laminar flow cabinet. The soaked, dried discs were then placed in the middle of the plates and incubated for 24 h. Antimicrobial activity was measured as the diameter (mm) of the clear zone of growth inhibition. Negative controls were prepared using the same solvents employed to dissolve the plant extracts.

In order to evaluate morphological and agronomic performance of transgenic *R. glutinosa*, plants of each PCR positive T1 transgenic lines and seed derived control plants were transferred to field containing bed soil and evaluated for morphological characters.

2.2 Results and discussion

2.2.1 Biological activities of transgenic *R. glutinosa*

2.2.1.1 Scavenging of DPPH radicals of transgenic *R. glutinosa*

The free radical scavenging activities of non-transgenic control and transgenic R. glutinosa extracts, α-tocopherol, are presented in Fig.1. A solution of each extract at a concentration of 1.0 mg/ml was prepared. The activities of transgenic sample extracts were between 16.00 and 20.00 µg/ml at 1.0 mg/ml. Most of the transgenic leaves samples showed high antioxidant activity using DPPH as compared to non-transgenic control plants. With regard to RC50 values (the concentration of antioxidant required to achieve absorbance equal to 50% that of a control containing no antioxidants), RS3 transgenic lines showed highest radical-scavenging abilities (RC50 = 16.00 ± 2.00 µm). The DPPH free radical scavenging and

LDL peroxidation activities of trans-3′-H-Rglu and trans-resveratrol isolated from transgenic R. glutinosa evaluated (Fig. 2 & 3). DPPH activity of trans-resveratrol were significantly higher (72 ± 4.5 µm) than trans-3′-H-Rglu (198 ± 6.8 µm). This could be attributed to the higher level of accumulation of resveratrol compounds in the transgenic R. glutinosa (Fig. 4).

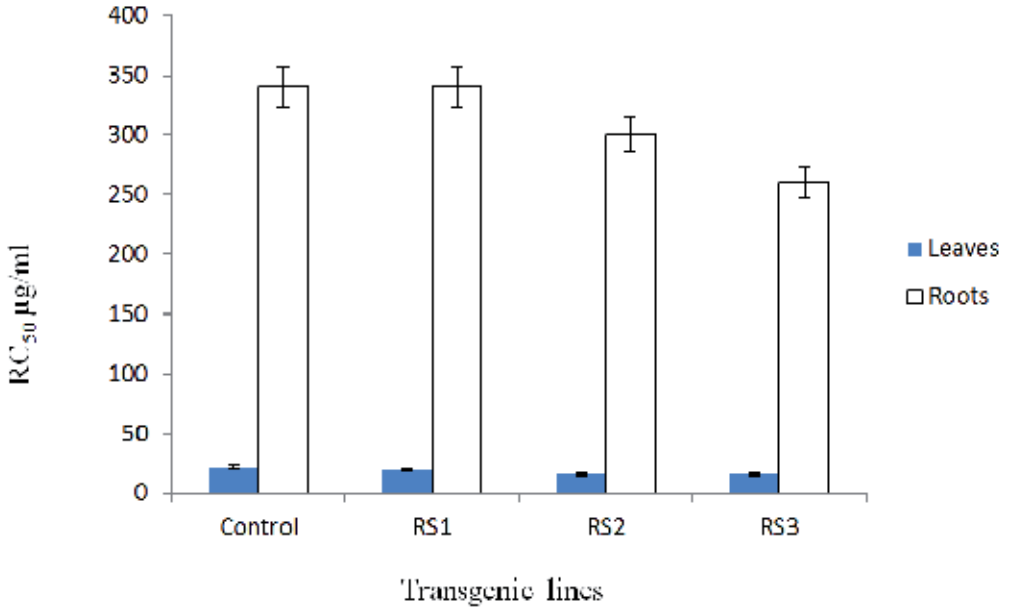

Fig. 1. DPPH free radical scavenging activity of extract in transgenic and non-transgenic *Rehmannia glutinosa*

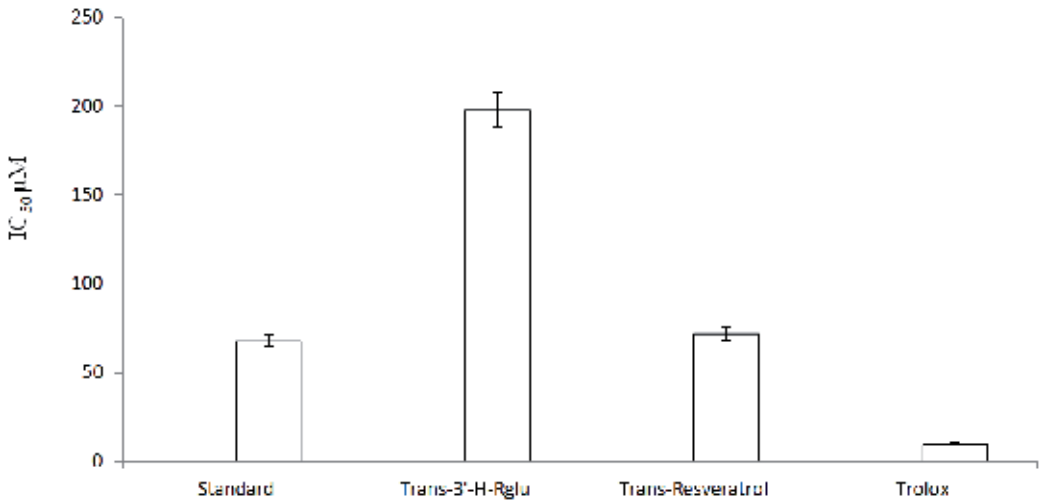

Fig. 2. DPPH scavenging activities of stilbenes isolated from transgenic *Rehmannia glutinosa.*

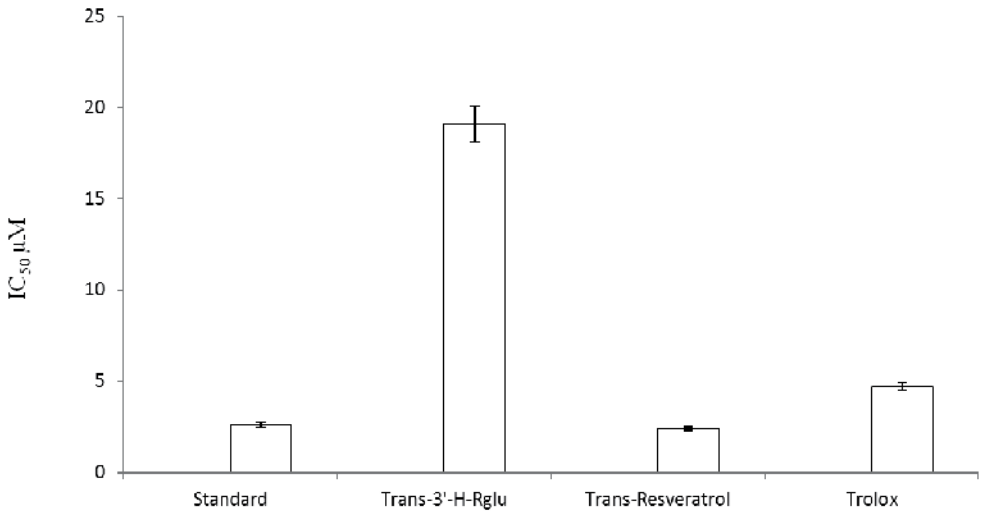

Fig. 3. LDL peroxidation inhibition activities of stilbenes isolated from transgenic *Rehmannia glutinosa.*

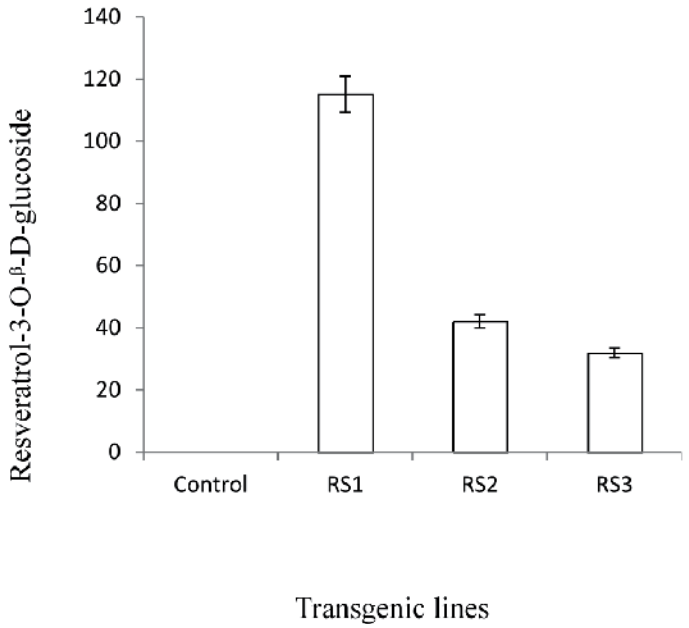

Fig. 4. Accumulation of Resveratrol-3-O-β-D-glucoside levels observed in leaves of RS3-transformed transgenic *Rehmannia glutinosa.*

2.2.2 Superoxide Dismutase (SOD) activity of transgenic *R. glutinosa*

The SOD activities non transgenic plant and transgenic plants (without water stress) were 13.81 and 11.23% respectively. In contrast, the SOD activities non transgenic plant and transgenic plants (with water stress) were 24.59 and 3.8 % respectively (Fig. 5).

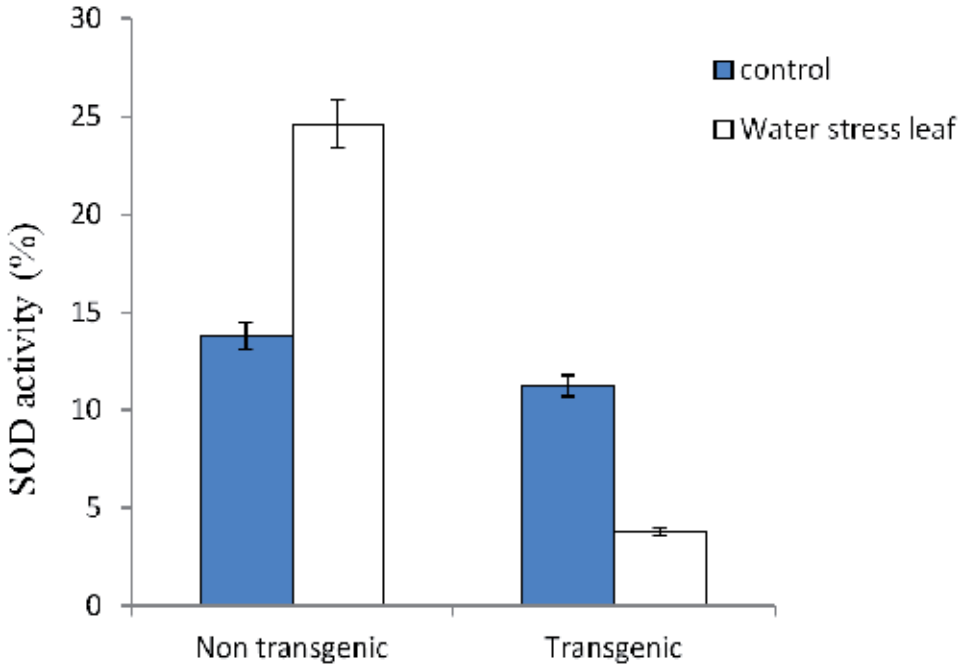

Fig. 5. SOD activities of stilbenes isolated from transgenic *Rehmannia glutinosa.*

2.2.3 Phenolic compound analysis of transgenic *R. glutinosa*

The quantitative analysis of phenolic compounds of non-transgenic and transgenic *R. glutinosa* extract performed using HPLC is given in Table 1. We found quantitative differences in total phenolic compounds between transgenic and control plants stem and root samples under hyper irrigation treatment (Table 2).

Lines	Plant parts	Hyd[1]	Chl[2]	Cat[3]	Caf[4]	Syr[5]	Sal[6]	Cou[7]	Fer[8]	Hes[9]	Nar[10]	Hyr[11]	Cin[12]	Que[13]	Nan[14]	Total
		ug/g														
Control	S	9.12	37.3	1.33	13.25	25.68	0.25	97.19	67.02	54.11	52.02	19.07	82.77	0	0	412.69
	R	4.13	0	0	0	2.19	0	7.21	12.35	6.71	0	53.45	98.53	26.39	0	210.94
RS1	S	3.71	34.13	1.14	0.25	20.81	3.35	90.84	76.28	47.66	55.17	16.99	185.22	0	0	497.72
	R	1.48	0	0	0	10.21	0	18.04	8.53	3.23	0	14.42	198.69	14.57	0	269.18
RS2	S	0.25	29.77	0.25	0.25	11.26	0.25	74.26	83.06	34.41	176.93	73.53	83.69	0	0	537.88
	R	9.48	0	0	0	4.84	0	7.75	7.41	0	10.96	23.88	114.2	19.99	0	189.01
RS3	S	0.25	43.08	0.25	0.25	12.48	0.25	117.69	60.2	48.63	84.61	43	100.91	0	0	468.27
	R	14.61	6.47	0	0	10.02	0	23.32	17.95	0.77	0	24.18	264.23	19.57	0	360.01

S: shoot, R: root, Hyd[1]: *p*-hydroxybenzoic acid, Chl[2]: Chlorogenic acid, Cat[3]: Cathechin, Caf[4]: Caffeic acid, Syr[5]: Syringic acid, Sal[6]: Salicylic acid, Cou[7]: *p*-coumeric acid, Fer[8]: Ferulic acid, Hes[9]: Hesperidin, Nar[10]: Narigen, Hyr[11]: Hyricetin, Cin[12]: trans-cinnamic acid, Que[13]: Quercitin, Nan[14]: Narigenin.

Table 1. Distribution of major phenolic compounds in control and transgenic plants transformed by resveratrol synthase in *R. glutinosa* under hyper irrigation.

The average total concentrations of phenolic compounds in control plant stem and roots were 412.69 and 210.94 µg/g dry weight (DW), respectively; in comparison, transgenic stem and

root samples had higher concentrations of 468.27–537.88 and 189.01–360.01µg/g DW, respectively. The phenolic compounds that increased in the transgenic lines were *p*-hydroybenzoic acid, *p*-coumaric acid, ferulic acid, narigenin, trans-cinnamic acid, chlorogenic acid. Similarly, we found quantitative differences in total phenolic compounds between transgenic and control plants stem and root samples under pathogen treatment (Table 2). The average total concentrations of phenolic compounds in control plant stem and roots were 364.58 and 181.20 µg/g DW, respectively; in comparison, transgenic stem and root samples had higher concentrations of 555.00–919.16 and 312.70–677.26 µg/g DW, respectively.

Lines		Hyd[1]	Chl[2]	Cat[3]	Caf[4]	Syr[5]	Sal[6]	Cou[7]	Fer[8]	Hes[9]	Nar[10]	Hyr[11]	Cin[12]	Que[13]	Nan[114]	Total
		ug/g														
Control	S	7.08	5.75	0	0	0	0.25	0.25	22.8	0	293.04	26.14	0	0	22.11	364.58
	R	21.4	14.28	0	9.82	0	7.78	5.73	12.53	0	0	33.23	94.47	17.66	0	181.2
RS1	S	27.98	39.58	2.57	0	53.45	22.22	28.97	195.6	0	195.35	56.84	0	0	0	555
	R	8.97	10.64	0	0	21.45	0	10.21	6.93	0	3.35	33.91	211.4	20.16	0	318.05
RS2	S	35.27	78.8	1.18	0.25	60.42	240.9	38.61	137.3	15.97	245.74	38.84	6.87	0	133.01	919.16
	R	0	0	0	0	36.87	0	9.04	4.25	8.37	0	8.35	223.25	22.58	0	312.7
RS3	S	0	0	0	0	0	0	153.91	353.5	25.04	97.34	47.42	0	0	0	677.26
	R	30.54	14.61	0	7.01	0	6.02	46.33	21.62	9.93	0	26.56	464.94	19.7	0	602.11

S: shoot, R: root, Hyd[1]: *p*-hydroxybenzoic acid, Chl[2]: Chlorogenic acid, Cat[3]: Cathechin, Caf[4]: Caffeic acid, Syr[5]: Syringic acid, Sal[6]: Salicylic acid, Cou[7]: *p*-coumeric acid, Fer[8]: Ferulic acid, Hes[9]: Hesperidin, Nar[10]: Narigen, Hyr[11]: Hyricetin, Cin[12]: trans-cinnamic acid, Que[13]: Quercitin, Nan[14]: Narigenin.

Table 2. Distribution of major phenolic compounds in control and transgenic plants transformed by resveratrol synthase in *R. glutinosa* under infected pathogen (*Fusarium oxysporum*).

2.2.4 Antimicrobial activities transgenic *R. glutinosa*

Antimicrobial activities of the non-transgenic and transgenic plants were assessed by a paper disc diffusion assay. The results indicated variation in the antimicrobial properties of the resveratrol-3-O-B-D glucoside and resveratrol extracted from the transgenic *R. glutinosa* (Table 3). In general, the resveratrol was more effective than the resveratrol-3-O-B-D glucoside against all the microbes tested. The strongest inhibitory effect was against *E. coli* and *S. typhimurium* at concentration of 1 mg/ml. Antimicrobial activity in plant extracts depends not only on the presence of phenolic compounds, but also on the presence of various secondary metabolites (Gordana et al., 2007). These observations suggest that the antimicrobial activity of transgenic *R. glutinosa* enhanced by the RS3 genes transformed into the *R. glutinosa* genome. However, other phenolic acid-like phenols are thought to contribute to plant defences against pests and pathogens (Awika & Rooney, 2004).

Compounds	Clear zone (mm)							
	Conc. (ppm)	*P. jadinii*	*C. albicans*	*S. aureus*	*B. subtilis*	*K. pneumonia*	*E. coli*	*S. typhimurium*
Resveratrol -3-O-B-D-glucoside	20000	11	10.8	10.6	10.1	9.8	12.4	12.7
Resveratrol	20000	13.7	12.8	12.9	14.2	11.5	19.8	18.6

Table 3. Antimicrobial activity of stilbenes compounds isolated from transgenic *R. glutinosa.*

2.2.5 Morphological characterization of transgenic *R. glutinosa*

Phenotypic differences were observed within the different transgenic lines and between the transgenic and non-transgenic control plants (Table 4). However, there were no apparent differences in terms of root length and root diameter. Significant differences in root weight were observed between transgenic and non-transgenic lines and showed reduced weight over control plants.

Line	Root length (cm)	root diameter	Root weight
Control	24.3	20	330.1
RS1	20.3	14	142
RS2	23.6	18	226.3
RS3	21.9	15	159.5
RS4	21.7	17	273.1

Table 4. Growth characteristics of transgenic *R. glutinosa.*

2.2.6 Analysis of catapol content of transgenic *R. glutinosa*

The catapol contents and composition in subterranean parts of the transgenic lines and non-transformed plants were investigated using HPLC (Fig. 6). Overexpression of RS3 gene significantly increased the catapol, compared to that of wild type *R. glutinosa.*

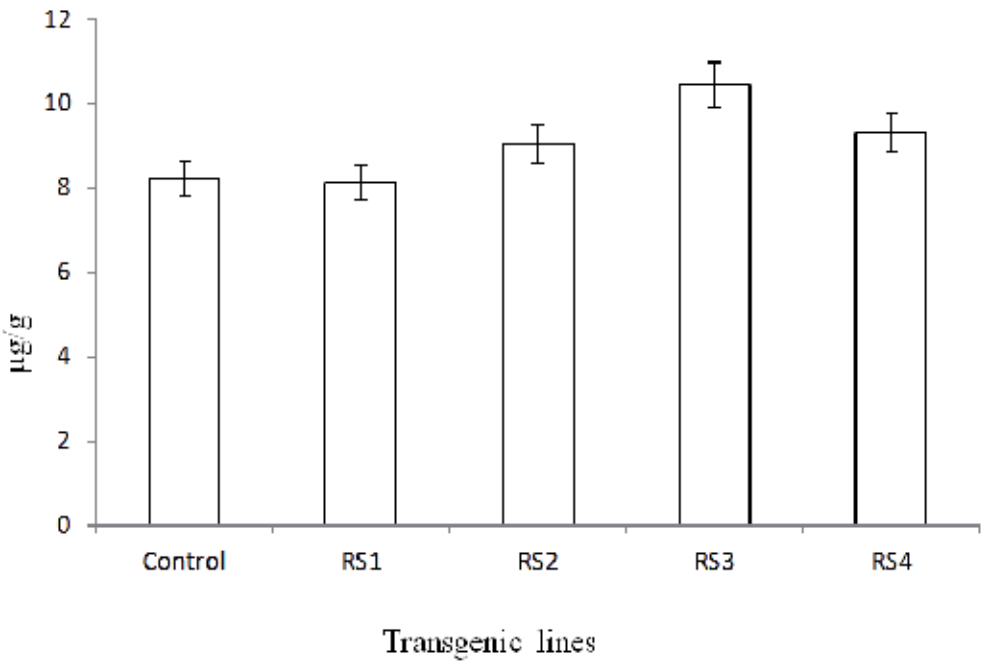

Fig. 6. Content of catapol in root of *R. glutinosa*

2.2.7 Effect of the photosynthesis rate in transgenic *R. glutinosa*

To compare the effect of RS3 gene overexpression on the photosynthesis rate and yield of transgenic and control plants, we measured stomatal conductance (gs), CO_2 concentration (CI), and photosynthesis rate (A) and found significant differences in these factors between transgenic and control plants (Table 5). The photosynthesis rate increased progressively with increasing CO_2 concentration. Photosynthesis rate of both non-transgenic and transgenic plants reduced by the increased duration of dry stress, being much lower at 15 days. Comparatively, transgenic lines showed higher photosynthetic control plants. Therefore, it is very possible that the higher level of RS3 gene in the transgenic plant is responsible for its enhanced photosynthetic performance.

Treatment	Days	Photosynthetic rate					
		Non-transgenic plant			Transgenic plant		
		A (μmol $m^{-2}s^{-1}$)	gs (μmol $m^{-2}s^{-1}$)	Ci (ppm)	A (μmol $m^{-2}s^{-1}$)	Gs (μmol m-2s-1)	Ci (ppm)
Control-		14.63 ± 0.1	0.29 ± 0.0	241.67 ± 0.85	17.42 ± 0.14	0.27 ± 0.02	212.47 ± 0.17
	3D	9.68 ± 0.06	0.11 ± 0.01	195.8 ± 3.14	17.1 ± 0.06	0.32 ± 0.0	223.98 ± 1.88
Dry stress	9D	3.63 ± 0.14	0.02 ± 0.0	45.52 ± 5.92	14.39 ± 0.04	0.23 ± 0.0	220.13 ± 1.24
	15D	1.13 ± 0.01	0.0	215.82 ± 0.78	1.69 ± 0.31	0.0	194.13 ± 4.21

A: Photosynthetic rate; gs: Stomatal conductance; Ci: Intercellular; CO_2concentration.

Table 5. Photosynthetic rate of non-trangenic and transgenic *R. glutinosa* under water stress.

It can be concluded that introduction of RS3 gene into the *R. glutinosa* genome resulted into increased production of stilbenes compounds that enhances the biological activity of plants. Increased in the resveratrol compounds further enhanced the disease resistance capacity of plant. This may cause beneficial effects on human and plant defence system.

3. References

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9

Methods to Transfer Foreign Genes to Plants

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1. Introduction

Genome sequencing of several organisms has resulted in the rapid progress of genomic studies. Genetic transformation is a powerful tool and an important technique for the study of plant functional genomics, i.e., gene discovery, new insights into gene function, and investigation of genetically controlled characteristics. In addition, the function of genes isolated using map-based cloning of mutant alleles has been confirmed by functional complementation using genetic transformation. Furthermore, genetic transformation enables the introduction of foreign genes into crop plants, expeditiously creating new genetically modified organisms. Gene transformation and genetic engineering contribute to an overall increase in crop productivity (Sinclair et al., 2004).

This review outlines general methods for plant transformation and focuses on the development of the *Arabidopsis* transformation system.

2. Plant transformation methods

Plant transformation was first described in tobacco in 1984 (De Block et al., 1984; Horsch et al., 1984; Paszkowski et al., 1984). Since that time, rapid developments in transformation technology have resulted in the genetic modification of many plant species. Methods for introducing diverse genes into plant cells include *Agrobacterium tumefaciens*-mediated transformation (De la Riva, 1998; Hooykaas & Schilperoort, 1992; Sun et al., 2006; Tepfer, 1990; Zupan & Zambryski, 1995), recently reclassified as *Rhizobium radiobacter*, direct gene transfer into protoplasts (Gad et al., 1990; Karesch et al., 1991; Negrutiu et al., 1990; Neuhaus & Spangenberg, 1990), and particle bombardment (Birch & Franks, 1991; Christou, 1992; Seki et al., 1991; Takeuchi et al., 1992, 1995; Yao et al., 2006).

2.1 Gene transformation

Several gene transformation techniques utilize DNA uptake into isolated protoplasts mediated by chemical procedures, electroporation, or the use of high-velocity particles (particle bombardment). Direct DNA uptake is useful for both stable transformation and transient gene expression. However, the frequency of stable transformation is low, and it takes a long time to regenerate whole transgenic plants.

2.1.1 Chemical procedures

Plant protoplasts treated with polyethylene glycol more readily take up DNA from their surrounding medium, and this DNA can be stably integrated into the plant's chromosomal DNA (Mathur & Koncz, 1997). Protoplasts are then cultured under conditions that allowed them to grow cell walls, start dividing to form a callus, develop shoots and roots, and regenerate whole plants.

2.1.2 Electroporation

Plant cell electroporation generally utilizes the protoplast because thick plant cell walls restrict macromolecule movement (Bates, 1999). Electrical pulses are applied to a suspension of protoplasts with DNA placed between electrodes in an electroporation cuvette. Short high-voltage electrical pulses induce the formation of transient micropores in cell membranes allowing DNA to enter the cell and then the nucleus.

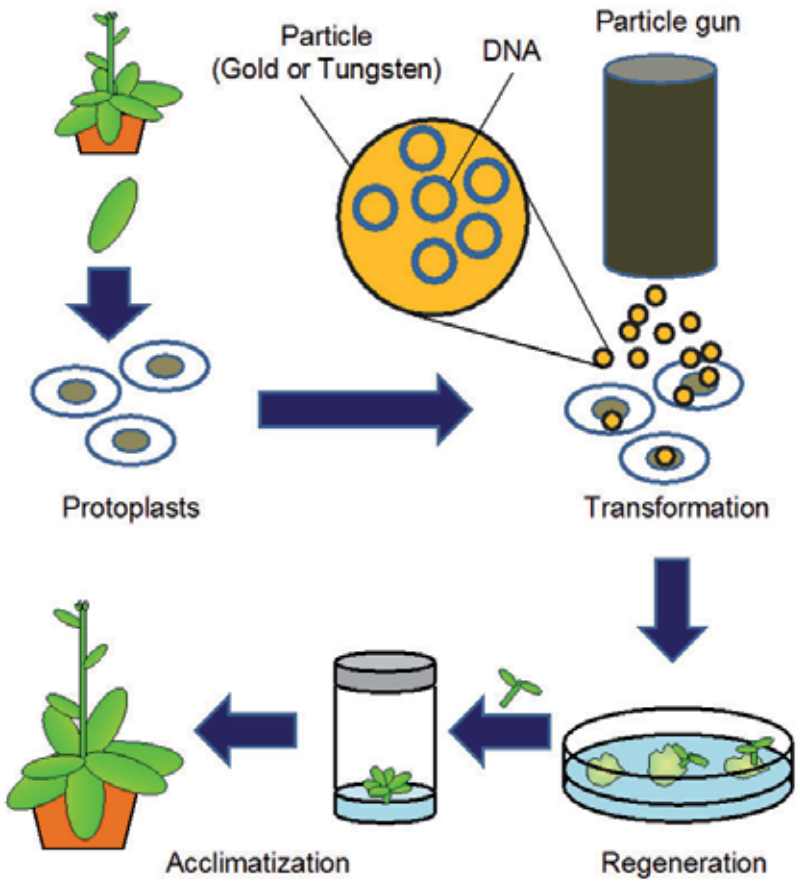

Fig. 1. Plant transformation process using particle bombardment includes the following steps: (1) Isolate protoplasts from leaf tissues. (2) Inject DNA-coated particles into the protoplasts using particle gun. (3) Regenerate into whole plants. (4) Acclimate the transgenic plants in a greenhouse.

2.1.3 Particle (microprojectile) bombardment

Particle bombardment is a technique used to introduce foreign DNA into plant cells (Birch & Franks, 1991; Christou, 1992, 1995; Gan, 1989; Takeuchi et al., 1992; Yao et al., 2006) (Figure 1). Gold or tungsten particles (1–2 μm) are coated with the DNA to be used for transformation. The coated particles are loaded into a particle gun and accelerated to high speed either by the electrostatic energy released from a droplet of water exposed to high voltage or using pressurized helium gas; the target could be plant cell suspensions, callus cultures, or tissues. The projectiles penetrate the plant cell walls and membranes. As the microprojectiles enter the cells, transgenes are released from the particle surface for subsequent incorporation into the plant's chromosomal DNA.

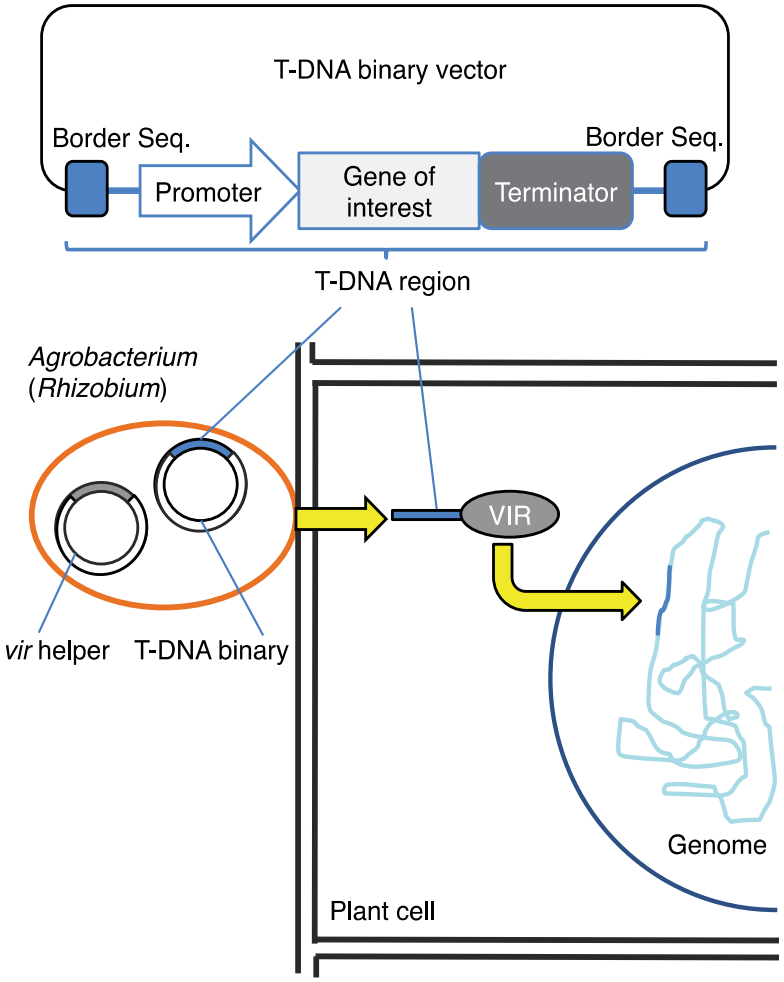

Fig. 2. The *Agrobacterium*-mediated transformation process includes the following steps: (1) Isolate genes of interest from the source organism. (2) Insert the transgene into the Ti-plasmid. (3) Introduce the T-DNA containing-plasmid into *Agrobacterium*. (4) Attach the bacterium to the host cell. (5) Excise the T-strand from the T-DNA region. (6) Transfer and integrate T-DNA into the plant genome.

2.2 Using *Agrobacterium* for plant transformation

Agrobacterium-mediated transformation is the most commonly used method for plant genetic engineering (Bartlett et al., 2008; Leplé et al., 1992; May et al., 1995; Sun et al., 2006; Tsai et al., 1994; Tzfira et al., 1997). The pathogenic soil bacteria *Agrobacterium tumefaciens* that causes crown gall disease has the ability to introduce part of its plasmid DNA (called transfer DNA or T-DNA) into the nuclear genome of infected plant cells (Figure 2) (Binns & Thomashaw, 1988; Gelvin, 2000; Nester et al., 1984; Tzfira et al., 2004; Zupan & Zambryski, 1995).

2.3 Transforming *Arabidopsis thaliana*

Arabidopsis thaliana, a small flowering plant, is a model organism widely used in plant molecular biology. The first *in planta* transformation of *Arabidopsis* included the use of tissue culture and plant regeneration (Feldmann & Marks, 1987). The *Agrobacterium* vacuum (Bechtold et al., 1993) and floral dipping (Clough & Bent, 1998) are efficient methods to generate transgenic plants. They allow for plant transformation without the need for tissue culture. The floral dipping method markedly advanced the ease of creating *Arabidopsis* transformants, and it is the most widely used transformation method. These methods were later simplified and substantially improved (Davis et al., 2009; Zhang et al., 2006), significantly reduced the required labor, cost, and time, as compared with earlier procedures.

However, these transformation methods have some problems. The floral dipping method involves dipping *Arabidopsis* flower buds into an *Agrobacterium* cell suspension, requiring large volumes of bacterial culture grown in liquid media. The large shakers and centrifuges, necessary to house the media, require sufficient experimental space. These factors limit transformation quantities. Here, we describe an improved method for *Agrobacterium*-mediated transformation that does not require the large volumes of liquid culture necessary for floral dipping.

2.3.1 Improved method for *Agrobacterium*-mediated transformation

A. thaliana can be stably transformed with high efficiency using T-DNA transfer by *Agrobacterium tumefaciens. Agrobacterium*-mediated transformation using the floral dipping method is the most widely used method for transforming *Arabidopsis*. We have showed that *A. thaliana* can be transformed by inoculating flower buds with 5 µl of *Agrobacterium* cell suspension, thus avoiding the use of large volumes of *Agrobacterium* culture (Narusaka et al., 2010). Using this floral inoculating method, we obtained 15–50 transgenic plants per three transformed *A. thaliana* plants. The floral inoculating method can be satisfactorily used in subsequent analyses. This simplified method, without floral dipping, offers an equally efficient transformation as previously reported methods. This method reduces overall labor, cost, time, and space. Another important aspect of this modified method is that it allows many independent transformations to be performed at once.

2.3.2 *Agrobacterium* strains

The *Agrobacterium* strain GV3101 (C58 derivative) is frequently used to transform many binary vectors, e.g., pBI121, pGPTV, pCB301, pCAMBIA, and pGreen, into *Arabidopsis*. It carries rifampicin resistance (10 mg l^{-1}) on the chromosome (Koncz & Schell, 1986). On the other hand, LBA4404 is a popular strain for tobacco transformation but is less effective for *Arabidopsis*.

2.3.3 *Agrobacterium* transformation—freeze/thaw and electroporation procedures

Agrobacterium can be transformed with plasmid DNA using the freeze/thaw (Höfgen & Willmitzer, 1998; Holsters et al., 1978) and electroporation (den Dulk-Ras & Hooykaas, 1995; Mersereau et al., 1990; Shen & Forde, 1989) procedures. The freeze/thaw procedure is very simple and does not require special equipment.

Reagents

- *Agrobacterium* strain
- 20 mM $CaCl_2$
- Liquid nitrogen
- Luria–Bertani (LB) agar plate
- Liquid LB medium

Equipments

- Microcentrifuge
- Water bath
- Eppendorf tube (1.5 ml)

1. Pellet 1.5 ml of overnight-grown *Agrobacterium* (GV3101) cells by centrifugation in an Eppendorf tube at 14,000 rpm for 1 min at 4°C.
2. Resuspend in 1 ml of ice-cold 20 mM $CaCl_2$.
3. Recentrifuge at 14,000 rpm for 1 min at 4°C.
4. Resuspend in 200 µl of ice-cold 20 mM $CaCl_2$.
5. Add binary vector DNA (500 ng or 5–10 µl from an alkaline lysis miniprep) to the suspension. Mix by pipetting.
6. Freeze the Eppendorf tube in liquid nitrogen for 5 min and thaw at 37°C in a water bath for 5 min. Repeat two times.
7. Cool on ice.
8. Add 1 ml LB liquid medium to the Eppendorf tube and incubate at 28°C for 2–5 hrs with gentle agitation (150 rpm; water bath).
9. Spread 50–200 µl of the cells onto LB agar medium containing appropriate antibiotics and incubate at 28°C for two days.

2.3.4 Selecting transformed *Agrobacterium* using polymerase chain reaction (PCR)

This method is designed to quickly screen for plasmid inserts directly from *Agrobacterium* colonies. Alternatively, the insert presence can be determined by DNA sequencing.

Reagents

- PCR components (one reaction):
  Autoclaved, distilled water - 11.625 µl
  10× PCR buffer - 1.5 µl
  2.5 mM dNTPs - 1.2 µl
  10 pmol μl^{-1} Primer #1 - 0.3 µl
  10 pmol μl^{-1} Primer #2 - 0.3 µl
  Taq DNA polymerase (5 U/µl) - 0.075 µl
  Total PCR master mix volume - 15.0 µl

- *Taq* DNA polymerase: Takara EX *Taq* (Takara, Otsu, Japan) (recommended)
- TBE (Tris/Borate/EDTA) buffer

Equipments

- PCR tubes (0.2 ml)
- Thermocycler
- Electrophoresis system

1. Prepare sufficient PCR master mix for the number of samples tested.
2. Add 15 µl of PCR master mix to each PCR tube.
3. Select *Agrobacterium* colonies from the plate using a sterile toothpick or pipette tip.
4. Insert selected colony sample into the PCR master mix and mix with a sterile toothpick or pipette tip.
   (Note: Sufficient mixing results in complete cell lysis and high yields.)
5. Briefly centrifuge tubes to collect all liquid and insert them into the PCR.
6. Set the thermocycler conditions and start PCR.
   Conditions: Preliminary denaturation at 95°C for 3 min then 40 cycles at 95°C for 20 sec, 55°C for 30 sec, and 72°C for 30 sec.
   (Note: Preliminary denaturation is very important for initial cell breakage.)
7. Run 8–10 µl of each PCR sample on 1.0% agarose gel in 1× TBE buffer at 100 V for 30 min to visualize the PCR results. Stain gels according to your lab method.

2.3.5 Simplified *Arabidopsis* transformation: Floral inoculating method

Until now, a limited number of constructs could be transformed into *Arabidopsis* because of difficulty growing large volumes of *Agrobacterium*. Therefore, we focused on improvements to the floral dipping method (Figure 3) (Narusaka et al., 2010). The problem of space and volume can be solved by using a small culture volume. Each plant is transformed using only 30–50 µl of bacteria grown in 2 ml of liquid culture. Our present method, as described below, is a simple modification of the method reported by Clough & Bent (1998).

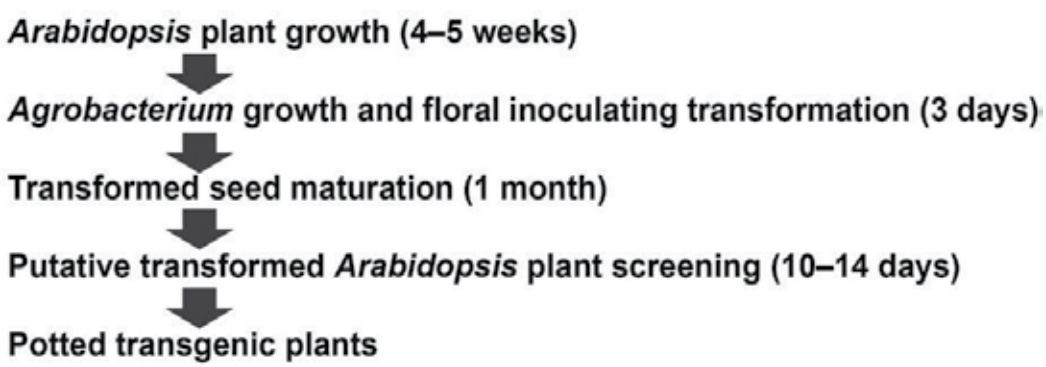

Fig. 3. Transformation using *Agrobacterium* and the floral inoculating method

Recent papers (Liu et al., 2008; Zhang et al., 2006) illustrate the floral dipping process. Clough and Bent (1998) reported that neither Murashige and Skoog (MS) salts and hormones nor optical density (OD) makes a difference in transformation efficiency. An *Agrobacterium* cell suspension containing 0.01–0.05% Silwet L-77 (vol/vol) was used in the uptake of *Agrobacterium* into female gametes, instead of vacuum-aided infiltration of inflorescences.

Reagents

- *A. thaliana*: There are marked differences in transformation efficiency between various ecotypes. For floral dipping transformation, efficiency in the Landsberg *erecta* (L*er*-0) ecotype is lower than that in the Columbia (Col-0) ecotype. Transformation efficiency in Wassilewskija (Ws-0) is very high among *Arabidopsis* ecotypes.
- *Agrobacterium* strain: GV3101 (Koncz & Schell, 1986) (recommended) or others.
- 0.1% (wt/vol) agar solution
- 70% (vol/vol) ethanol
- Sodium hypochlorite solution containing 1% available chlorine and 0.02% (vol/vol) Tween 20
- Distilled water
- MS medium: 1× MS plant salt mixture (Wako Pure Chemical Industries, Osaka, Japan), 1× Gamborg's vitamin solution (Sigma-Aldrich, St. Louis, MO, USA), 1% (wt/vol) sucrose, 0.05% (wt/vol) MES, and pH 5.7 adjusted with 1 N KOH
- Bacto agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) (recommended)
- LB agar plate
- Liquid LB
- Glycerol
- Transformation buffer: 1/2× MS plant salt mixture, 1× Gamborg's vitamin solution, 5% (wt/vol) sucrose, and pH 5.7, adjusted with 1 N KOH
- 5% (wt/vol) sucrose solution
- Silwet L-77
- 6-Benzylaminopurine (BAP) (final concentration 0.01 μg ml^{-1})
- Claforan (Aventis Pharma AG, Zürich, Switzerland) (final concentration 2 mg ml^{-1})
- Kanamycin (final concentration 30 μg ml^{-1})
- Hygromycin (final concentration 20 μg ml^{-1})
- Bialaphos (final concentration 7.5 μg ml^{-1})
- Peat moss (Soil Mix, Sakata Seed Corp., Yokohama, Japan)
- Expanded vermiculite granules

Equipments

- Growth chamber
- Plant pot (3-inch)
- Conical tube (15 ml)
- Eppendorf tubes (2 ml)

1. Grow *A. thaliana* plants. (Note: Plant health is an important factor. Healthy *A. thaliana* plants should be grown until they are flowering.) There are two different procedures: standard (A) and quick (B) (Zhang et al., 2006). We generally use the quick procedure, which is useful for rare seeds and seeds with low germination frequency. It is also used to retransform a transgenic line with a second construct.

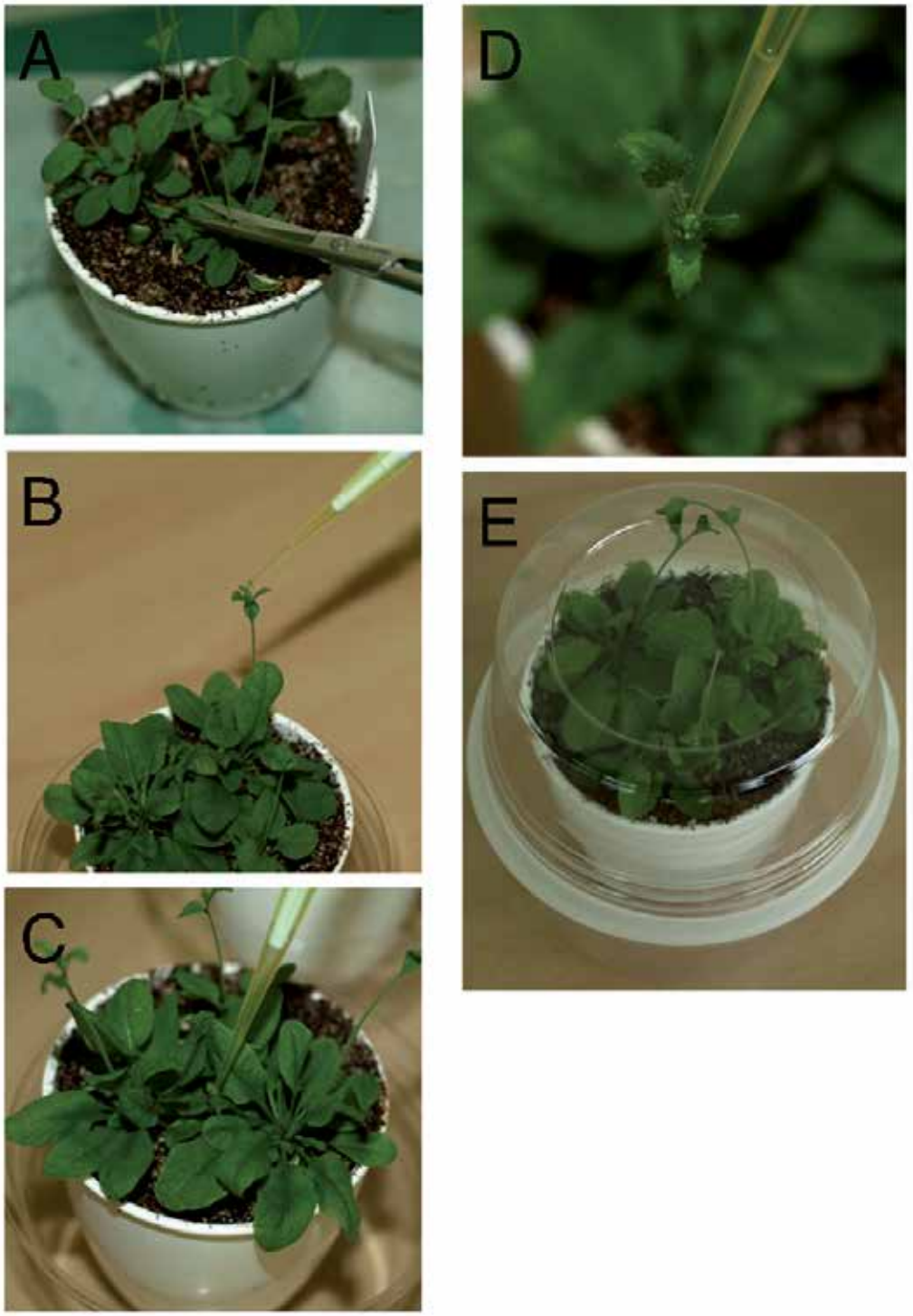

Fig. 4. Part 1.

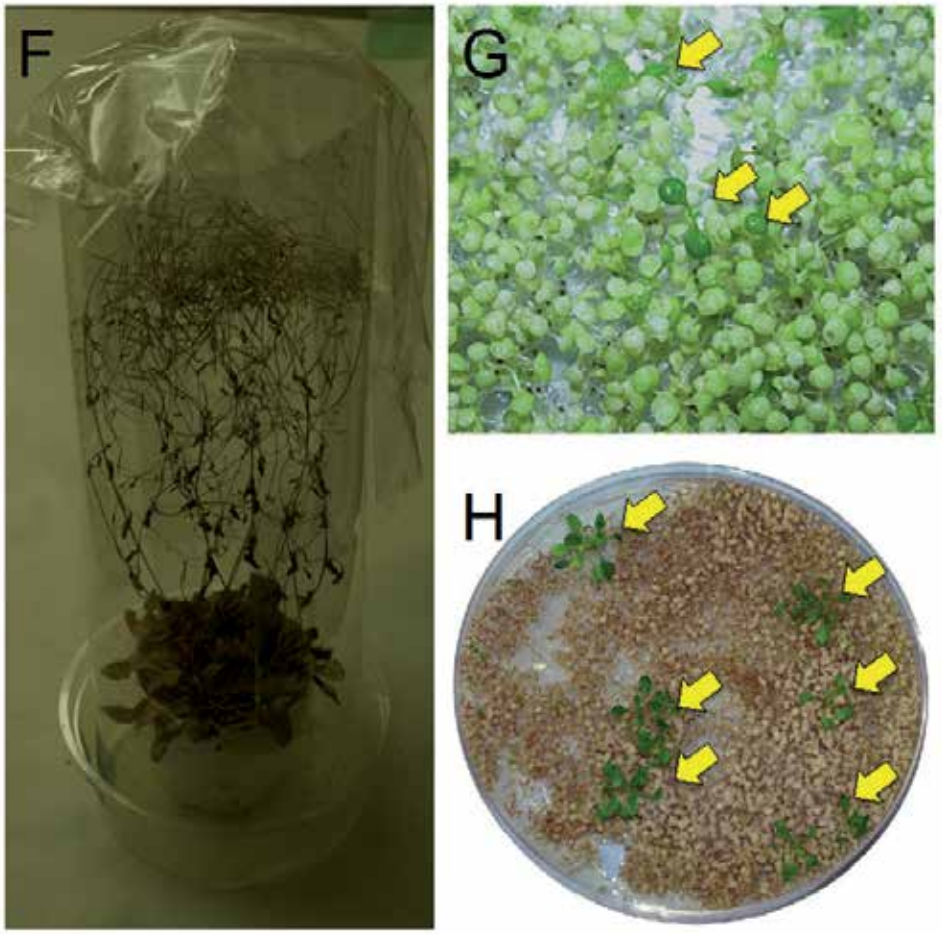

Fig. 4. part 2. Floral inoculating transformation of *Arabidopsis thaliana*. (A) Clipping primary bolts. (B, C, and D) Using a micropipette, inoculate flower buds with 5 µl of *Agrobacterium* when plants have just started to flower after clipping primary bolts. (E) Place inoculated plants under a dome or cover for 16–24 hrs to maintain high humidity. (F) Remove the cover and grow the plants in a greenhouse or growth chamber until maturity. (G, H) Screening of putative transformed *Arabidopsis* plants. G: 10 days, H: 21 days. Arrows indicate putative transformed *Arabidopsis* plants.

1.a. Standard procedure (A): Suspend seeds in 0.1% (wt/vol) agar solution and keep in darkness for 2–4 days at 4°C to break dormancy. Spread seeds on wet soil (a mixture of peat moss and expanded vermiculite granules at a 1:2 ratio) in a 3-inch pot and grow under long-day conditions (16-hr light/8-hr dark) at 22°C. Thin to three seedlings per pot. Do not cover with a bridal veil, window screen, or cheesecloth.

1.b. Quick procedure (B): Sterilize seeds by treatment with 70% (vol/vol) ethanol for 1 min then immerse in sodium hypochlorite solution containing 1% available chlorine and 0.02% (vol/vol) Tween 20 for 7 min. Wash seeds five times with sterile distilled water. Place seeds on MS medium containing 0.8% (wt/vol) Bacto agar. Keep seeds in

darkness for 2–4 days at 4°C to break dormancy. Grow under long-day conditions (16-hr light/8-hr dark) for 3 weeks at 22°C. Transfer to pots per Step 1a. Do not cover with a bridal veil, window screen, or cheesecloth.

2. Clip primary bolts to encourage proliferation of secondary bolts (Figure 4A). Plants will be ready approximately 4–6 days after clipping.
3. Prepare the *Agrobacterium* strain carrying the gene of interest. Spread a single *Agrobacterium* colony on an LB agar plate with suitable antibiotics. Incubate the culture at 28°C for two days.
4. Use feeder culture to inoculate a 2-ml liquid culture in LB with suitable antibiotics to select for the binary plasmid in a 15-ml Conical tube at 28°C for 16–24 hrs. Mid-log cells or a freshly saturated culture (Clough and Bent 1998) can be used. (Optional: If needed, keep 500 µl of *Agrobacterium* culture in a 25% (vol/vol) glycerol stock at -80°C.)
5. Spin down 1.5 ml of the *Agrobacterium* cell suspension in 2-ml Eppendorf tubes and resuspend in 1 ml transformation buffer. OD_{600} value adjustment is not required. Each small pot containing three plants requires approximately 150 µl of culture. (Optional: 5% (wt/vol) sucrose solution may be used instead of transformation buffer.)

   Just before inoculation, add Silwet L-77 to a concentration of 0.02% (vol/vol) and immediately mix well. (Optional: If using transformation buffer, add 0.01 µg ml^{-1} BAP just before transformation.)

6. Apply 5 µl of *Agrobacterium* inoculum to the flower buds (Figures 4B, C, and D), inoculating each plant with a total of 30–50 µl of inoculum.
7. Place inoculated plants under a dome or cover for 16–24 hrs to maintain high humidity (Figure 4E). Avoid excessive exposure to light. (Optional: For higher rates of transformation, inoculate newly forming flower buds with *Agrobacterium* 2–3 times at 7-day intervals.)
8. Water and grow plants normally, tying up loose bolts with wax paper, tape, stakes, twist-ties, or other means. Stop watering as seeds become mature (Figure 4F).
9. Harvest dry seeds. Though transformants are usually independent, independence can be guaranteed if seeds come from separate plants.
10. Surface-sterilize seeds by immersion in 70% (vol/vol) ethanol for 1 min, followed by immersion in sodium hypochlorite solution containing 1% available chlorine and 0.02% (vol/vol) Tween 20 for 10 min. Then, wash seeds five times with sterile distilled water.

    To select for transformed plants, resuspend liquid-sterilized seeds in approximately 8 ml of 0.1% (wt/vol) agar solution containing 2 mg ml^{-1} Claforan. Sow seeds per Step 1b in MS medium containing 0.8% Bacto agar and appropriate antibiotics or herbicide selective markers at the following concentrations: kanamycin (final concentration 30 µg ml^{-1}), hygromycin (20 µg ml^{-1}), and bialaphos (7.5 µg ml^{-1}). Claforan is necessary for *Agrobacterium* decontamination (Figures 4G and H).

11. Transplant putative transformants to soil per Step 1a. Grow, test, and use.

2.3.6 Screening of transgenic plants by PCR

Transgenes can be detected by plant genome DNA analysis with PCR (Figure 5). Although transgenes can be distinguished from their surrounding host plant genome, their presence should be determined by DNA sequencing.

PCR-based transgene detection is a simple and highly sensitive process. Subsequent PCR tests are assessed by agarose gel electrophoresis, and results are visualized by the presence or absence of the appropriately sized DNA fragment. If PCR shows a positive result, the transgene may be present. Transgene presence is confirmed by incorporating it into the genome by DNA sequencing. In contrast, a negative PCR result implies that the transgene is not present.

Simplified DNA isolation method

A small plant leaf disc (3–4 mm diameter) can be directly used as a PCR template. *Arabidopsis*, tomato, Chinese cabbage, Komatsuna (*Brassica rapa*), and tobacco leaf discs are good template candidates.

Reagents

Buffer A: 100 mM Tris-HCl (pH 9.5), 1 M KCl, 10 mM EDTA (ethylenediaminetetraacetic acid)

Equipments

- Cork borer (3–4 mm diameter)
- Disposable blade
- Eppendorf tube (1.5 ml)
- PCR tube (0.2 ml)
- Heat block

1. Cut each plant leaf disc using a cork borer (3–4 mm diameter) or disposable blade (leaf piece should be approximately 3 mm × 3 mm).
2. Place the leaf disc into an Eppendorf tube.
3. Add 100 µl of Buffer A.
4. Incubate for 10 min at 95°C.
5. Vortex thoroughly.
6. Transfer 0.5 µl of the template DNA supernatant to a PCR tube.

PCR detection method

Reagents

- PCR components (one reaction):

  Autoclaved, distilled water - 3.9 µl
  2× PCR buffer for KOD FX - 10.0 µl
  2 mM dNTPs - 4.0 µl
  10 pmol μl^{-1} Primer #1 - 0.6 µl
  10 pmol μl^{-1} Primer #2 - 0.6 µl
  KOD FX (1.0 U/µl) - 0.4 µl
  Total PCR master mix volume - 19.5 µl

  Add template DNA - 0.5 µl
  Total reaction volume - 20.0 µl

- DNA polymerase: KOD FX (Toyobo Co., Ltd, Osaka, Japan) (required)
- TBE buffer

Equipments

- PCR tubes (0.2 ml)
- Thermocycler
- Electrophoresis system

1. Prepare a PCR master mix for the number of samples tested.
2. Add 19.5 µl of PCR master mix to the template DNA and gently mix by pipetting.
3. Briefly centrifuge tubes to collect all liquid and insert into the PCR.
4. Set the Thermocycler condition and start PCR. Conditions: Preliminary denaturation step at 94°C for 2 min, followed by 40 cycles at 98°C for 10 sec, 55°C for 15 sec, and 68°C for 30 sec.
5. Run 8–10 PCR samples on 1.0% agarose gel in 1× TBE buffer at 100 V for 30 min to visualize the PCR results. Stain gels according to your lab method.

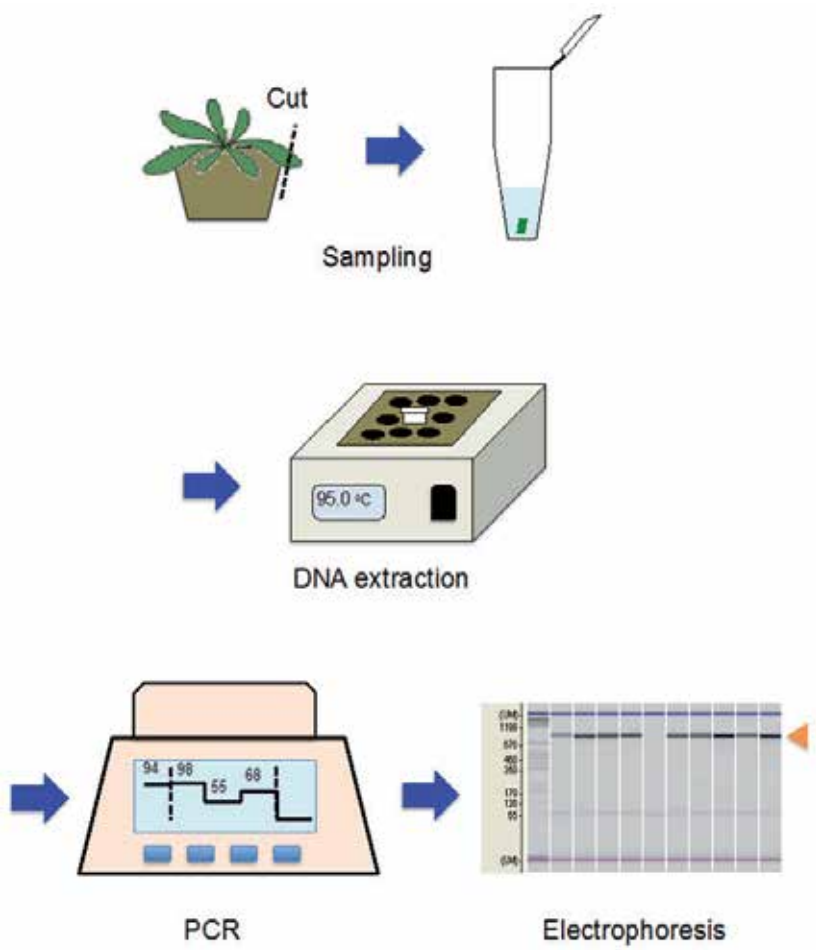

Fig. 5. Screening regimen for transgenic plants by PCR.

3. Conclusion

The floral inoculating method resulted in 15–50 transgenic plants per three transformed *A. thaliana* plants (Table 1). The method can be satisfactorily used for subsequent analyses. This simplified method does not utilize plant inversion or floral dipping, which requires large volumes of *Agrobacterium* culture. It offers equally efficient transformation as previously reported methods with the added benefit of reduced labor, cost, time, and space. Of further importance, this modified method allows many independent transformations to be performed at once.

Vector	Antibiotic marker (final concentration)	Ecotype	%Transformation[a]
pBI101	kanamycin (30 μg ml^{-1})	Columbia (Col-0)	0.32 ± 0.02
		Wassilewskija (Ws-0)	0.86 ± 0.12
pGWB1[b]	kanamycin (30 μg ml^{-1}) hygromycin (20 μg ml^{-1})	Wassilewskija (Ws-0)	0.31 ± 0.05
[a] Values are mean ± SE. [b] Refer to Nakagawa et al. (2007).			

Table 1. Transformation efficiency using floral inoculating method

4. Acknowledgment

We thank Ms. Mariko Miyashita for her excellent technical assistance. This work was supported in part by the Industrial Technology Research Grant Program in 2004 and 2009 from the New Energy and Industrial Technology Development Organization (NEDO) of Japan, by the Programme for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry to Y.N., and by a Grant-in-Aid for Scientific Research (KAKENHI) (21580060 to Y.N. and 21780038 to M.N.).

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Part 2

Crop Improvement

10

Genetic Enhancement of Grain Quality-Related Traits in Maize

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1. Introduction

Maize (*Zea mays*) is a major food and animal feed worldwide and occupies a relevant place in the world economy and trade as an industrial grain crop (White & Johnson, 2003). Currently more than 70% of maize production is used for food and feed; therefore, knowledge of genes involved in grain structure and chemical is important for improving the nutritional and food-making properties of maize.

Although, plant breeding has been extremely successful at improving the yield of maize, quality has received less attention. However, important advances were made by breeders in this area as well, resulting in maize with a wide range of compositions. In fact, by exploiting genetic variation, the composition of the kernel was altered for both the quantity and quality (structure and chemical diversity) of starch, protein, and oil throughout kernel development. Furthermore, the ability of plant scientists to use existing genetic variation and to identify and manipulate commercially important genes will open new avenues to design novel variation in grain composition. This will provide the basis for the development of the next generation of speciality in maize and of new products to meet future needs.

This chapter focuses on gene discovery, exploitation, and genetic variation known to affect the development and chemical composition of maize kernel. Throughout the chapter we have attempted to summarize the current status in these areas with a particular reference to deposition of storage proteins, starches, lipids, and carotenoids, and research pertinent to enhance kernel quality-related traits. Finally, we provide a brief outlook on future developments in this field and the resultant opportunities and application of conventional and molecular breeding for the development of new maize products better suited to its various end uses.

2. Kernel growth and development

The great economical and nutritional value of the maize kernel is mainly due to its high starch content, as it represents approximately 75% of the mature seed weight. However, the protein complement (ca. 10% of the mature seed weight), mainly found in the form of zeins (storage proteins) is essential for human and animal nutrition. Yet the question remains of why the selection for higher starch level irremediably results in less protein content, as

illustrated by the Illinois Long-Term Selection Experiment, which is spanning over more than 100 generations of classical breeding (Mooses et al., 2004).

As a typical angiosperm, the maize kernel comprise two zygotic tissues, namely the embryo (germ) and the endosperm, that are embedded in the testa (or seed coat) and the pericarp (or fruit wall), which fuse into a thin protective envelope. The endosperm is the main storage site of starches and proteins, whereas the embryo reserves mainly lipids. However, the economical and nutritional value of the kernel is mostly derived from the endosperm, a starch-rich tissue, that supports the embryo at germination.

In maize, endosperm makes up the majority of kernel dry matter (70-90%) and is the predominant sink of photosynthates and other assimilates during reproductive growth; therefore, factors that mediate endosperm development to a large extent also determine grain yield. Furthermore, the endosperm of seed can serve as a valuable system to address fundamental questions related to the improvement of seed size in crops.

High-throughput genomics and post-genomics approaches are now providing new tools for a better understanding of the genetic and biochemical networks operating during kernel development. Recently, large databases of maize gene expressed sequence tags (ESTs) have been made available (i.e http://www.maizegdb.org), and transcriptome analyses aimed at identifying genes involved in endosperm development and metabolism have been published, along with computer software to systematically characterize them, has made possible to analyze gene expression in developing maize endosperm more thoroughly to identify tissue-specific genes involved in endosperm development and metabolism (Lai et al., 2004; Verza et al., 2005; Liu et al., 2008; Prioul et al., 2008). These studies have shown that in maize, at least 5000 different genes could be expressed during development. However, about 35% of them are orphan genes, whose functions remain enigmatic, possibly corresponding to endosperm specific genes (Liu et al., 2008), as also observed in wheat (Wan et al., 2008). Furthermore, Mèchin and co-workers (2004) have established a proteome reference map for the maize endosperm. They found that metabolic processes, protein destination and synthesis, cell rescue, defence, cell death and ageing were the most abundant functional categories detected in the maize endosperm.

Collectively, the transcriptome and proteome maps constitute a powerful tool for physiological studies and are the first step for investigating maize endosperm development and metabolism. Although, mRNAs are the primary products of gene expression and their levels are often weakly correlated to corresponding protein levels (Gygi et al., 1999), the analyses of the changes in the transcription profiles of endosperm mutants may allow to formulate predictions regarding the biological role of these loci in endosperm development and metabolism. This information is useful for identifying distinctive, previously uncharacterised, endosperm-specific genes; in addition, it provides both further research material for academic laboratories, and material for plant breeders and food processors to include in their respective research or product pipelines.

3. Accumulation of storage products

The structure and biochemical properties of seed storage compounds have been widely investigated over the past 30 years due to their abundance, complexity, and impact on the

overall nutritional value of the maize seed. A great deal is now known about the compounds that are made and stored in seeds, as well as how they are hydrolyzed and absorbed by the embryo. For more detailed reviews describing the nature and biochemistry of maize endosperm and embryo storage products, we refer the reader to a number of recent reviews (i.e. Hannah, 2007; Holding & Larkins, 2009; Motto et al., 2009; Val et al. 2009).

3.1 Storage protein

The primary storage proteins in the maize grain are prolamines called "zeins". Specifically, the zeins are the most abundant protein storage component (>60%) in developing endosperm tissues and are constituted by alcohol-soluble compounds with a characteristic amino acid composition, being rich in glutamine, proline, alanine, and leucine, and almost completely devoid of lysine and tryptophan (Gibbon & Larkins, 2005). From a nutritional point of view, the exceedingly large proportion of codons for hydrophobic amino acids in α-zeins is mostly responsible for the imbalance of maize protein reserves. Therefore, the reduction in α-zein protein accumulation with biased amino acid content could provide a correction to this imbalance. Zeins have also unique functional and biochemical properties that make them suitable for a variety of food, pharmaceutical, and manufactured goods (Lawton, 2002).

Based on their evolutionary relationships, zeins are divided into four protein subfamily of α- (19 and 22-kDa), β- (15 kDa), γ- (16-, 27-, and 50-kDa), and δ-zeins (10- and 18-kDa), that are encoded by distinct classes of structural genes (Holding & Larkins, 2009). Miclaus et al. (2011) have recently reported that α-zein genes have evolved from a common ancestral copy, located on the short arm of chromosome 1, to become a 41-member gene family in the reference maize genome, B73. According to these workers once genes are copied, expression of donor genes is reduced relative to new copies. In particular, epigenetic processes that modify the information content of the genome without changing the DNA sequence, seems to contribute to silencing older copies: some of them can be reactivated when endosperm is maintained as cultured cells, indicating that copy number variation might contribute to a reserve of gene copies.

The proper deposition of zeins inside subcellular structures called protein bodies (PBs) confers the normal vitreous phenotype to the endosperm. PBs are specialized endosperm organelles that form as an extension of the membrane of the rough endoplasmic reticulum (RER), into which zeins are secreted as the signal peptide is processed. After being secreted into the RER, the β- and γ-zeins form a matrix, which is penetrated by the α- and δ-zeins, enlarging the PB and making it a spherical structure of 1-2 μm (Lending & Larkins 1989). Alterations in size, shape or number of PBs generally determine the opaque phenotype (Holding and Larkins 2009), the sole exception being *floury1 (fl1)*, an opaque mutant with no alterations in PB size or shape (Holding et al. 2008). Recently, maize storage protein mutants created through RNAi showed that γ- zein RNA interference (RNAi) maize mutant lines exhibited slightly altered PB body formation and that a more drastic effect was observed in the β-γ- combined mutant, where protein bodies showed an irregular shape, particularly in their periphery (Wu & Messing, 2010). Further studies reported by Llop-Touset et al., (2010) have indicated that the N-terminal proline-rich domain of γ-zein plays an important role in PB formation. To gain a deeper insight into the relationship between RNA and protein

localization in plants, Washida et al. (2009) have identified that the cis-localization elements of the 10-kDa δ-zein are responsible for PB-ER targeting. Their results indicate that there is a close relationship between RNA and protein localization in plant cells and that RNA localization may be an important process in mediating the deposition of storage protein in the endomembrane system in plants.

3.1.1 Endosperm mutants altering storage protein synthesis

As highlighted before, endosperm growth and development is a complex phenomenon that may be driven by the coordinate expression of numerous genes. Strategies using spontaneous and induced mutants allow the characterization of the complex underlying gene expression system integrating carbohydrate, amino acid, and storage protein metabolisms and operating during endosperm growth and development. In this respect several endosperm mutants altering the timing and the rate of zein synthesis have been described (reviewed by Motto et al., 2009). The mutants altering the rate of zein synthesis exhibit a more or less defective endosperm and have a lower than normal zein content at maturity. Many of these genes have been mapped to chromosomes and their effect on zein synthesis has been described (Table 1). All mutants confer an opaque phenotype to the endosperm, and, as zein synthesis is reduced, the overall lysine content is elevated, giving potential for use in the development of "high-lysine" maize.

Genotype	**Inheritance**	**Effect on zein accumulation**	**Molecular bases**
Opaque-2 (o2)	Recessive	22-kDa elimination, 20-kDa reduction,	Transcriptional activator
Opaque-5 (o5)	Recessive	No reduction	MGD1
Opaque-6 (o6)	Recessive	General reduction	
Opaque-7 (o7)	Recessive	General reduction 20 and 22-kDa	ACS-like protein
Opaque-15 (o15)	Recessive	27-kDa reduction, reduction γ-zein	
Opaque-2 modifiers	Semidominant	27-kDa overproduction	
Floury-1 (fl1)	Semidominant	General reduction	Transmembrane protein
Floury-2 (fl2)	Semidominant	General reduction	Defect 22-kDa zein
Floury-3 (fl3)	Semidominant	General reduction	
*Defective-endospermB30 (De*B30)*	Dominant	General reduction	Defect 20-kDa zein
Mucronate (Mc1)	Dominant	General reduction	Abnormal 16-kDa γ-zein
Zpr10(22)	Recessive	10-kDa reduction	

Table 1. Some features of maize mutans affecting zein accumulation.

Genetics has played an important role in discovering a series of opaque endosperm mutants and demonstrating their effects on genes mediating zein deposition (Motto et al., 2009). For

example, the recessive mutation *opaque-2* (*o2*) induce a specific decrease in the accumulation of 22-kDa α-zeins, while the *opaque-15* (*o15*) mutation exerts its effect primarily on the 27-kDa γ-zeins. The *floury1* (*fl1*) mutation is somewhat different, since it does not affect the amount or composition of zein proteins but rather results in the abnormal placement of α-zeins within the PB: *Fl1* encodes a transmembrane protein that is located in the protein body ER membrane. Similarly, Myers et al. (2011) have found that the *opaque5* (*o5*) mutant phenotype is caused by a reduction in the galactolipid content of the maize endosperm, with no change in zein proteins. Furthermore, these workers reported that *O5* locus encodes the *monogalactosyldiacylglycerol synthase (MGD1)* and specifically affects galactolipids necessary for amyloplast and chloroplast function. A further interesting maize opaque endosperm mutant, termed *mto140*, which also shows retarded vegetative growth has been studied by Holding et al., (2010). The seeds showed a general reduction in zein storage protein accumulation and an elevated lysine phenotype typical of other opaque endosperm mutants; however, it is distinct from the other opaque mutants because it does not result from quantitative or qualitative defects in the accumulation of specific zeins but rather from a disruption in amino acid biosynthesis. Because the opaque phenotype co-segregated with a Mutator transposon insertion in an *arogenate dehydrogenase* gene (*zmAroDH-1*), this has led the previous authors the characterization of the four-member family of maize *arogenate dehydrogenase* genes (*zmAroDH-1–4*) which share highly similar sequences. Their differential expression patterns, as well as subtle mutant effects on the accumulation of tyrosine and phenylalanine in endosperm, embryo, and leaf tissues, suggested that the functional redundancy of this gene family provides metabolic plasticity for the synthesis of these important amino acids.

The *o2* mutation has been widely studied at the genetic, biochemical and molecular levels. *O2* encodes a basic leucine zipper (*bZIP*) transcriptional regulator that is specifically expressed in the endosperm (reviewed in Motto et al., 2009). These studies showed that *O2* activates the expression of 22-kDa α-zein and 15-kDa β-zein genes by interacting with the TC-CACGT(a/c)R(a/t) and GATGYRRTGG sequences of their promoters, therefore displaying a broad binding specificity and recognizing a variety of target sites in several distinct genes. *O2* also regulates directly or indirectly a number of other non-storage protein genes, including *b-32*, encoding a type I ribosome-inactivating protein, one of the two cytosolic isoforms of the *pyruvate orthophosphate dikinase* gene (*cyPPDK1*), and *b-70*, encoding a heat shock protein 70 analogue, possibly acting as a chaperonin during PB formation. *O2* also regulates the levels of *lysine-ketoglutarate reductase* (Brochetto-Braga et al., 1992) and *aspartate kinase1* (Azevedo et al., 1997). These broad effects suggest that *O2* plays an important role in the developing grain as a coordinator of the expression of genes controlling storage protein, and nitrogen (N) and carbon (C) metabolism.

The *O7* gene was recently cloned by two different groups, using a combination of map-based cloning and transposon tagging and confirmed by transgenic functional complementation (Miclaus et al., 2011; Wang et al., 2011). Moreover, these last workers via sequence analysis indicated that the *O7* gene showed similarities with members of the larger family of *acyl-CoA synthetase*-like genes (*ACS*), although its exact enzymatic activity is uncertain. In particular, Miclaus et al (2011), have hypothesized a mechanism in which the O7 protein functions in post-translational modification of zein proteins, thus contributing to membrane biogenesis and stability of PBs and conferring the normal vitreous phenotype of

the kernel. Alternatively, Wang et al. (2011) have suggested, by analysis of amino acids and key metabolites, that *O7* gene function might affect amino acid biosynthesis by affecting α-ketoglutaric acid and oxaloacetic acid phenotype, indicating a conserved biological function of *O7* in cereal crops. In this respect, Hartings et al. (2011), in a study to clarify the role that *O2* and *O7* play in endosperm gene expression through transcriptomic analyses, indicated that the *o2* and *o7* mutants alter gene expression in a number of enzymatic steps in the tricarboxylic acid cycle (TCA) and glycolysis pathways that are of central importance for the amino acid metabolism in developing seeds. Although, a systematic characterization of such enzymes will be necessary before any inferences are warranted, the cloning of *O7* revealed a novel regulatory mechanism for storage protein synthesis and highlighted an effective target for the genetic manipulation of storage protein contents in cereal seeds, maize included.

An alternative approach to understand the relationship between zein synthesis and the origin of the opaque endosperm phenotype is to perturb zein accumulation transgenically. In this respect, a number of laboratories have reported a reduction in 22-kDa (Segal et al., 2003) and 19-kDa α-zeins (Huang et al., 2004) by RNAi and by seed-specific expression of lysine rich protein (Rascon-Cruz et al., 2004; Yu et al., 2004).

3.1.2 Regulation of storage protein synthesis

The expression of zein genes is regulated coordinately and zein mRNAs accumulate at high concentrations during early stages of endosperm development (reviewed in Motto et al., 2009). From these studies it was also noted that the coordinate expression of zein genes in maize is controlled primarily at the level of transcription according to specific spatial/temporal patterns. Therefore, attention has turned to understanding the regulatory mechanisms responsible for zein gene expression. Highly conserved *cis*-regulatory sequences have been identified in the promoter of prolamine genes and corresponding *trans*-activity factors (cf Motto et al., 2009). Zein gene expression can also be affected by other regulatory mechanisms, such as methylation, aminoacid supply and phosphorylation. In this context, Locatelli et al. (2009) have provided evidence that O2-mediated transcriptional activation occurs in two-phases, first a potentiated and second a transcriptional activated phase, both characterized by a specific profile of chromatin modifications. The dependency on O2 activity in the establishment of these chromatin states was different for distinct sub-sets of *O2* targets, indicating a gene-specific interaction of *O2* with chromatin modifying mechanisms in driving transcription.

3.1.3 Practical applications and perspectives

Despite efforts to develop opaque mutations that are commercially useful, its inherent phenotypic deficiencies, such as soft endosperm texture, lower yield, increased seed susceptibility to pathogens and mechanical damages, have limited their use. To overcome these drawbacks Quality Protein Maize (QPM) strains were created by selecting genetic modifiers that convert the starchy endosperm of an o2 mutant to a hard, vitreous phenotype. Genetic studies have shown that there are multiple, unlinked *o2* modifiers (*Opm*), (review in Gibbon and Larkins, 2005). Genetic analysis of *o2* modifiers identified several disperse quantitative trait loci (QTLs). Although their molecular identities have remained unknown, QTLs could be correlated with observed increases in 27-kDa γ-zein

transcript and protein in QPM (Holding et al. 2008, and references therein). Two different QTLs, which are candidates for *o2* modifier genes, affect 27-kDa γ-zein gene expression. The first of these is associated with increased expression and the other is linked to *o15*, a mutation at a different chromosome 7 location, which causes decreased 27-kDa γ-zein expression suggesting that the amount of γ-zeins would become critical to keep starch granules embedded in the vitreous area. To examine the role of γ-zeins in QPM, Wu et al., (2010) have used an RNAi construct, designed from the inverted coding sequences of the 27-kDa γ-zein gene, to knock down both 27- and 16-kDa γ-zeins by taking advantage of their DNA sequence conservation. Their findings reinforce the fact that different zeins have evolved to play distinct roles in the development of the endosperm.

Although maize endosperm storage protein genes have been studied for many years, many questions regarding their sequence relationships and expression levels have not been solved, such as structure, synthesis and assembly into protein bodies, and their genetic regulation (Holding and Larkins, 2009). The development of tools for genome-wide studies of gene families makes a comprehensive analysis of storage protein gene expression in maize endosperm possible with the identification of novel seed proteins that were not described previously (Woo et al., 2001). For example, to advance our understanding of the nature of the mutations associated with an opaque phenotype, Hunter et al. (2002) assayed the patterns of gene expression in a series of opaque endosperm mutants by profiling endosperm mRNA transcripts with an Affimetrix GeneChip containing approximately 1,400 selected maize gene sequences. Their results revealed distinct, as well as shared, gene expression patterns in these mutants. Similar research on the pattern of gene expression in *o2*, *o7*, and in the *o2o7* endosperm mutants was carried out by Hartings et al. (2011) by profiling endosperm mRNA transcripts at 14 DAP. Their results, based on a unigene set composed of 7,250 ESTs, allowed to identify a series of mutant related up-regulated (17.1%) and down-regulated (3.2%) transcripts. In addition, the same authors identified several differentially expressed ESTs, homologous to gene encoding enzymes involved in amino acid synthesis, C metabolism (TCA cycle and glycolysis), storage protein and starch metabolism, gene transcription and translation processes, signal transduction, and in protein, fatty acid, and lipid synthesis. Those analyses demonstrate that the mutants investigated are pleiotropic and play a critical role in several endosperm metabolic processes. Although, by necessity, these data are descriptive and more work is required to define gene functions and dissect the complex regulation of gene expression, the genes isolated and characterized to date give us an intriguing insight into the mechanisms underlying amino acid metabolim in the endosperm.

A useful strategy to develop more quickly new QPM varieties has been proposed by Wu and Messing (2011). In fact, conversion of QPM into local germplasm is a lengthy process that discourages the spread of the benefits of QPM because breeders have to monitor a high-lysine level, the recessive *o2* mutant allele, and the modifiers *o2*, (M*o2*s). Accordingly, to overcome this problem these last authors presented a simpler and accelerated QPM selection. Instead of using the recessive *o2* mutation, they used an RNAi construct directed against both 22- and 19-kDa zeins, but linked to the visible green fluorescent protein (GFP) marker gene. Indeed, when such a green and nonvitreous phenotype was crossed with QPM lines, the Mo2s produced a vitreous green kernel, demonstrating that high lysine and kernel hardness can be selected in a dominant fashion.

3.2 Starch synthesis

Maize, like other cereals, accumulate starch in the seed endosperm as an energy reserve. Moreover, its starch is one of the most important plant products and has various direct and indirect applications in food, feed, and industries. For this reason attempts to increase starch accumulation have received a great deal of attention by plant breeders and plant scientists. Starch biosynthesis is a central function in plant metabolism that is accomplished by a multiplicity of conserved enzymatic activities (see Hannah & James 2008, for a review). Roughly three-quarters of the total starch is amylopectin, which consists of branched glucose chains that form insoluble, semi-crystalline granules. The remainder of the starch is amylose, which is composed of linear chains of glucose that adopt a helical configuration within the granule (Myers et al., 2000). Briefly starch synthesis has two fundamental activities represented by starch synthase, which catalyzes the polymerization of glucosyl units into α(1/4)-linked "linear" chains, and starch-branching enzyme, which catalyzes the formation of α(1/6)-glycoside bond branches that join linear chains. Acting together, the starch synthases and starch-branching enzymes assemble the relatively highly branched polymer amylopectin, with approximately 5% of the glucosyl residues participating in α(1/6)-bonds, and the lightly branched molecule amylose. A third activity necessary for normal starch biosynthesis is provided by starch-debranching enzyme (DBE), which hydrolyzes α(1/6)-linkages. Two DBE classes have been conserved separately in plants (Beatty et al., 1999). These are referred as pullulanase-type DBE (PUL) and isoamylase-type DBE (ISA), based on similarity to prokaryotic enzymes with particular substrate specificity. ISA functions in starch production are implied from genetic observations that mutations typically result in reduced starch content, abnormal amylopectin structure, altered granule morphology, and accumulation of abnormally highly branched polysaccharides similar to glycogen.

3.2.1 Genes affecting starch biosynthesis

Starch biosynthesis in seeds is dependent upon several environmental, physiological, and genetic factors (reviewed in Boyer and Hannah, 2001). Moreover, the maize kernel is a suitable system for studying the genetic control of starch biosynthesis. A large number of mutations that cause defects in various steps in the pathway of starch biosynthesis in the kernel have been described. Their analysis has contributed greatly to the understanding of starch synthesis (reviewed in Boyer and Hannah, 2001). In addition, these mutations have facilitated the identification of many genes involved in starch biosynthetic production. As there seems little point in reviewing these data, we will simply summarize in Table 2 cloned maize genes and their gross phenotypes. Although, the effects shown in this table may not necessarily be the primary effect of a mutant, these are the ones presently known. More recently, Kubo et al. (2010) have described novel mutations of *sugary1 (su1*) and *isa2* loci, coding for isoamylase-type starch-DE enzyme (ISA) ISA1 and ISA2, respectively: Their data indicate that in maize endosperm these enzymes function to support starch synthesis either as a heteromeric multisubunit complex containing both ISA1 and the noncatalytic protein ISA2 or as a homomeric complex containing only ISA1. In particular, it was found that i) homomeric ISA has specific functions that determine amylopectin structure that are not provided by heteromeric ISA and ii) tissue-specific changes in relative levels of ISA1 and ISA2 transcripts, or functional changes in the ISA1 protein, could explain how maize endosperm acquired the homomeric enzyme.

Genotype	Mayor biochemical changes[a]	Enzyme affected
Shrunken-1 (sh1)	↑ Sugars, ↓ Starch	↓ Sucrose synthase
Shrunken-2 (sh2)	↑ Sugars, ↓ Starch	↓ ADPG-pyrophosphorylase, ↑ Hexokinase
Brittle-1 (bt1)	↑ Sugars, ↓ Starch	↓ Starch granule-bound phospho-oligosaccharide synthase
Brittle-2 (bt2)	↑ Sugars, ↓ Starch	↓ ADPG-pyrophosphorylase
Shrunken-4 (sh4)	↑ Sugars, ↓ Starch	↓ Pyridoxal phosphate
Sugary-1 (su)	↑ Sugars, ↓ Starch	↑ Phytoglycogen branching enzyme, ↓ Phytoglycogen debranching enzyme
Waxy (wx)	↑ 100% Amylopectin	↓ Starch-bound starch syntase, ↑ Phytoglycogen branching enzyme
Amylose-extender(ae)	↑ Apparent amylose, ↑ Loosely branched polysaccharide	↓ Branching enzyme IIb
Dull-1 (du1)	↑ Apparent amylase	↓ Starch synthase II, ↓ Branching enzyme Iia,↑ Phytoglycogen branching enzyme

Table 2. Summary of mutant effects in maize where an associated enzyme lesion has been reported.[a] Changes relative to normal. ↑, ↓ = increase or decrease, respectively. Sugars = the alcohol-soluble sugars.

Many biochemical and molecular studies on starch synthesis have been also focused on identifying the rate limiting enzymes to control metabolism. In this context, ADP-glucose pyrophosphorylase (AGPase) plays a key role in regulating starch biosynthesis in cereal seeds. The AGPase in the maize endosperm is a heterotetramer of two small subunits encoded by *Brittle2* (*Bt*2) gene, and two large subunits, encoded by the *Shrunken2* (*Sh2*) gene. Transgenic approaches focused on allosteric regulation of AGPase, although studies of the kinetic mechanism of maize endosperm AGPase has uncovered complex regulatory properties (Kubo et al., 2010), increase starch content and caused an increased seed weight than lines expressing wild-types (Giroux et al., 1996; Wang et al., 2007). Additional research has been also devoted to the over-expression of the wide-type genes encoding maize AGPase. For example Li et al. (2011), have transferred the *Bt*2 and *Sh2* genes from maize, with an endosperm-specific promoter from 27-kDa zein or an endosperm-specific promoter from 22-kDa zein, into elite inbred lines, solely and in tandem, by *Agrobacterium tumefaciens*-mediated transformation. They found that developing transgenic maize kernels exhibited higher *Bt2* and *Sh2* gene expression, higher AGPase activity, higher seed weight, and the kernels accumulated more starch compared with non-transgenic plants. The over-expression of either gene enhanced AGPase activity, seed weight (+15%) and starch content compared with the wild type, but the amounts were lower than plants with over-expression of both *Bt2* and *Sh2*. Collectively, these results indicate that over-expression of those genes in transgenic maize plants could improve kernel traits and provide a feasible approach for enhancing starch content and seed weight in maize.

3.2.2 Regulation of starch synthesis

In spite of the above mentioned studies as a complex metabolic pathway, the regulation of starch biosynthesis is still poorly understood. This is surprising, considering the number and variety of starch mutations identified so far, which may indicate that nutrient flow is the key regulatory stimulus in carbohydrate interconversion. In this connection, it has been argued that glucose also serves as a signal molecule in regulating gene expression, in some cases, different sugars or sugar metabolites might act as the actual signal molecules (reviewed in Koch, 2004). There is evidence that regulation of major grain-filling pathway is highly integrated in endosperm. Gene responses to sugars and C/N balance have been implicated. For example, Sousa et al. (2008) have recently identified in maize a gene for *Sorbitol dehydrogenase1* (*Sdh1).* They showed that this gene is highly expressed early in seed development throughout the endosperm, with greatest levels in the basal region, compatible with SDH involvement in the initial metabolic steps of carbohydrate metabolism. The same authors also presented genetic, kinetic, and transient expression evidence for regulation at the transcriptional level by sugars and hypoxia. Moreover, many pleiotropic defective kernel (*dek*) mutations that fail to initiative or complete grain-filling have been identified, but not studied in detail. These are likely to include mutations in "housekeeping genes" as well as important developmental mutants or transcription factors. In this respect, a key challenge is to devise molecular and genetic strategies that can be used to effectively analyse this large, complex phenotypic class. As far as transcription factors are concerned, Fu & Xue (2010) have recently identified in rice candidate regulators for starch biosynthesis by gene coexpression analysis. Among these genes, *Rice Starch Regulator1 (RSR1*), an APETALA2/ethylene-responsive element binding protein family transcription factor, was found to negatively regulate the expression of type I starch synthesis genes; moreover, *RSR1* deficiency results in the enhanced expression of starch synthesis genes in seeds. Collectively these results demonstrate the potential of co-expression analysis for studying rice starch biosynthesis and the regulation of a complex metabolic pathway and provide informative clues, including the characterization of *RSR1*, to facilitate the improvement of seed quality and nutrition. It is expected that similar orthologous loci will be soon identified in maize; this will allow us to deeper our knowledge on regulatory mechanisms affecting starch biosynthesis in maize.

Different approaches in this area are needed to identify direct interaction among starch biosynthetic enzymes, as well as modifying factors that regulate enzyme activity. In this respect, Wang et al. (2007) described a study in which a bacterial *glgC16* gene, which encodes a catalytically active allosteric-insensitive enzyme, was introduced into maize. The results of this study showed that developing transgenic maize seeds exhibited higher AGPase activity (a rate limiting step in glycogenesis and starch synthesis), in the presence of an inhibitory level of Pi in vitro, compared with the untransformed control. More interestingly, the same authors fuond the seed weight of transgenic plants was increased significantly. Furthermore, tools for genome-based analyses of starch biosynthesis pathway are now available for maize and other cereals. This may eventually help to explain species differences in starch granule shape and size, and thus provide the potential for agricultural advances. Recently, Prioul et al. (2008) have provided information on carbohydrate metabolism by comparing gene expression at three levels - transcripts, proteins and enzyme activities - in relation to substrate or product in developing kernels from 10 to 40 DAP. Their

study have identified two distinct patterns: during endosperm development: invertases and hexoses are predominant at the beginning, whereas enzyme patterns in the starch pathway, at the three levels, anticipate and parallel starch accumulation, suggesting that, in most cases, transcriptional control is responsible for the regulation of starch biosynthesis.

3.3 Lipids

While intensive agricultural and industrial uses of the maize kernel is widely due to its high starch content, the oil stored in the maize kernel also has considerable importance. Moreover, its oil is the most valuable co-product from industrial processing of maize grain through wet milling or dry milling and is high-quality oil for human.

Research in this field (for review see Val et al., 2009) indicate that i) the mature embryo is approximately 33% lipid in standard hybrids and contains about 80% of the kernel lipids; ii) high-oil maize shows a greater feed efficiency than normal-oil maize in animal feed trials: the caloric content of oil is 2.25 times greater than that of starch on a weight basis and its fatty acid composition, mainly oleic and linoleic acids; iii) maize oil is highly regarded for its low level of saturated fatty acids, on average 11% palmitic acid and 2% stearic acid, and its relatively high levels of polyunsaturated fatty acids such as linoleic acid (24%); and iv) maize oil is relatively stable, since it contains only small amounts of linolenic acid (0.7%) and high levels of natural antioxidants. Additionally, it was found that oil and starch are accumulated in different compartments of the maize kernel: 85% of the oil is stored in the embryo, whereas 98% of the starch is located in the endosperm. Therefore, the relative amounts of oil and starch are correlated with the relative sizes of the embryo and endosperm and successful breeding for high oil content in the Illinois High Oil strains has mainly been achieved through an increase in embryo size (Moose et al., 2004). Whereas the embryo represents less than 10% of the kernel weight in normal or high-protein lines, it can contribute more than 20% in high-oil lines. However, genetic components may also modulate oil content in the embryo, independently of its size, as shown by the cloning of a high-oil QTL in maize that is caused by an amino acid insertion in an acyl-CoA:diacylglycerol acyltransferase catalyzing the last step of oil biosynthesis (Zheng et al., 2008).

3.3.1 Lipid biosynthetic pathway and genetic inheritance

The primary determinant of amount of lipids in maize kernels is the genetic makeup (Lambert, 2001). In maize studies through genetic mapping of oil traits reported that multiple (>50) QTLs are involved in lipid accumulation (Laurie et al., 2004), making yield improvement through conventional breeding difficulty. High-oil varieties of maize were developed at the University of Illinois through successive cycles of recurrent selection (Dudley and Lambert, 1992). Although these lines have an improved energy content for animal feeding applications, the poor agronomic characteristics, including disease susceptibility and poor standability. These deficiencies precluded their commercial introduction on broad hectarage.

In spite of a good understanding of the oil biosynthetic pathway in plants and of the many genes involved in oil pathway have been isolated, the molecular basis for oil QTL is largely unknown. However, Zheng et al. (2008) have recently found that a oil QTL (*qHO6*) affecting

maize seed oil and oleic-acid content, encodes an acyl-CoA:diaglycerol acytransferase (DGAT1-2), which catalyze the final step of oil synthesis.

As far as the composition in concerned, maize oil is mainly composed of palmitic, stearic, oleic, linoleic, and linolenic fatty acids. Evidence has shown that genetic variation existed also for the fatty acid composition of the kernel (Lambert, 2001). In essentially all studies, researchers suggested that major gene effects were being modulated by modifier genes for oil composition. Although it seems that sources of major genes for composition of maize oil can be utilized, other studies indicate that the inheritance of oleic, linoleic, palmitic, and stearic acid content when considered together is complex and under multigenic control (Sun et al., 1978). Molecular characterization of fatty acid desaturase-2 (*fad2*) and fatty acid desaturase-6 (*fad6*) in this plant indicates that *fad2* and *fad6* clones are not associated with QTLs for the ratio of oleic/linoleic acid, suggesting that some of the QTLs for the oleic/linoleic acid ratio do not involved variants of *fad2* and *fad6*, but rather involved other gene that may influence flux via enzymes encoded by *fad2* or *fad6*. Additional studies are needed to more precisely identify the genes and enzymes involved in determining the composition of maize oil. Application of powerful new technologies, such as transcription profiling, metabolic profiling, and flux analyses, should prove valuable to achieving this scope. In addition, identification of transcription factors or other regulatory proteins that exert higher level control of oil biosynthesis or embryo development will be particularly attractive candidate for biotechnology approaches in the future.

In maize, Pouvreau et al. (2011) have recently identified orthologs related, respectively, to the master regulators *LEAFY COTYLEDON1-2 (i.e. ZmLEC1),* that directly activate in Arabidopsis genes involved in TAG metabolism and storage, and to the transcription factor *WRINKLED1* (i.e.. *ZmWRI1a* and *ZmWRI1b)*, necessary to mediate the regulatory action of the master regulators towards late glycolytic and oil metabolism. In this crop, both genes are preferentially expressed in the embryo and exhibit a peak of expression at the onset of kernel maturation. *ZmWRI1a* is induced by *ZmLEC1* (Shen et al., 2010). Additionally, transcriptomic analyses carried out on *ZmWRI1a* over-expressing lines have allowed to the previous workers to identify putative target genes of *ZmWRI1a* involved in late glycolysis, fatty acid or oil metabolism. Though not fully overlapping, the sets of *AtWRI1* and *ZmWRI1a* target genes are very resembling. Exhaustive analyses relying on ChIP experiments would allow determining whether these sets are identical. Interestingly, the DNA AW-box proposed to be bound by *AtWRI1* (Maeo et al., 2009) was also identified in promoter sequences of putative target genes of *ZmWRIa*, suggesting that even the *cis*-regulatory element recognized by *WRI1* seems have been conserved between dicots and monocots. Additionally this study has shown that transgenic *ZmWri1a-OE* kernels did not only induce a significant increase in saturated and unsaturated fatty acids with 16 to 18 C atoms but also cause a significant increase for several free amino acids (Lys, Glu, Phe, Ala, Val), intermediates or cofactors of amino acid biosynthesis (pyro-Glu, aminoadipic acid, Orn, nor- Leu), and intermediates of the TCA cycle (citric acid, succinic acid). Since the transcriptome analysis suggests that *ZmWri1a* essentially activates genes coding for enzymes in late glycolysis, fatty acid, CoA, and TAG biosynthesis, and considering that no misregulated candidates participate in any additional pathways, the increase in amino acids and TCA intermediates probably reflects secondary adjustments of the C and N metabolism to the increased oil biosynthesis triggered by *ZmWri1a*. The three amino acids Phe, Ala, and

Val are derived from PEP or pyruvate, and their increase may simply be a byproduct of a strongly increased C flux through glycolysis.

3.4 Carotenoid pigments

Along with their essential role in photosynthesis, carotenoids are of significant economic interest as natural pigments and food additives (reviewed in Botella-Pavía & Rodríguez-Concepción, 2006). Their presence in the human diet provides health benefits as nontoxic precursors of vitamin A and antioxidants, including protection against cancer and other chronic diseases (review by Fraser & Bramley 2004). These motives have promoted scientists to explore ways to improve carotenoid content and composition in staple crops (reviewed in Sandmann et al. 2006; Zhu et al. 2009). Analyses of genotypes with yellow to dark orange kernels exhibits considerable natural variation for kernel carotenoids, with some lines accumulating as much as 66 µg/g (e.g. Harjes et al., 2008), with provitamin A activity (*β*-cryptoxanthin, *a*- and *β*-carotene is typically small (15% to 18% of the total carotenoids fraction) compared to lutein or zeaxanthin (45% and 35%, respectively; Kurlich & Juvik, 1999; Brenna and Berardo, 2004). Moreover, a moderate to high heritability estimates indicate that breeding for increased levels of both carotenes and xanthophylls should be feasible.

3.4.1 Carotenoid biosynthesis and genetic control

Carotenoids are derived from the isoprenoid biosynthetic pathway and are precursors of the plant hormone abscisic acid (ABA) and of other apocarotenoids (Matthews and Wurtzel, 2007). In maize characterization of the carotenoid biosynthetic pathway has been facilitated by the analysis of mutants associated with reduced levels of carotenoids. In fact, by using this approach in maize three genes controlling early steps in the carotenoid pathway have been cloned. The use of these cloned genes as probes on mapping populations will enable the candidate gene approach to be used for studying the genetic control of quantitative variation in carotenoids. Accordingly, Wurtzel et al.. (2004) detect major QTLs affecting accumulation of *β*-carotene and *β*-cryptoxanthin indicating that these QTLs could be selected to increase levels of pro-vitamin A structures. Chander et al. (2007), using a RIL population found 31 QTL including 23 for individual and 8 for total carotenoid accumulations. Moreover, Harjes et al. (2008), via association mapping, linkage mapping, expression analysis, and mutagenesis, showed that variation in *lycopene epsil cyclase* (*lcyE*) locus alters flux down *a*-carotene versus *β*-carotene branches of the carotenoid pathway. Additional experimental evidence obtained by Yan et al. (2010) have documented that also the gene encoding *β-carotene hydroxylase1 (crtRB1)* underlies a principal QTL associated with *β*-carotene concentration and conversion in maize kernels. Moreover, the same workers noted that the *crtRB1* alleles associated with reduced transcript expression correlate with higher β-carotene concentrations. Genetic variation at *crtRB1* also affects hydroxylation efficiency among encoded allozymes, as observed by resultant carotenoid profiles in recombinant expression assays. Similarly, studies on natural maize genetic diversity carried out by Vallabhaneni et al (2009), have provided the identification of hydroxylation genes associated with reduced endosperm provitamin A content. In particular transcript profiling led to discovery of the *Hydroxylase3* locus that coincidently mapped to a carotene QTL, thereby prompting investigation of allelic variation in a broader collection. Vallabhaneni & Wurtzel (2009) have sampled a maize germplasm collection via statistical testing of the

correlation between carotenoid content and candidate gene transcript levels. They observed multiple pathway bottlenecks for isoprenoid biosynthesis and carotenoid biosynthesis acting in specific temporal windows of endosperm development. Transcript levels of paralogs encoding *isoprenoid isopentenyl diphosphate* and *geranylgeranyl diphosphate*-producing enzymes, such as DXS3 (*1-deoxy-D-xylulose-5-phosphate synthase3*), *DXR* (*DXP reductoisomerase*), *HDR* (*4-hydroxy-3-methylbut-2-enyl diphosphate reductase*), and *GGPPS1* (*geranylgeranyl pyrophosphate synthase1*), were found to positively correlate with endosperm carotenoid content. Toledo-Ortiz et al. (2010) have recently identified in *Arabidopsis* seedlings that *phytochrome-interacting factor1* (*PIF1*) and other transcription factors of the *phytochrome-interacting factor* (*PIF*) family down-regulate the accumulation of carotenoids by specifically repressing the gene encoding *PSY*, the main rate-determining enzyme of the pathway. Their results also suggest a role for *PIF1* and other *PIFs* in transducing light signals to regulate *PSY* gene expression and carotenoid accumulation during daily cycles of light and dark in mature plants. In this context, manipulating the levels of *PIF* transcription factors by transgenic or marker-assisted breeding approaches might help improve carotenoid accumulation in plants for the production of varieties with enhanced agronomical, industrial, or nutritional value.

4. New strategies for creating variation

The use of molecular biology to isolate, characterize, and modify individual genes followed by plant transformation and trait analysis will introduce new traits and more diversity into maize database. For example, maize-based diets (animals or humans) require lysine and tryptophan supplementation for adequate protein synthesis. The development of high-lysine maize to use in improved animal feeds illustrates the challenges that continually interlace metabolic engineering projects. From a biochemical standpoint, the metabolic pathway for lysine biosynthesis in plants is very similar to that in many bacteria. The key enzymes in the biosynthetic pathway are aspartakinase (AK) and dihydrodipicolinic acid synthase (DHDPS), both of which are feedback inhibited by lysine (Galili, 2004). Falco et al. (1995) isolated bacterial genes encoding lysine-insensitive forms of AK and DHDPS from *Escherichia coli* and *Corynebacterium*, respectively. A deregulated form of the plant DHDPS was created by site-specific mutagenesis (Shaver et al., 1996). The expression of the bacterial DHPS in maize seeds overproduced lysine, but they also contained higher level of lysine catabolic products then their wild-type parents (Mazur et al., 1999), despite the fact that lysine catabolism was suggested to be minimal in this tissue (Arruda et al., 2000). Likewise, a gene corresponding to a feedback-resistant form of the enzyme anthranilate synthase (AS) has been cloned from maize and re-introduced via transformation under the control of seed-specific promoters. This altered AS has reduced sensitivity to feedback inhibition by tryptophan; thus, tryptophan is overproduced and accumulates to higher than normal levels in the grain. This strategy has been successful in reaching commercially valuable levels of tryptophan in the grain (Anderson et al., 1997). More recently, Houmard et al. (2007) reported the increase in maize grains by specific suppression of lysine catabolism via RNAi. An important observation from these studies was that the lysine content was increased in the transgenic lines by 15-20% to 54.8%. These experiments showed that transgenic approaches, in addition to investigating relationships between zein synthesis and opaque endosperm, could be useful to increase kernel lysine content. Similarly, Reyes et al. (2008), using RNAi, have produced transgenic maize lines that had LKR/SDH suppressed in the

embryo, endosperm or both. These authors noted a synergist increase in free lysine content in the mature kernel when LKR/SDH was suppressed in both embryo and endosperm; these results have also suggested new insights into how free lysine level is regulated and distributed in developing grains.

A different approach to enhance the level of a given amino acid in kernels is to improve the protein sink for this amino acid (Kriz, 2009). This goal can be achieved by transforming plants with genes encoding stable proteins that are rich in the desired amino acid(s) and that can accumulate to high levels. Among a variety of natural, modified or synthetic genes that were tested, the most significant increases in seed lysine levels were obtained by expressing a genetically-engineered hordothionine (HT12) or a barley high-lysine protein 8 (BHL8), containing 28 and 24% lysine, respectively (Jung and Falco, 2000). These proteins accumulated in transgenic maize to 3-6% of total grain proteins and when introduced together with a bacterial DHPS, resulted in a very high elevation of a total lysine to over 0.7% of seed dry weight (Jung and Falco, 2000) compared to around 0.2% in wild-type maize. Similarly, Rascon-Cruz et al. (2004) have found that the introduction of a gene encoding amarantin-protein from *Amaranth* plants, which is known to be balanced in its amino acid content, increases from 8 to 44% essential amino acid content. Bicar et al. (2008) have developed transgenic maize lines that produce milk *a*-lactalbumin in the endosperm. They noted that the lysine content of the lines examined was 29-47% greater in endosperm from transgene positive kernels. Furthermore, Wu et al. (2007) provided a novel approach to enrich the lysine content (up to 26%) in the maize grain by endosperm-specific expression of an *Arabidopsis* lysyl tRNA synthate. Combining these traits with seed-specific reduction of lysine catabolism offers an optimistic future for commercial application of high-lysine maize.

Single mutations in starch biosynthesis have been commercially used for the production of some specialty maize. For example, specialty varieties such as waxy can result in 99% amylopectins, while the use of "amylomaize varieties" (*amylose extender* endosperm mutants) have kernels up to 20% amylopectin and 80% amylose. These varieties are of interest for commercial purposes in starch industry, such as food ingredients, sweeteners, adhesives, and for the development of thermoplastics and polyurhetanes. However, advances in understanding the starch biosynthetic pathway provide new ways to redesign starch for specific purposes, such for ethanol production. Alteration in starch structure can be achieved by modifying genes encoding the enzymes responsible for starch synthesis, many of which have more than one isoform (Boyer and Hannah, 2001). Transgenic lines with modified expression of specific starch synthases, starch branching enzymes or starch debranching enzymes are being generated in attempts to produce starch granules with increased or decreased crystallinity, and thus altered susceptibility to enzymatic digestion. Another strategy is to reduce the energy requirements for the starch to ethanol conversion process. For example, gelatinization is the first step in bioethanol production from starch. It is conceivable that a modified starch with decreased gelatinization temperature might require less energy for the conversion process. Research showed that expression of a recombinant amylopullulanase in rice resulted in starch that when heated to 85°C was completely converted into soluble sugars (Chiang, 2005). The expression of microbial genes in transgenic plants represents also an opportunity to produce renewable resources of fructans. Transgenic maize expressing the *Bacillus amyloliquefaciens SacB* gene accumulates high-molecular weight fructose in mature seed (Caimi et al., 1996). This could potentially be exploited within the high-fructose maize syrup market. Moreover, Zhang et al. (2007) have

developed transgenic maize endosperm, via the introduction of a *Streptococcus mutans gtfD* gene, that accumulates novel glucan (oligo- and polysaccharides composed solely of glucose molecules) polymers at levels relevant to commercial production. The expression of that gene yielded fully functional GTF-D enzyme as shown by accumulation of novel soluble α-(1→6)-linked glucan at high levels in the mature maize kernels (up to 14% of their dry weight). These findings suggest that the introduction of greater diversity in linkages within α-glucan polymers will enable the generation of specialty glucans to replace modified starches used for several products (e.g. thickening reagents, adhesives, textile modification, and papermaking polymers with economical and environmental benefits).

Efforts to increase oil content and composition in maize kernels through breeding have considerable success, but high oil lines have significant reduced yield (cf Moose et al., 2004). Several and complementary approaches might be considered to try and enhance oil content in maize kernels. This goal may be achieved by increasing the relative proportion of the oil-rich embryonic tissue within the grain. It has been recently reported that embryo size and oil content could be increased in transgenic maize by ectopic expression of the wheat *Purindoline a* and *b* (*PINA* and *PINB*) genes (Zhang, et al., 2010). While total oil content of the kernel was increased by 25% in these transgenic lines, the molecular mechanism responsible for the increase remains to be clarified. If no modification of kernel size was observed in these transgenic lines, other agronomic characteristics remain to be studied to evaluate the economic potential of such material. Another strategy to increase oil accumulation in the grain may consist in improving both oil content of embryonic tissues. A close examination of C metabolism in maize embryos suggested that the flux of C through NADP-ME may constitute a metabolic bottleneck (Alonso et al., 2010). Accordingly, the oil content of the kernel was positively correlated with malic enzyme activities in maturing embryos (Doehlert & Lambert, 1991), which makes NADP-ME an attractive target for engineering high oil concentrations in embryos of maize. Furthermore, in oilseed species, numerous biotechnological approaches have been carried out that were aimed at maximizing the flow of C into oil by overexpression of enzymes of the TAG assembling network. For example in maize, several attempts have been made to over-express *diacylglycerol acyltransferases* (*DGAT*). *DGAT* catalyses the transfer of an acyl chain from the acyl-CoA pool to the *sn-3* position of a diacylglycerol molecule, resulting in the synthesis of TAG. The embryospecific over-expression of both maize *DGAT1-2* and of fungal *DGAT2 (*Zheng et al., 2008; Oakes et al., 2011) resulted in limited (1.25 fold) but statistically significant increases in kernel oil content. Whereas it has been shown that grain yield was not affected by expression of fungal *DGAT2*, data concerning the putative incidence of the over-expression of maize *DGAT1-2* on yield and other agronomic characteristics of the modified lines are missing. Nevertheless these works provide insights into the molecular basis of natural variation of oil and oleic-acid contents in plants and highlight *DAGT* as a promising target for increasing oil and oleic-acid content in other crops.

The recent identification of transcriptional regulators of the oil biosynthetic network in maize has opened the way for designing and testing new original biotechnological strategies. A study has shown that seed-specific expression *ZmWRI1*, a WRI1-like gene of maize, enhanced oil accumulation in transgenic maize without detectable abnormalities. However, expression of *ZmLEC1* under similar conditions severely affected growth and development of the resulting transgenic maize plants (Shen, et al., 2010). Similar results

were obtained by constitutive overexpression of the *ZmWRI1* gene in the transgenic maize plants (Pouvreau et al., 2011*)*. It was also found that *ZmWri1* not only increases the fatty acid content of the mature maize grain but also the content of certain amino acids (Lys, Glu, Phe, Ala, Val) of several compounds involved in amino acid biosynthesis (pyro-Glu, aminoadipic acid, Orn, nor- Leu), and of two intermediates of the TCA cycle(citric acid, succinic acid) (Pouvreau et al., 2011). Finally, a third approach to increase oil content in maize grains may consist in diverting C flux from starch to oil in the endosperm. Considering both the elevated amounts of ATP consumed in futile cycling processes and the rates of reductant production in endosperm tissues of maize kernels, Alonso et al (2010) have speculated that increasing biomass synthesis and redirecting part of the C flux toward fatty acid production by metabolic engineering could theoretically be obtained. This would require inhibiting futile cycling whilst overexpressing the whole set of enzymes involved in TAG production. To date, no successful attempt has been reported. If the use of *ZmWRI1* as a biotechnological tool for improving oil content in embryos of maize seems promising (see above), over-expression of *ZmWRIa* in the starchy endosperm was not sufficient to trigger oil accumulation in this compartment (Shen et al., 2010). Since there is no evidence that WRI1 regulates TAG assembly, it is not surprising that over-expression of ZmWRI1a only proves to be efficient in tissues already accumulating oil, and thus already expressing the TAG biosynthetic machinery. What is more, the structure and size of maize kernels may impair large accumulation of oil in the endosperm.

The cloning of carotenogenic genes in maize and in other organisms have opening up the possibility of modifying and engineering the carotenoid biosynthetic pathways in plants, although question remains about the rate-controlling steps that limit the predictability of metabolic engineering in plants. Engineering high levels of specific carotenoid structures requires controlled enhancement of total carotenoid levels (enhancing pathway flux, minimizing degradation, and optimizing sequestration) plus controlled composition for specific pathway end products. While most of the nuclear genes for the plastid-localized pathway are available (Li et al. 2007) and/or can be identified, questions remain about the rate-controlling steps that limit the predictability of metabolic engineering in plants. Predictable manipulation of the seed carotenoid biosynthetic pathway in diverse maize genotypes necessitates the elucidation of biosynthetic step(s) that control carotenoid accumulation in endosperm tissue. Studies have implicated PSY, the first committed enzyme, as rate controlling for endosperm carotenoids (e.g. Pozniak et al., 2007; Li et al., 2008). However, upstream precursor pathways may also positively influence carotenoid accumulation (Matthews and Wurtzel, 2000; Mahmoud & Croteau, 2001), while downstream degradative pathways may deplete the carotenoid pool (Galpaz et al., 2008).

Transgenic strategies can also be used as tools to complement breeding techniques in meeting the estimated levels of provitamin A. In this respect, Aluru et al. (2008) reported that the overexpression of the bacterial genes *crtB* (for PS) and *crtI* (for the four desaturation steps of the carotenoid pathway catalyzed by PDS and β-carotene desaturase in plants), resulted in an increase of total carotenoids of up to 34-fold with a preferential accumulation of β-carotene in the maize endosperm. The levels attained approach those estimated to have a significant impact on the nutritional status of target populations in developing countries. Furthermore, the same authors, via gene expression analyses, suggested that increased accumulation of β-carotene is due to an up-regulation of the endogenous lycopene β-cylase.

These experiments set the stage for the design of transgenic approaches to generate provitamin A-rich maize that will help alleviate vitamin A deficiency in developing countries. Similarly, Naqvi et al. (2009) produced transgenic maize plants with significantly increased contents for *β*-carotene, ascorbate, and folate in the endosperm via that simultaneous modification of 3 separate metabolic pathways. The transgenic kernels contained 169-fold the normal amount of *β*-carotene, 6-fold, and 2-fold the normal amount of ascorbate and folate, respectively. This finding, which largely exceeds any realized thus far by conventional breeding alone, opens the way for the development of nutritional complete cereals to benefits the consumers in developing countries. Moreover, this is a very important proof of concept for genetic manipulation of distinct metabolic pathways.

There is evidence indicating that tocophenols, in particular γ-tocophenol the predominant form of vitamine E in plant seeds, are indispensable for protection of the polyunsaturated fatty acid in addition to have benefits to the meet industry (Rocheford et al., 2002). The same authors have also shown that considerable variation is present among different maize inbreds from tocophenol levels, as well as different ratios of α-tocophenol to γ-tocophenol. This result suggested that breeders can use natural varieties, molecular marker assisted selection strategies and transgenic technologies to alter overall level of tocophenols and ratio of α- to γ-tocophenol. However, current nutritional research on the relative and unique benefits of α- to γ-tocophenol should be considered in developing breeding strategies to alter levels of these vitamin E compounds.

Another area in which transgenic approaches may help solve an important problem with maize as a feed grain is in the reduction of phytic acid levels. In maize, 80% of the total phosphorous (P) is found as phytic acid, and most of that is in the germ (O'Dell et al., 1972). Phytate P is very poorly digested by non-ruminant animals, therefore inorganic supplementation is necessary. Phytate is also a strong chelator that reduces the bioavailability of several other essential minerals such as Ca, Zn, Cu, Mn, and Fe. In addition, since the phytate in the diet is poorly digested, the excrement of monogastric animals (e.g. poultry and pigs), is rich in P and this contributes significantly to environmental pollution. *Low phytic acid* mutants (*lpa*) of maize are available; these have received considerable attention by breeders in order to develop commercially acceptable hybrids with reduced levels of phytic acid (Raboy, 2009).

In maize, several mutants with low levels of phytate have been isolated and mapped; this includes *lpa 1-1*, *lpa 2-1,* and *lpa 241*, (Raboy, 2009). The *lpa1* mutant does not accumulate *myo*-inositol monophosphate or polyphosphate intermediates. It has been proposed that *lpa1* is a mutation in *myo*-inositol supply, the first part of the phytic acid biosynthesis pathway (Raboy et al., 2000). The *lpa2* mutant has reduced phytic acid content in seeds and accumulates *myo*-inositol phosphate intermediates. Maize *lpa2* gene encodes a *myo*-inositol phosphate kinase that belongs to the Ins(1,3,4)P3 5/6-kinase gene family (Shi et al., 2003). The *lpa3* mutant seeds have reduced phytic acid content and accumulate *myo*-inositol, but not *myo*-inositol phosphate intermediates was found to encode *myo*-inositol kinase (Shi et al., 2005).

Despite efforts to elucidate and manipulate phytic acid biosynthesis, *low phytic acid* mutants have limited value to breeders because of adverse effects on agronomic traits such as low germination rates, reduced seed weight (*lpa1-1*), stunted vegetative growth and impaired

seed development (*lpa241*). However, Shi et al. (2007) have recently identified the gene disrupted in maize *lpa1* mutants as a multidrug resistance-associated protein (MRP) ATP-binding cassette (ABC) transporter. Silencing expression of this transporter using the embryo-specific globuline promoter produced low-phytic acid, high phosphate transgenic maize seeds that germinate normally and do not show any significant reduction in seed dry weight.

To increase the amount of bioavailable iron in maize, Drakakaki et al. (2005) have generated transgenic maize plants expressing aspergillus phytase and iron-binding protein ferritin. This strategy has proven effective for increasing iron availability and enhancing its absorption. However, much work is still to be done to transfer this technology to tropical and subtropical maize genotypes normally grown in the areas of greatest need for enhanced iron content maize.

A relatively new area in plant biotechnology is the use of genetically-engineered maize to produce high-value end products such as vaccines, therapeutic proteins, industrial enzymes and specialty chemicals (see Hood & Howard, 2009 for a review). The long-term commercial expectations for this use of "plants as factories", often also called "molecular farming", are large. Transgenic maize seed has many attractive features for this purpose, including: i) well-suited for the production and storage of recombinant proteins; ii) ease of scale-up to essentially an infinite capacity; iii) well-established infrastructure for producing, harvesting, transporting, storing, and processing; iv) low cost of production; v) freedom from animal pathogenic contaminants; vi) relative ease of producing transgenic plants which express foreign proteins of interest. However, there is a need, apart the public issues related with the acceptance of genetically-engineered maize, for continued efforts in increasing expression in order to reduce cost effectiveness for products at protein accumulation levels in transgenic plants to broaden this new uses.

5. Conclusion and future perspectives

Two prominent features of agriculture in the 20th century have been the use of breeding and genetics to boost crop productivity and the use of agricultural chemicals to protect crops and enhance plant growth. In the 21st century, crops must produce good yields while conserving land, water, and labor resources. At the same time, industries and consumers require plants with an improved and novel variation in grain composition. We expect that genomics will bolster plant biochemistry as researchers seek to understand the metabolic pathways for the synthesis of these compounds. Identifying rate-limiting steps in synthesis could provide targets for genetically engineering biochemical pathways to produce augmented amounts of compounds and new compounds. Targeted expression will be used to channel metabolic flow into new pathways, while gene-silencing tools will reduce or eliminate undesirable compounds or traits. Therefore, developing plants with improved grain quality traits involves overcoming a variety of technical challenges inherent in metabolic engineering programs.

Metabolism is one of the most important and best recognized networks within biological systems. However, advances in the understanding of metabolic regulation still suffer from insufficient research concerning the modular operation of such networks. Elucidation of metabolic regulation within the context of the entire system, including transcriptional, translational and posttranslational mechanisms, is rarely attempted (Sweetlove et al., 2008).

Instead, to date, studies on metabolic regulation have mostly been limited to regulatory interactions within the metabolic pathways themselves (Sweetlove & Fernie, 2005; Sweetlove et al., 2008). Strategies to detect intermediary metabolic fluxes can now be estimated by computer- aided modeling of the central metabolic network and by mapping the pattern of metabolic fluxes underlying, via the possibility of labeling data collected by NMR and GC-MS and the biomass composition. For example Alonso et al., (2011), to map the pattern of metabolic fluxes underlying this efficiency, have labeled maize embryos to isotopic steady state using a combination of labeled ^{13}C-substrates. The resultant flux map reveals that even though 36% of the entering C goes through the oxidative pentose-phosphate pathway; this does not fully meet the NADPH demands for fatty acid synthesis. Metabolic flux analysis and enzyme activities have highlighted the importance of plastidic NADP-dependent malic enzyme, which provides one-third of the C and NADPH required for fatty acid synthesis in developing maize embryos.

It should worth to be mentioned that metabolic engineering of maize has been relatively slow due to the difficulty of maize transformation. Maize transformation with *Agrobacterium* is now more efficient than currently used particle gun transformation (reviewed in Jones, 2009; Reyes et al., 2010). In addition, larger DNA fragments can be inserted with *Agrobacterium* than those previously reported by other methods. The ability to routinely insert metabolic pathway quantities of DNA into the maize genome will further speed up maize metabolic engineering. Furthermore, site-directed mutagenesis via gene targeting, based on homologous recombination such as the application of designed zinc finger nucleases that induce a double stranded break at their target locus, are promising tools for genetic applications (Shukla et al., 2009; Saika et al., 2011). The use of these technologies may lead to both targeted mutagenesis and targeted gene replacement at remarkably high frequencies and enable to modify useful information, acquired from structural- and computational-based protein engineering, to be applied directly to molecular breeding of crops, including metabolic engineering.

Advances in plant genetics and genomic technologies are also contributing to the acceleration of gene discovery for maize product development. In the past few years there has been much progress in the development of strategies to discover new plant genes. In large part, these developments derive from four experimental approaches: firstly, genetic and physical mapping in plants and the associated ability to use map-based gene isolation strategies; secondly, transposon tagging which allows the direct isolation of a gene via forward and reverse genetic strategies as well as the development of the Targeting Induced Local Lesions IN Genomes (TILLING) technique; thirdly, protein-protein interaction cloning, that permits the isolation of multiple genes contributing to a single pathway or metabolic process. Finally, through bioinformatics/genomics, the development and use of large ESTs databases (http://www.maizegdb.org) and, DNA microarray technology to investigate mRNA-level controls of complex pathways. Moreover, new technologies and information continue to increase our understanding of maize; for instance, the complete DNA sequence of the maize genome, along with comprehensive transcriptome, proteome, metabolome, and epigenome information, is also a key resource for advancing fundamental knowledge of the biology of development seed quality-related traits to be applied in molecular breeding and biotechnology. These additional layers of information should help to further unravel the complexities of how genes and gene networks function to give plants

including quality-traits. This knowledge will drive to improved predictions and capacities to assemble gene variation through molecular breeding as well as more optimal gene selection and regulation in the development of future biotechnology products.

In conclusion, although, conventional breeding, molecular marker assisted breeding, and genetic engineering have already had, and will continue to have, important roles in maize improvement, the rapidly expanding information from genomics and genetics combined with improved genetic engineering technology offer a wide range of possibilities for the improvement of the maize grain.

6. Acknowledgements

We apologize to all those whose contributions we did not include in this review because of space constraints, personal preferences, or simple oversight. In addition, we would like to thank the members of the laboratory for their research contributions which are described here. The work was supported by Ministero per le Politiche Agricole, Alimentari e Forestali, Rome.

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Stability of Transgenic Resistance Against Plant Viruses

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1. Introduction

Plant viruses constitute one of the main problems of the agricultural production worldwide (Kang et al., 2005). To date, there are not therapeutical measures available for the control of plant-virus diseases in the field and the main control strategy used in practice is based on prevention measures. Genetic resistance is by far the most effective way to control plant viruses. However, 'traditional' genetic sources of resistance to viruses are rare (Lecoq et al., 2004) and due to the high rate of mutation of the viral genomes this resistance even when applicable, is frequently broken under field conditions. The era of *Agrobacterium*-mediated genetic transformation of plants which started at the 80s (Thomashow et al., 1980; Zambryskiet al., 1980) offered new promising prospects for engineered genetic resistance to viruses with numerous following studies reporting a successful use of the transgenic technology against almost all genera of plant viruses or even viroids (Lin et al., 2007; Prins et al., 2008; Ritzenthaler, 2005; Schwind et al., 2009). However, mainly due to public concerns for the safety of using transgenic plants in agriculture only in a relatively small number of virus diseases transgenic technology has been used in the field and in these cases it was proved an efficient and safe way of control (Fuchs et al., 2007). The mechanism of resistance in the vast majority of the applications of transgenic-plant strategy is based on RNA-silencing. RNA-silencing is a sequence specific RNA degradation mechanism, highly conserved between kingdoms, which in plants, among other functions, operates as a natural antiviral defense system (Eamens et al., 2008). The role of RNA-silencing as an antiviral weapon has been further supported by the fact that almost every known plant virus species encodes for at least one protein with RNA-silencing suppression activity (Dıaz-Pendon & Ding, 2008). This knowledge raised the first concerns regarding the efficiency of RNA-silencing based resistance against viruses under field conditions. As silencing is sequence specific, the resistance of transgenic plants engineered to be resistant to typically one virus could be broken by a different, heterologous virus that could infect the plants in the field. The hypothesis was that the heterologous virus through its silencing suppressor protein(s) could repress the RNA silencing machinery of the plant as a whole, resulting in the loss of the initially engineered resistance. In addition, the extensive research on RNA-silencing that is going on for over a decade has revealed a number of environmental and plant physiological factors that can influence the silencing mechanism and consequently the effectiveness of RNA-silencing based transgenic resistance to viruses under field conditions.

This review summarizes a fair amount of data that have been produced during the last decade in studies that have examined the role of heterologous viruses, the effect of temperature, the influence of the developmental stage of the plants in the stability of the transgenic resistance to viruses as well as recent findings for a direct effect of light intensity on the RNA silencing machinery. Moreover, new approaches for the implementation of RNA silencing in transgenic plant virus resistance are discussed as possible ways to overcome constrains of the current applications.

2. Strategies for engineering resistance to plant viruses

After the revolutionary work that was carried on *Agrobacterium* as a vector for plant transformation, the breakthrough for the creation of transgenic resistance to plant viruses came by Beachy's group which showed that the expression of the coat protein gene of *Tobacco mosaic virus* (TMV) in transgenic plants is conferring resistance to TMV (Abel et al., 1986). This discovery led the way for the production of an enormous number of transgenic plants resistant to viruses, using most types of viral genes. This genetically engineered resistance, referred to as pathogen-derived resistance (PDR) (Sanford & Johnston, 1985), mechanistically was divided into two categories; protein mediated and RNA-mediated. In protein mediated resistance the transformation cassette is designed in such a manner that the introduced viral gene, most commonly either of the coat protein, the replicase or a defective movement protein gene, would be able to be translated and expressed into the plant and somehow interfere with the disassembly, the replication or the movement respectively, of the intruding virus. However, this division is rather simplistic as in most cases of resistance which were designed to be protein mediated, it was proved that multiple mechanisms were involved, most frequently the RNA-mediated one (Lin et al., 2007; Prins et al., 2008; Ritzenthaler, 2005). RNA-mediated resistance is related to RNA-silencing which is probably the most important and common strategy for engineered resistance to plant viruses and will be discussed more extensively below.

Besides the PDR strategy, alternative biotechnological approaches for the manufacturing of plants resistant to viruses include the expression of plant virus-resistance genes in other plants than those from which they were isolated (Farnham, 2006; Seo et al., 2006; Spassova et al., 2001) and the expression of peptides (Lopez-Ochoa et al., 2006; Rudolph et al., 2003; Uhrig, 2003) or antibodies. After the first successful application of the later strategy in 1993 by Tavladoraki and co-workers, with antibodies that reduced the susceptibility to *Artichoke mottle crinkle virus* using a single-chain variable fragment (scFv) directed against the CP of the virus, technical difficulties hampered a wider application of this methodology. Nevertheless, several studies have reported the creation of plants resistant to viruses by expressing scFvs targeting structural as well as non-structural viral proteins (Binz & Plückthun, 2005; Prins et al., 1995; Prins et al., 2005; Ziegler & Torrance, 2002). The mechanisms of protein mediated resistance and of alternative methodologies are out of the scope of this review and will not be discussed further.

3. RNA-silencing based transgenic resistance against plant viruses

RNA silencing constitutes a vital element of the innate antiviral 'immune' response in plants. It uses cytoplasm-associated small interfering RNAs (siRNAs) to specifically target

and inactivate invading nucleic acids. Besides siRNAs, a vast population of small RNAs (sRNAs) accumulates in plant tissues, which includes microRNAs (miRNAs), *trans*-acting siRNAs (ta-siRNAs), heterochromatin-associated siRNAs (also referred to as *cis*-acting siRNAs that are linked to transcriptional gene silencing) and natural antisense transcript siRNAs. These sRNAs through RNA silencing mediate repressive gene regulation and play important role in developmental control, preservation of genome integrity and plant responses to adverse environmental conditions, including biotic stress (Brodersen & Voinnet, 2006; Chapman & Carrington, 2007; Pasquinelli et al., 2005; Ruiz-Ferrer & Voinnet, 2009; Vaucheret, 2006). To date, it has primarily been the cytoplasmic siRNA silencing pathway (also referred to as post transcriptional gene silencing, PTGS) that has been exploited by genetic engineering to confer resistance to plant viruses (Mlotshwa et al., 2008; Tenllado et al., 2004).

RNA silencing, is activated as a response to double-stranded RNA (dsRNA). Viruses, as well as transgenes, arranged as inverted repeats (IR), can directly produce dsRNA (which at a subsequent stage will give rise to primary siRNAs), whereas highly transcribed, sense orientated, single copy transgenes produce aberrant transcripts that serve as a substrate for producing dsRNA (subsequently processed to secondary siRNAs). In the latter case dsRNA is synthesized by one member of a family of cellular RNA-depended RNA polymerases (RdRPs) which counts six members in *Arabidopsis* (RDR1-6). Subsequently, the dsRNA can be targeted by a member of a group of Dicer-like ribonucleases (DCL1-4 in *Arabidopsis*) with each of them being involved in specific sRNA pathway(s) and generating specific size of sRNA duplexes (18-25nt in length). All four *Arabidopsis* DCL enzymes appear to be involved – directly or indirectly – in the production of siRNAs from DNA plant viruses, whereas the activities of DCL-4 and DCL-2 are mainly related to the production of siRNAs from single stranded RNA (ssRNA) viruses (Blevins et al., 2006; Ruiz-Ferrer & Voinnet, 2009; Vaucheret, 2006, and references therein). dsRNA cleavage is facilitated by another group of dsRNA-binding proteins (HYPONASTIC 1or HYL 1 and DRB2-5 in *Arabidopsis*). Then, siRNAs are stabilized by 2'O-methylation in their overhanging 3'ends and exported to cytoplasm for PTGS. One selected sRNA strand together with one member of the ARGONAUTE (AGO) family of proteins form the core of a nuclease complex (RNA induced silencing complex, RISC) that targets and cleaves sequence-specifically homologous ssRNA (Ronemus et al., 2006; Ruiz-Ferrer & Voinnet, 2009). The AGO family in *Arabidopsis* is predicted to contain ten members and for some of them a RNA slicer activity has been verified (Brodersen & Voinnet, 2006; Chapman & Carrington, 2007, and references therein). Many excellent reviews cover the functions of sRNAs and their role in RNA-silencing pathways in plants in great detail (Brodersen & Voinnet, 2006; Chapman & Carrington, 2007; Pasquinelli et al., 2005; Ruiz-Ferrer & Voinnet, 2009; Vaucheret, 2006; Mlotshwa et al., 2008).

RNA silencing impedes viral multiplication in plants by two major ways. First it degrades the dsRNA intermediates of virus replication themselves as well as the cognate mRNAs (referred to as cell-autonomous silencing), a procedure that leads to the increase of accumulation of the respective siRNAs. Second, it generates a mobile signal that triggers the degradation of homologous mRNAs in distant cells (systemic silencing). This systemic branch of antiviral RNA silencing is related to siRNA population or their dsRNA precursors that move between neighboring cells through plasmodesmata and over long distances through the phloem (Kalantidis et al., 2008).

RNA-silencing based resistance against viruses was first reported by Lindbo et al. (1993) and was shown to be related to the previously observed co-suppression mechanism (Napoli et al., 1990; Van der Krol et al., 1990). The following years, engineering of transgenic plants to harbor single-stranded sense and to a less extend antisense viral sequences became a common strategy to pre-activate the silencing machinery and obtain resistance against the homologous virus from which the introduced sequence has derived (Ritzenthaler, 2005). Further exploiting this knowledge led to constructing IR transgenes from which long double-stranded (ds) RNA precursors of siRNAs were directly generated. The utilization of such IR transgene constructs has become the method of choice for providing genetically engineered resistance to viruses because a single copy is sufficient to provide immunity, there is no expression of viral proteins, short genome incomplete sequences can be used and efficiencies of up to 90% of all transgenic plants produced to be resistant to the homologous virus were achieved (Lin et al., 2007; Tenllado et al., 2004; Ritzenthaler, 2005). In contrast to the situation with RNA viruses, the use of RNA silencing against DNA viruses most often resulted in delays in symptom development and did not always prevent virus replication (Lin et al., 2007). However, immune lines against *Tomato yellow leaf curl virus* (TYLCV) have been reported by Yang and co-workers (2004), and Fuentes and associates (2006).

In order to overcome the weakness of RNA-silencing based resistance [ineffective against viruses whose sequence differs from that of the transgene by more than 10% (Bau et al., 2003; Jones et al., 1998)], Bucher et al. (2006) fused 150-nt fragments of viral sequences of four tospoviruses in a single small chimeric IR construct. This strategy resulted in a high frequency of produced resistant plants. A most recent approach used modified plant miRNA cistrons to produce a range of antiviral artificial miRNAs (amiRNAs) (Niu et al., 2006; Qu et al., 2007; Schwab et al., 2006; Simon-Mateo & Antonio Garcıa, 2007; Zhang et al, 2011).

4. Factors that influence the RNA-silencing based transgenic resistance

4.1 Heterologous viruses

Since 1998 where the first viral suppressor of silencing was discovered it has been established that most known virus species carry at least one RNA silencing suppressor (Dıaz-Pendon & Ding, 2008; Ding & Voinnet, 2007). The awareness of this viral counter-defensive strategy against the innate antiviral defense system of plants guided several groups to investigate the effect that could invoke on transgenic resistance of plants that were immune to a virus, the infection with a different virus carrying a strong silencing suppressor.

The first studies were presented in 2001 by Savenkov and Valkonen, and Mitter and co-workers. Savenkov and Valkonen produced transgenic tobacco plants resistant to *Potato virus A* (PVA, genus *Potyvirus*) and examined whether the resistance to PVA was affected by infection of the transgenic plants with *Potato virus Y* (PVY), another potyvirus that was known to suppress RNA silencing through its HC-Pro protein (Dıaz-Pendon & Ding, 2008; Ding & Voinnet, 2007). The PVY infection resulted in increased steady-state levels of the transgene mRNA in the transgenic plants. PVA challenge was followed 15 days after inoculation with PVY. In contrast to healthy (non-PVY inoculated) transgenic plants, in

which no detectable infection with PVA was observed following challenge with PVA, all the PVY-infected transgenic plants were readily systemically infected by PVA. Moreover, in all PVA-infected plants, new leaves continued to display the severe symptoms, indicating no recovery from disease up to 90 days post inoculation. It was concluded that RNA-silencing mediated resistance in transgenic plants against viruses may be suppressed by infection of the plants with heterologous viruses that encode suppressors of gene silencing (Savenkov & Valkonen, 2001). Not equally definite was the outcome from the studies of Mitter et al. (2001; 2003) which showed that in transgenic tobacco plants, infection with *Cucumber mosaic virus* (CMV, genus *Cucumovirus*) expressing the silencing suppressor 2b protein could transiently suppress the silencing mediated immunity to PVY but solely in new leaves that emerged after CMV inoculation and for a limited period of time. The experiments were carried out for six months and different time intervals were examined between the two virus inoculations. It was shown that longer periods of time between CMV inoculation and challenge of transgenic plants with PVY led to a larger proportion of PVY-susceptible plants. Nevertheless, in these plants the relative PVY titers tended to be lower as compared with untransformed control plants and the movement of PVY in the transgenic plants was restricted relatively to the controls. Most importantly, CMV infection supported only a transient PVY infection and did not prevent recovery of the transgenic plants. Moreover, re-inoculation with PVY of the recovered plants or of plants that had been infected with CMV nine weeks earlier, failed to establish a PVY infection. Finally, although CMV infection resulted in increased transgene-derived mRNA levels in the leaves where breakdown of immunity had been recorded, the transgene-specific siRNAs levels were left unaffected.

Simon-Meteo et al. (2003) performed similar experiments on *Nicotiana benthamiana* plants that displayed RNA-silencing based resistance and were regenerated from recovered tissue of plants which showed a delayed resistance to *Plum pox virus* (PPV, genus *Potyvirus*). They used two heterologous viruses with distinct silencing suppressors, CMV and *Tobacco vein mottling virus* (TVMV, genus *Potyvirus* carrying an HC-Pro silencing suppressor). Each heterologous virus and PPV were inoculated either simultaneously or sequentially with an interval of two to four weeks onto transgenic plants. Both viruses, when applied sequentially, were able to reactivate transgene expression, but surprisingly, only the silencing suppression caused by CMV and not that originating from TVMV, was able to revert the transgenic resistant plants to a PPV-susceptible phenotype.

Taking into consideration these first studies several of the numerous succeeding reports (Fuentes et al, 2006; Germundsson & Valkonen, 2006; Praveen et al, 2010; Kawazu et al, 2009; Yang et al, 2004) of engineered transgenic resistance to plant viruses have examined the possible effect of heterologous virus infection in the resistance. However, not always an influence on resistance was observed. Missiou et al. (2004) in transgenic potato plants resistant to PVY examined the effect on the resistance of *Potato virus X* (PVX, genus *Potexvirus*, carrying the P25 silencing suppressor) infection simultaneously with PVY or one week prior to the challenge with PVY. In either of the two variations, infections with PVX occurred without a PVY infection to be detected. Similarly, resistance of transgenic cucumbers incorporating the 54K polymerase domain of *Cucumber fruit mottle mosaic virus* (CFMMV) was not influenced by infection with the potyviruses *Zucchini green mottle mosaic virus* (ZYMV), *Zucchini fleck mosaic virus* (ZFMV), the ipomovirus *Cucumber vein yellowing*

virus (CVYV) or CMV (Gal-On et al., 2005). In a different work, Lennefors et al. (2007) tested whether the high levels of RNA silencing-based resistance to *Beet necrotic yellow vein virus* (BNYVV) in transgenic sugar beet roots could be reduced by co-infection with common soil-borne and aphid-borne beet viruses. The plants were first inoculated with the aphid transmitted *Beet mild yellowing virus* (BMYV), *Beet yellows virus* (BYV), or both viruses. Four weeks later, the plants were transplanted to soil infested with BNYVV, *Beet soil borne virus* (BSBV) and *Beet virus Q* (BVQ) and their fungal vector, *Polymyxa betae*. The effectiveness of the resistance was not detectably compromised even following co-infection with all five viruses. Most recently, transgenic tobacco plants were produced, transformed with an IR construct corresponding to sequences of the TMV movement protein gene and the exhibited resistance to TMV was not affected by infection with CMV regardless of the order that the latter was inoculated (prior to or simultaneously with TMV) (Hu et al., 2011). In a different approach, amiRNAs expressed in tomato plants against CMV coding sequences resulted in resistance against the virus which was not noticeably affected by infection with TMV or TYLCV (Zhang et al., 2011). Moreover, the stability of transgenic resistance of tobacco plants against *Tobacco rattle virus* (TRV) (Vassilakos et al., 2008) remained largely unaffected by infection with CMV, PVY or *Tomato spotted wilt virus* (TSWV) (Vassilakos, unpublished results).

In contrast, in *N. benthamiana* plants expressing a *Grapevine virus A* (GVA) minireplicon and displaying high resistance to GVA, infection with *Grapevine virus B* (GVB, genus *Vitivirus*, carrying a P10 silencing suppressor) or PVY resulted in suppression of the GVA-specific defense (Brumin et al., 2009). Interestingly, in these tests GVA and GVB or PVY inocula were applied simultaneously as a mixture of saps derived from plants infected with the respective viruses, unlike previous studies, in which only sequential inoculations with the heterologous viruses resulted in reduced resistance. Finally, sweetpotato transgenic plants transformed with an IR construct targeting the replicase encoding sequences of *Sweetpotato chlorotic stunt virus* (SPCSV, genus *Crinivirus*) and *Sweetpotato feathery mottle virus* (SPFMV, genus *Potyvirus*) exhibited mild or no symptoms and virus accumulation was significantly reduced following SPCSV infection. However, development of severe sweetpotato virus disease symptoms (attributed to infection by both viruses) occurred in transgenic plants infected with a SPFMV isolate with a limited sequence similarity to the sequence used in the transgene (Kreuze et al., 2008).

The results from the studies that examined the effect of heterologous virus infection on the silencing-based transgenic resistance indicated that this kind of resistance, despite the immunity that can confer to the plants against a specific virus, could be compromised to some degree if applied in the field where mixed virus infections occur frequently. However, it became evident that the outcome of the interference between the heterologous viruses and the silencing machinery of the plant is not so easily predictable (Table 1).

The reasons for the discrepancies are unclear, but could be related to the mode of action of the viral suppression proteins of the different virus tested. Viral silencing suppressors are highly diverse in sequence, structure and activity, and could target multiple points in RNA silencing pathways whereas viruses with large genomes may encode several functionally distinct proteins to achieve silencing suppression (Dıaz-Pendon & Ding, 2008; Ding &

Voinnet, 2007). It is considered that suppressor proteins interfere either with siRNAs biogenesis or siRNA function without a multifunctional nature to be excluded. For instance, most studies agree that the potyviral HC-Pro probably specifically blocks accumulation of secondary siRNAs and leaves primary siRNA accumulation unimpaired, whereas P25 blocks accumulation of primary siRNAs (Dıaz-Pendon & Ding, 2008). In contrast, the 2b protein of cucumoviruses directly sequestrate siRNAs duplexes using a pair of hook-like structures that interact more promiscuously with long and short dsRNA (Dıaz-Pendon & Ding, 2008; Ding & Voinnet, 2007; Ruiz-Ferrer & Voinnet, 2009). Additionally, it binds AGO1 and blocks slicing without interfering with sRNA loading *in vitro*. Although apparently contradictory, these two anti-silencing 2b activities are reconcilable, because 2b's affinity for dsRNA is weak and its interaction with AGO1 could increase 2b local concentrations and enhance specific binding to siRNAs (Ruiz-Ferrer & Voinnet, 2009). Besides, Buchmann et al. (2009) reported that geminivirus AL2 and L2 proteins act as inhibitors of transcriptional gene silencing, which is the branch of silencing that targets DNA viruses.

Additional antiviral plant defense pathways could also be involved in the interference between the heterologous virus infection and the transgenic resistance or as yet unknown factors involved in specific virus species interactions. Thus, the CMV 2b protein has been shown also to block silencing indirectly by interfering with the salicylic acid mediated defense pathway (Li & Ding, 2001). Moreover, *N. benthamiana* plants transformed with an IR construct containing partial *N* gene sequences from five tospoviruses [TSWV, *Groundnut ring spot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV), *Watermelon silver mottle virus* (WSMoV) *Tomato yellow ring virus* (TYRV-t)] displayed resistance against all five viruses. However, co-infection of one of the tospoviruses with a genetically distant strain of the same species (TYRV-s), resulted in specific intraspecies breakdown of resistance through a procedure that involved complementation of the silencing suppressors of the two viruses (Hassani-Mehraban et al., 2009) (Table 1).

4.2 Temperature

It has been well known to plant virologists that temperature strongly influences plant-virus interactions. In high temperature, symptoms are frequently attenuated and virus titers in infected plants are decreased. In contrast, outbreaks of virus diseases are frequently associated with low temperatures (Hull, 2002).

Kalantidis and co-workers (2002) examined the influence of elevated temperature on siRNAs in CMV-resistant transgenic tobacco plants. Two transgenic lines, one expressing very high and the other very low levels of siRNAs, were tested for siRNAs concentration at 25°C and 32°C and at two time points, 20 and 30 days post-germination. At the early time point, transgene derived siRNAs could be detected only in the first line at 25°C and in both lines at 32°C. However, in the first line transgene specific siRNAs were at 32°C in a significantly higher concentration compared to that of 25°C. The analysis of samples taken at the second time point revealed the presence of transgene derived siRNAs in both lines at 25°C. However, at 32°C, siRNAs were detected in both plant lines at a higher concentration. Apparently, in these experiments, except for temperature the developmental stage of the plants also influenced the siRNA concentration (discussed further below).

Factor		Transgenic Plant	Engineered resistance against	Effect on the resistance	Reference
Heterologous viruses	PVY	*N. tabacum*	PVA	Suppressed	Savenkov & Valkonen, 2001
	CMV	*N. tabacum*	PVY	Reduced	Mitter et al., 2001; 2003
	CMV TVMV	*N. benthamiana*	PPV	Suppressed Unaltered	Simón-Mateo et al., 2003
	PVX	Potato	PVY	Unaltered	Missiou et al., 2004
	ZYMV ZFMV CVYV CMV	Cucumber	CFMMV	Unaltered	Gal-On et al., 2005
	BMYV BYV BSBV BVQ	Sugar beet	BNYVV	Unaltered	Lennefors et al., 2007
	SPFMV-C	Sweetpotato	SPCSV SPFMV-Uganda	Suppressed	Kreuze et al., 2008
	GVB, PVY	*N. benthamiana*	GVA	Suppressed	Brumin et al., 2009
	TYRV-s	*N. benthamiana*	TSWV GRSV TCSV WSMoV TYRV-t	Suppressed	Hassani-Mehraban et al., 2009
	CMV	*N. tabacum*	TMV	Unaltered	Hu et al, 2011
	TMV TYLCV	Tomato	CMV	Unaltered	Zhang et al, 2011
	CMV, PVY TSWV	*N. tabacum*	TRV	Unaltered	Vassilakos (unpublished)
Temperature	32^0C	*N. tabacum*	CMV	n/t	Kalantidis et al., 2002
	15^0C	*N. benthamiana*	CymRSV	Suppressed	Szittya et al., 2003
		N. tabacum	TMV CMV	Unaltered	Hu et al, 2011
		N. tabacum	TRV	Suppressed locally	Vassilakos (unpublished)
Light	High/Low Intensity	*N. benthamiana*	PPV	n/t	Kotakis et al., 2010
Early developmental stage		*N. benthamiana*	PMMoV	Reduced	Tenllado & Dıaz-Ruız, 1999
		Squash	SqMV	Suppressed	Jan at al., 2000
		Papaya	PRSV	Suppressed	Tennant at al., 2001
		N. tabacum	CMV	n/t	Kalantidis et al., 2002
		N. tabacum	TRV	Reduced	Vassilakos et al., 2008

Table 1. Synopsis of the studies described in the text that involved experiments with transgenic plants resistant to viruses and the influence to the resistance of the various factors examined; n/t, not tested.

Szittya and associates (2003) provided further insight into the mechanism that is involved in these observations. Through a set of delicate experiments they demonstrated that RNA silencing induced by viruses or transgenes is inhibited at low temperatures and enhanced with rising temperatures. They used wild type *Cymbidium ringspot virus* (CymRSV) encoding a p19 viral suppressor and a mutated one unable to express p19 (Cym19stop). In virus transfected *N. benthamiana* protoplasts, virus derived siRNA were undetectable at 15°C and gradually increased with temperature from 21 to 27°C indicating that virus-induced cell-autonomous silencing is temperature dependent. The effect of temperature on virus-induced systemic RNA silencing was also examined. *N.benthamiana* plants were inoculated with CymRSV and Cym19stop and grown at different temperatures. CymRSV infected plants died within 2 weeks at 15, 21 and 24°C whereas CymRSV symptoms were attenuated at 27°C and associated with reduced virus level. Confirming the role of p19 as a suppressor of systemic silencing, plants infected with the Cym19stop showed a recovery phenotype at 21 and 24°C. At 27°C, the mutant virus was unable to infect the plants, while at 15°C, Cym19stop-infected plants displayed strong viral symptoms demonstrating that at low temperature, RNA silencing failed to protect the plants even when the virus lacked the silencing suppressor. In addition, using a strain of *Agrobacterium tumefaciens* carrying a green fluorescent protein (GFP) gene construct which was infiltrated sole or together with p19, to wt *N.benthamiana* or *N.benthamiana* plants expressing GFP, it was shown that transgene-induced silencing is also temperature dependent. The stability of RNA silencing mediated transgenic virus resistance at different temperatures was examined using transgenic *N.benthamiana* plants expressing a CymRSV-derived RNA. After inoculation with CymRSV the plants displayed strong resistance at 24°C whereas at 15°C, severe symptoms were developed and CymRSV RNA accumulated to a high level demonstrating that the transgene-mediated virus resistance was broken at low temperature. A temperature effect was also observed on the antisense-mediated endogen gene inactivation of *Arabidopsis* and potato plants, in which antisense inhibition of genes involved in carbohydrate metabolism is broadly used. Interestingly, in contrast to siRNA production, miR157, miR169 and miR171 RNAs accumulated to equal levels at 15, 21 and 24°C in arabidopsis suggesting that accumulation of miRNAs is not affected by temperature.

Chellappan and co-workers (2005) expanding the above findings quantified gemini virus-derived siRNAs at different temperatures and evaluated their distribution along the virus genome for isolates of five species of cassava geminiviruses, consisting of recovery and non-recovery types. In cassava plants, geminivirus-induced RNA silencing increased by raising the temperature from 25°C to 30°C and the appearance of symptoms in newly developed leaves was reduced, irrespectively of the nature of the virus. Consequently, high temperature rendered non-recovery type geminiviruses to recovery-type viruses. The distribution of virus derived siRNAs on the respective virus genome at three temperatures (25°C, 25°C-30°C and 30°C) remained unaltered only for recovery-type viruses. siRNAs derived from recovery-type viruses accumulated at moderately higher levels during virus-induced silencing at higher temperatures. However, siRNAs from non-recovery-type viruses accumulated six times higher than those observed for infections with recovery-type viruses at high temperature. Thus, the decreased symptom severity and virus concentration that were recorded at higher temperature indicate a similar effect of temperature on ssDNA and RNA viruses although there was a differential effect of temperature on the level of virus-derived siRNAs between recovery and non-recovery types of ssDNA viruses.

As with the effect of heterologous viruses, inhibition of RNA silencing or decreasing of siRNAs concentration in low temperature has not always been observed. Thus, transgene anti-sense induced RNA silencing was not inhibited in potato plants at low temperature (Sos-Hegedus et al., 2005). Moreover, tomato plants carrying an IR construct derived from *Potato spindle tuber viroid* (PSTVd) sequences and exhibiting resistance to PSTVd infection, did not show an elevated IR-siRNA accumulation at 31°C in comparison to 21°C (Schwind et al., 2009). In a more recent study, transgenic tobacco plants transformed separately with IR constructs corresponding to sequences of TMV movement protein gene or CMV replication protein gene, exhibited at both 15°C and 24°C similar high levels of resistance to TMV or CMV, respectively (Hu et al, 2011). In addition, the resistance against TRV of transgenic tobacco plants (Vassilakos et al., 2008) grown at 15°C was influenced only in the inoculated leaves but not systemically (Vassilakos, unpublished results).

In summary (Table 1), the well-known temperature effect on the development of viral diseases is closely associated to the RNA silencing antiviral pathway and consequently influences the efficiency of silencing-based transgenic resistance. However, it appears that the low temperature effect on the transgenic resistance depends on additional factors that remain to be identified, fact supported by inconsistencies in the results of the diverse studies described here. Importantly, although at low temperature the siRNA-based silencing machinery is partially inactivated as an adaptive response of plants to adverse conditions, the miRNA-mediated, which is essential for regulatory functions, continues to operate ensuring plant growth (Szittya et al., 2003).

4.3 Light

Studies on the effect of light on transgenic resistance to viruses are not available, however light has been implicated as one of the factors that affect RNA silencing initiation and maintenance in several studies. Although in most of them light effect on silencing was not clearly isolated from that of temperature (Nethra et al., 2006; Vaucheret et al., 1997) recently, Kotakis et al. (2010) investigated solely the role of light intensity in physiological ranges on RNA silencing. They used as a system *N. benthamiana* transgenic lines engineered to express GFP, which exhibited spontaneously silencing at different frequencies and of different spreading intensities. The authors demonstrated that high light intensity increased the frequency of plants displaying both short range and systemic silencing. In contrast, plants grown under low light conditions, showed lower silencing frequencies. In addition, increased light intensity positively affected siRNA levels corresponding to the GFP transgene (sense) transcript. In a different set of experiments, *N. benthamiana* plants were used, incorporating an IR structure derived from the NIb gene of *Plum pox virus* (PPV) and it was shown that levels of all distinguishable siRNA classes corresponding to the IR transcript were also positively affected by high light intensity (Table 1). Although in the latter case, the effect of light intensity on virus resistance was not tested, the authors proposed that light conditions comprise an additional environmental factor that should be taken under consideration when transgenic technology against viral infections applies on the field.

4.4 Plant developmental stage

Quite a few studies with plants carrying sense transgenes and displaying RNA-silencing mediated resistance have suggested an influence of plant developmental stage on the degree

of the expressed resistance. Tenllado and Diaz-Ruiz (1999) reported that a higher percentage of transgenic *N. benthamiana* plants, transformed with the 54K read-through domain of the replicase gene of *Pepper mild mottle virus* (PMMoV), displayed complete virus resistance at maturity than at an earlier stage of development. Subsequently, Jan et al (2000) demonstrated that a recovery type of resistance, in squash genetically transformed with the coat protein genes of *Squash mosaic virus* (SqMV), was due to RNA silencing that was activated at a later developmental stage, independently of virus infection. However, a different phenotype of complete resistance was not altered after SqMV inoculation at early developmental stages. Moreover, analysis of crosses between lines exhibiting complete resistance, recovery and susceptible phenotypes revealed that the time of activation of silencing, besides the developmental stage, is affected by the interaction of transgene inserts. Similarly, transgenic papaya plants were susceptible to *Papaya ringspot virus* (PRSV) at a younger stage but resistant when inoculated at an older stage (Tennant et al., 2001).

As mentioned already, Kalantidis and associates (2002) showed that siRNA accumulation in transgenic tobacco, incorporating an IR construct carrying CMV sequences, was higher at later developmental stages. No significant differences in the siRNA concentration were observed between leaves of different age from a single plant or from the seven-leaf stage on, while the siRNA concentration reached a plateau that remained stable in the course of further development.

In a more recent work, *N. tabacum* plants were transformed with the 57-kDa read-through domain of the replicase gene of TRV and were highly resistant to homologous (to the transgene sequence) TRV isolates and moderately resistant to the genetically distinct TRV-GR. Very young transgenic plants with detectable levels of transgene transcript were resistant only systemically to homologous isolates and were susceptible to TRV-GR. Conversely, older plants (at a five-leaf stage) containing a low steady state level of transcripts were immune to homologous isolates and displayed moderate resistance against TRV-GR (Vassilakos et al., 2008).

In conclusion (Table 1), most studies agree that younger transgenic plants accumulate reduced amounts of transgene specific siRNAs compared to older ones, or correspondingly accumulate higher amount of transgene specific transcripts suggesting a reduced efficiency of transgenic resistance against plant viruses. However, the resistance phenotype was not always affected in younger plants, possibly due to reasons associated with the type of the transgene construct used, its integration into the plant genome or the viral sequences that are targeted.

5. Conclusion

A great deal of progress has been made towards comprehension of plant virus biology and the ways in which plants defend themselves against these pathogens. RNA silencing has provided a promising potential for generating virus-resistant transgenic plants and this potential is certainly not cancelled by the awareness of factors that may affect under specific conditions the acquired resistance. However, as with any other pathogen control strategy, RNA silencing does not constitute a panacea and a number of issues should be taken into consideration before being applied in the field. Noticeably, silencing based transgenic

resistance is not influenced solely by the factors that were presented in this review. However, planting into areas where endemic virus diseases occur and mixed virus infections are expected especially during early stages of the vegetation period, time intervals of low air temperature and greenhouse or open field cultivation practices could affect the stability of transgenic resistance against plant viruses.

Further exploitation of our knowledge on RNA-silencing pathways is essential to improve the efficiency of the existing strategies or for the development of potential new strategies which will hopefully lead to a better reception by the public. Recent advances like the construction of chimeric IR constructs incorporating sequences derived from different virus species if combined with epidemiological data and pest risk analyses could reduce the effect of mixed virus infections on the resistance (Bucher et al., 2006; Dafny-Yelin & Tzfira, 2007; Kung et al., 2009). Recently, virus resistance was achieved through expression of amiRNAS against viral coding sequences (Ding & Voinnet, 2007; Duan et al., 2008; Niu et al., 2006; Qu et al., 2007; Simon-Mateo & Antonio Garcia, 2006; Zhang et al, 2011). Although there was evidence that amiRNA-mediated virus resistance may not be inhibited by low temperature (Niu et al., 2006) this possibly depends on the plant species examined (Qu et al., 2007). Moreover, the durability of this approach, which resulted in relatively few antiviral small RNAs compared with those of the long dsRNA approach, needs to be further demonstrated (Duan et al., 2008; Simon-Mateo & Antonio Garcia, 2006).

6. Acknowledgement

The author wishes to thank Christina Varveri for helpful discussions and for critically reading the manuscript, and to express his apologies to all those, whose papers were not cited.

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12

Expression of Sweet Potato Senescence-Associated Cysteine Proteases Affect Seed and Silique Development and Stress Tolerance in Transgenic *Arabidopsis*

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1. Introduction

Leaf is in general the main site of photosynthesis and acts as a carbohydrate source for nutrients to support the growth in sink organs of plants. Therefore, its longevity and senescent level may affect the photosynthesis efficiency and thus crop yield. There are endogenous and exogenous factors affecting leaf senescence, including plant growth regulators, sucrose starvation, dark, cold, drought, salt, wound, pathogen infection and insect attack (Yoshida, 2003; Lim et al., 2007). Leaf senescence is the final developmental stage of leaves and has been considered as a type of programmed cell death. During leaf senescence, it is not only a degradative process but also a recycling one. Therefore, macromolecules and organelles can be degraded into small molecules, salvaged and mobilized from the senescent cells to other sinks, such as young leaves, developing seeds, or storage tissues (Buchanan-Wollaston, 1997; Quirino et al., 2000).

In sweet potato, several morphological, biochemical and physiological changes have also be observed during leaf senescence, including leaf yellowing, decrease of chlorophyll contents, reduction of photochemical Fv/Fm, elevation of H_2O_2 amount, increase of plastoglobuli number in chloroplast, activation of senescence-associated gene expression, and finally cell death (Chen et al., 2000; Chen et al., 2003; Chen et al. 2010a). Several full-length cDNAs encoding putative isocitrate lyase, papain-like cysteine proteases and asparaginyl endopeptidase, have been cloned from senescent leaves (Chen et al., 2000, 2004, 2006, 2008, 2009, 2010b), which likely play roles in association with lipid degradation and gluconeogenesis, and protein degradation and re-mobilization. These data support the occurrence of macromolecule and organelle degradation into small molecules for recycling and re-mobilization during sweet potato leaf senescence.

During senescence, breakdown of leaf proteins by proteases provides a large pool of cellular nitrogen for recycling (Makino & Osmond, 1991). In plants, different degradation pathways

have been described and the vacuolar degradation pathway is assumed to be involved in bulk protein degradation by virtue of the resident proteases in the vacuole (Vierstra, 1996). There are two types of vacuoles described in plants: the storage vacuole and the lytic vacuole (Marty, 1999). Protein storage vacuoles are found in seed tissues and accumulate proteins that are re-mobilized and used as the main nutrient resource for germination (Senyuk et al., 1998; Schlereth et al., 2001). Most cells in vegetative tissues have lytic vacuoles, containing a wide range of proteases in an acidic environment. Substrate proteins must be transported and sequestered into these lytic vacuoles before degradation. Therefore, senescence-associated vacuoles are lytic vacuoles and involved in the degradation of imported chloroplast proteins in tobacco leaves (Martı´nez et al., 2008).

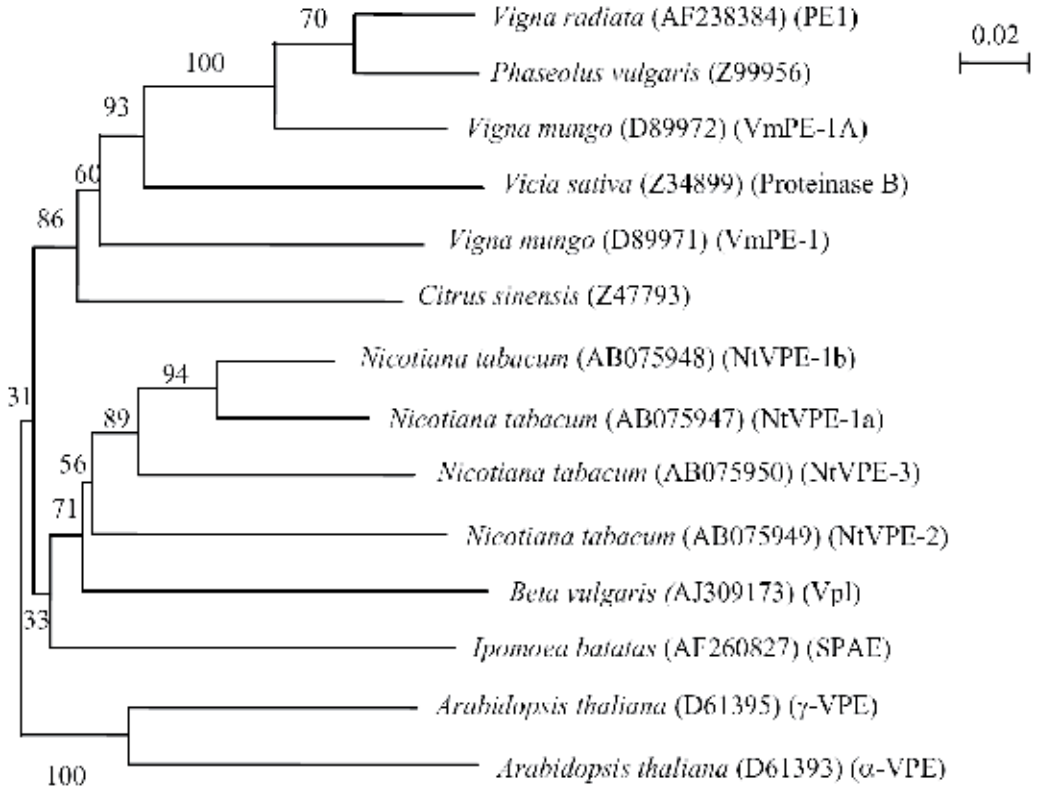

Fig. 1. Phylogenetic tree analysis of plant asparaginyl endopeptidases (Adapted and Modified from Chen et al., 2004).

The molecular mechanisms for vacuolar protein degradation and nutrient recycling pathway in senescent leaves are generally not clear. Phylogenetic tree analysis indicated that sweet potato asparaginyl endopeptidase (SPAE) exhibited high amino acid sequence identities and closely-related association with plant vacuolar processing enzymes (VPEs) or legumains, including legumain-like protease LLP of kidney bean (*Phaseolus vulgaris*), legumain-like protease VsPB2 of vetch (*Vicia sativa*), vacuolar processing enzymes of *Arabidopsis thaliana*, and asparaginyl endopeptidases VmPE-1 of *Vigna mungo* (Fig. 1). Sweet potato papain-like cysteine protease (SPCP2) also showed high amino acid sequence

identities and closely-related association with a subgroup of cysteine proteases, including *Actinidia deliciosa* CP3, *Arabidopsis thaliana* RD19, *Brassica oleracea* BoCP4, *Phaseolus vulgaris* CP2, *Solanum melongena* SmCP, *Vicia sativa* CPR2, and *Vigna mungo* SH-EP (Fig. 2). These data suggest the possible physiological roles and functions for *SPAE* and *SPCP2* related to these mentioned vacuolar processing enzymes and papain-like cysteine proteases, respectively.

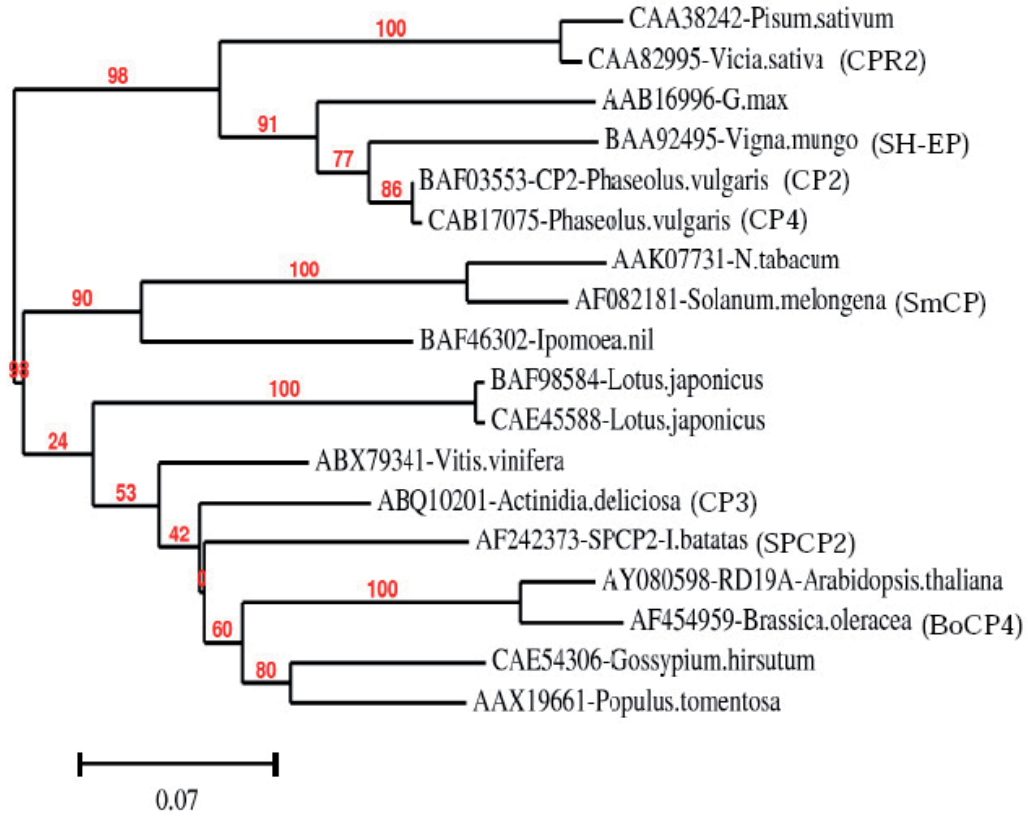

Fig. 2. Phylogenetic tree analysis of plant papain-like cysteine proteases (Adapted from Lin, 2010).

2. Association of vacuolar processing enzyme and papain-like cysteine protease with seed storage globulin protein degradation

Vacuolar processing enzyme is a novel group of cysteine endopeptidase and has recently been found in seeds. The enzyme exhibits strict cleavage specificity for the peptide bonds of seed globulin storage proteins with asparagines at the P1 position, and is called asparaginyl endopeptidase (Ishii, 1994). The substrate specificity was observed with purified asparaginyl endopeptidases from developing seeds of castor bean (Hara-Nishimura et al., 1991) and soybean (Scott et al., 1992; Hara-Nishimura et al., 1995), from mature seeds of jack bean (Abe et al., 1993), and from germinating seeds of vetch (Becker et al., 1995). Many seeds accumulate protein reserves in the storage vacuoles, and a number of these proteins

undergo proteolytic cleavage, including the 7S and 11S seed storage globulins (Müntz & Shutov, 2002). The 11S seed globulin storage proteins are synthesized as precursors and are cleaved post-translationally in storage vacuoles by an asparaginyl endopeptidase during seed development (Ishii, 1994). In castor bean and soybean seeds, vacuolar processing enzymes were found in the protein bodies and likely associated with the conversion of proproteins into their corresponding mature forms in vacuoles (Hara-Nishimura et al., 1991; Shimada et al., 1994).

Asparaginyl endopeptidases also play a role with bulk degradation and mobilization of storage proteins during seed germination and seedling growth. For example, the asparaginyl endopeptidase, which was also called "legumain-like proteinase" (LLP), was isolated from cotyledons of kidney bean (*Phaseolus vulgaris*) seedlings. It was the first proteinase ever known which *in vitro* extensively degraded native phaseolin, the major storage globulin of this grain legume (Senyuk et al., 1998). In vetch (*Vicia sativa*) seeds, the legumain-like VsPB2 and proteinase B together with additional papain-like cysteine proteinases were responsible for the bulk breakdown and mobilization of storage globulins during seed germination (Schlereth et al., 2000). In *Arabidopsis*, the seed protein profiles were compared between the wild type and a seed-type vacuolar processing enzyme β*VPE* mutant using a two dimensional gel/mass spectrometric analysis. A significant increase in accumulation of several legumin-type globulin propolypeptides was found in β*VPE* mutant seeds (Gruis et al., 2002).

For papain-like cysteine protease, the vacuolar SH-EP is synthesized in cotyledons of germinated *Vigna mungo* seeds and is responsible for the degradation of seed globulin storage proteins accumulated in protein bodies. In *Vicia faba* (vetch), globulins such as legumin and vicillin are major seed storage proteins present in the protein bodies of cotyledon, radicle, axis, and shoots. Papain-like cysteine protease such as CPR2 and CPR4 are found in cotyledon and axis of dry and imbibed seeds. Gene expression studies concluded that storage globulin mobilization in germinating vetch seeds is started by the stored cysteine proteases (CPRs), however, the bulk globulin mobilization is mediated by *de novo* synthesized CPRs (Schlereth et al., 2000; Schlereth et al., 2001; Tiedemann et al., 2001). These data suggest that papain-like cysteine proteases may play physiological roles and functions in association with seed storage globulin protein degradation and mobilization during seed germination and seedling growth. In addition to the possible physiological function with seed storage globulin protein degradation, papain-like cysteine proteases have also been implied to play a role in cope with environmental cues. For example, a dehydration-responsive papain-like cysteine protease RD19 was cloned and results showed that its expression was strongly induced under high-salt and osmotic stress conditions, which suggests a possible physiological role of RD19 in association with the regulation of plant cell osmotic potential in *Arabidopsis thaliana* (Koizumi et al., 1993; Xiong et al., 2002). In broccoli, the florets showed water loss during post-harvest storage. Gene expression of papain-like cysteine proteases BoCP4, which exhibited high amino acid sequence identity with *Arabidopsis* RD19, was also found to be dehydration-responsive and was repressed by water and sucrose (Coupe et al., 2003).

Many vacuolar enzymes are synthesized as pro-proteins and become active after proteolytically processed. In seed storage tissues, specific endoplasmic reticulum (ER)-derived compartments containing precursors of cysteine proteases have been described

(Chrispeels & Herman, 2000; Toyooka et al., 2000; Hayashi et al., 2001; Schmid et al., 2001). Germination of the seeds induces the expression and processing of those proteases into the mature active forms, which in turn participate in the degradation of cellular materials in storage tissues and provide nutrients to the growing embryo. The mechanism of asparaginyl endopeptidases (VmPE-1) and papain-like cysteine protease (SH-EP) associated with bulk seed storage globulin protein degradation has been studied in *Vigna mungo*. The vacuolar cysteine protease SH-EP is synthesized in cotyledons of germinated *Vigna mungo* seeds with an N-terminal and a C-terminal prosegments (Okamoto & Minamikawa, 1999; Okamoto et al., 1999). Okamoto & Minamikawa (1995) isolated a processing enzyme, designated VmPE-1. VmPE-1 is a member of the asparaginyl endopeptidases and is involved in the post-translational processing of SH-EP. In addition, the cleavage sites of the *in vitro* processed intermediates and the mature form of SH-EP were identical to those of SH-EP purified from germinated cotyledons of *V. mungo*. Therefore, it is proposed that the asparaginyl endopeptidase (VmPE-1)-mediated processing functions mainly in the activation of proSH-EP during seed germination (Okamoto et al., 1999). The activated SH-EP plays a major role in the degradation of seed storage proteins accumulated in cotyledonary vacuoles of *Vigna mungo* seedlings (Mitsuhashi et al., 1986). These reports demonstrate a role of asparaginyl endopeptidase associated with papain-like cysteine protease in the bulk breakdown and mobilization of storage globulins during seed germination.

3. Characterization of sweet potato asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2*

Recently, similar compartments have also been described in vegetative tissues of *Arabidopsis* (Hayashi et al., 2001). These precursor protease vesicles derived from ER are plant specific compartments and contain vesicle-localized vacuolar processing enzyme (γVPE) precursor, which is critical for maturation of the vacuolar protease AtCPY. The vacuolar protease AtCPY in turn participates in the degradation of cellular components including vacuolar invertase AtFruct4 and various proteins in organs undergoing senescence in *Arabidopsis* (Rojo et al., 2003). A mechanism of senescence-induced activation of vesicle-localized vacuolar processing enzyme precursor by releasing its inactive form from the precursor protease vesicle into the acidic lumen of the vacuole is suggested. This activation triggers the processing of downstream proteases for protein degradation and recycling in senescing tissues (Rojo et al., 2003). These data suggest sweet potato asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* may also play roles with functions related to protein degradation for nutrient remobilization during leaf senescence.

3.1 *SPAE*

SPAE had been cloned from senescent leaves with PCR-selective subtractive hybridization and exhibited high amino acid sequence homologies to seed vacuolar legumains/asparaginyl endopeptidases of kidney bean (*Phaseolus vulgaris*), spring vetch (*Vicia sativa*) and jack bean (*Canavalia ensiformis*) (Chen et al., 2004). The conserved catalytic residues (His and Cys) and central β-strands that supported the catalytic residues of human and mouse legumains (Chen et al., 1998) were also found in SPAE, plant legumain/asparaginyl endopeptidase, vacuolar processing enzymes, and the other cysteine proteases (Chen et al., 2004).

Asparaginyl endopeptidase *SPAE* encoded a pre-proprotein precursor, which contained a putative mature protein (325 amino acid residues) and an N-glycosylation site at its C-terminus. The deduced molecular mass of mature SPAE protein was, thus, likely between 33 and 36 kDa that detected by protein gel blot with polyclonal antibody against putative SPAE protein (Chen et al., 2004). Asparaginyl endopeptidase is an atypical cysteine endopeptidase with a reported insensitivity to the inhibitor L-3-carboxy-2,3-trans-epoxypropionyl-leucyl-amino(4-guanidino)butane (E-64) (Okamoto & Minamikawa, 1999). A cysteine protease activity band with a molecular mass near 36 kDa similar to the protein gel blot results was also detected and exhibited insensitivity to E-64 inhibitor (Chen et al., 2004). These data provide indirect evidence to support the existence of asparaginyl endopeptidase in senescent leaves.

In sweet potato, *SPAE* gene expression level is higher in dark- or ethephon-treated leaves similar to that in natural senescent leaves. Hormones such as jasmonic acid (JA) and abscisic acid (ABA) also caused the decrease of chlorophyll contents in treated leaves; whereas, did not significantly alter *SPAE* gene expression level compared to that of untreated dark control in mature green leaves within a 3-day period (Chen et al., 2004). These data suggest that *SPAE* is a senescence-associated gene and its expression in natural or induced senescent leaves is likely controlled by ethylene, but not by JA and ABA.

3.2 *SPCP2*

SPCP2 had been cloned from senescent leaves with PCR-selective subtractive hybridization. The open reading frame of *SPCP2* contained 1101 nucleotides (366 amino acids) and exhibited high amino acid sequence identities with a subgroup of vacuolar cysteine proteases including *Actinidia deliciosa* CP3, *Arabidopsis thaliana* RD19, *Brassica oleracea* BoCP4, *Phaseolus vulgaris* CP2, *Vicia sativa* CPR2, and *Vigna mungo* SH-EP (Chen et al., 2010). These data suggest an intracellular localization of SPCP2 in the vacuole. For SH-EP, a C-terminal KDEL sequence (endoplasmatic retention signal) was proved to be associated with its vacuole-targeting (Okamoto et al., 2003). However, no significant C-terminal KDEL sequence was found for SPCP2. For RD-19, a vacuolar localization was also suggested. However, it can be re-localized to nucleus in the presence of PopP2, an avirulent gene product of *R. solanacearum* (Bernoux et al., 2008; Poueymiro & Genin, 2009). Therefore, it is possible to assume that different vacuolar targeting mechanisms and signal peptides are involved and associated with different related cysteine protease genes.

SPCP2 gene expression was enhanced in natural senescent leaves and can be induced by dark, ethephon, ABA and JA (Chen et al., 2010). Buchanan-Wollaston et al. (2005) analyzed gene expression patterns and signal transduction pathways of senescence in *Arabidopsis* induced by different factors. Transcriptome analysis demonstrated that pathways such as dark, ethylene, and JA are all required for gene expression during developmental senescence. Genes associated with essential metabolic processes such as degradation of proteins and peptides and nitrogen mobilization can utilize alternative pathways for induction (Buchanan-Wallaston et al., 2005). Therefore, a possible explanation which is likely associated with multiple signal transduction pathways is suggested for the induction of sweet potato *SPCP2* gene expression by different factors, including development, dark, ABA, ethephon and JA.

4. Ectopic expression of asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* in transgenic *Arabidopsis*

Sweet potato full-length cDNAs of asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* were individually constructed in the T-DNA portion of recombinant pBI121 vector under the control of *CaMV 35S* promoter for transformation of *Arabidopsis* with *Agrobacterium*–mediated floral dip transformation method (Clough & Bent, 1998). Transgenic *Arabidopsis* plants ectopically expressing sweet potato asparaginyl endopeptidase *SPAE* (Chen et al., 2008) or papain-like cysteine protease *SPCP2* (Chen et al., 2010) were produced, identified and characterized.

4.1 Expression of sweet potato asparaginyl endopeptidase *SPAE* altered seed and silique development in transgenic *Arabidopsis*

Three transgenic *Arabidopsis* plants were isolated and identified with floral dip transformation method (Clough & Bent, 1998). Genomic PCR and protein gel blot analysis confirmed that these *Arabidopsis* plants (YP1, YP2 and YP3) were transgenic and sweet potato *SPAE* gene was expressed and properly processed into mature form with a predicted molecular mass near 36 kDa (Chen et al., 2008). Similar results have also been observed and reported for various plant vacuolar processing enzymes, including *Vigna mungo* VmPE-1 (Okamoto et al., 1999), *Arabidopsis* βVPE (Gruis et al., 2002), *Arabidopsis* γVPE (Kuroyanagi et al., 2002; Rojo et al., 2003). These data suggest that transgenic *Arabidopsis* plants may use similar mechanisms for sweet potato SPAE processing, and thus can produce mature sweet potato SPAE protein products.

Transgenic *Arabidopsis* plants exhibited earlier floral transition from vegetative growth and leaf senescence (Chen et al., 2008). Early transition of vegetative phase to reproductive phase has been considered as a type of senescence. The reasons and mechanisms that sweet potato *SPAE* gene expression can promote earlier floral transition and enhance senescence in transgenic *Arabidopsis* plants are not clear. However, Raper et al. (1988) and Rideout et al. (1992) hypothesized that floral transition is stimulated by an imbalance in the relative availability of carbohydrate and nitrogen in the shoot apical meristem. Barth et al. (2006) suggest that the flowering phenotype is likely linked to the endogenous ascorbic acid content. Degradation and removal of flowering repressor(s) by ectopic SPAE expression in transgenic *Arabidopsis* plants provides another possibility.

Expression of sweet potato *SPAE* in transgenic *Arabidopsis* plants caused altered development of seed and silique, elevated percentage of incompletely-developed siliques, and fewer silique numbers per plant than that of control (Figs. 3 and 4). The reasons for altered phenotypic characteristics in transgenic *Arabidopsis* by sweet potato SPAE expression are not clear. However, sweet potato SPAE is in close association with plant vacuolar processing enzymes of seeds from phylogenetic analysis (Chen et al., 2004). Vacuolar processing enzymes have been reported to be in association with the degradation and mobilization of globulin storage proteins during seed germination and seedling growth in *Phaseolus vulgaris* (Senyuk et al., 1998), *Vigna mungo* (Okamoto et al., 1999), *Vicia sativa* (Schlereth et al., 2000; Schlereth et al., 2001), and *Arabidopsis thaliana* (Gruis et al., 2002). In *Vigna mungo*, VmPE-1 has been demonstrated to increase in the cotyledons of germinating seeds and was involved in the post-translational processing of a vacuolar cysteine endopeptidase, designated SH-EP, which degraded seed storage proteins (Okamoto &

Minamikawa, 1999). A possible explanation that inappropriate pre-degradation of globulin-type storage protein during seed development and maturation by constitutively expressed sweet potato SPAE in transgenic *Arabidopsis* is suggested. The inappropriate pre-degradation of globulin-type storage protein may result in partial repression of seed and silique development which in turn leads to higher incompletely-developed silique percentage and lower silique numbers per plant. These data also suggest that sweet potato asparaginyl endopeptidase SPAE may have enzymatic function similar to seed vacuolar processing enzymes for protein degradation and nutrient recycling during leaf senescence.

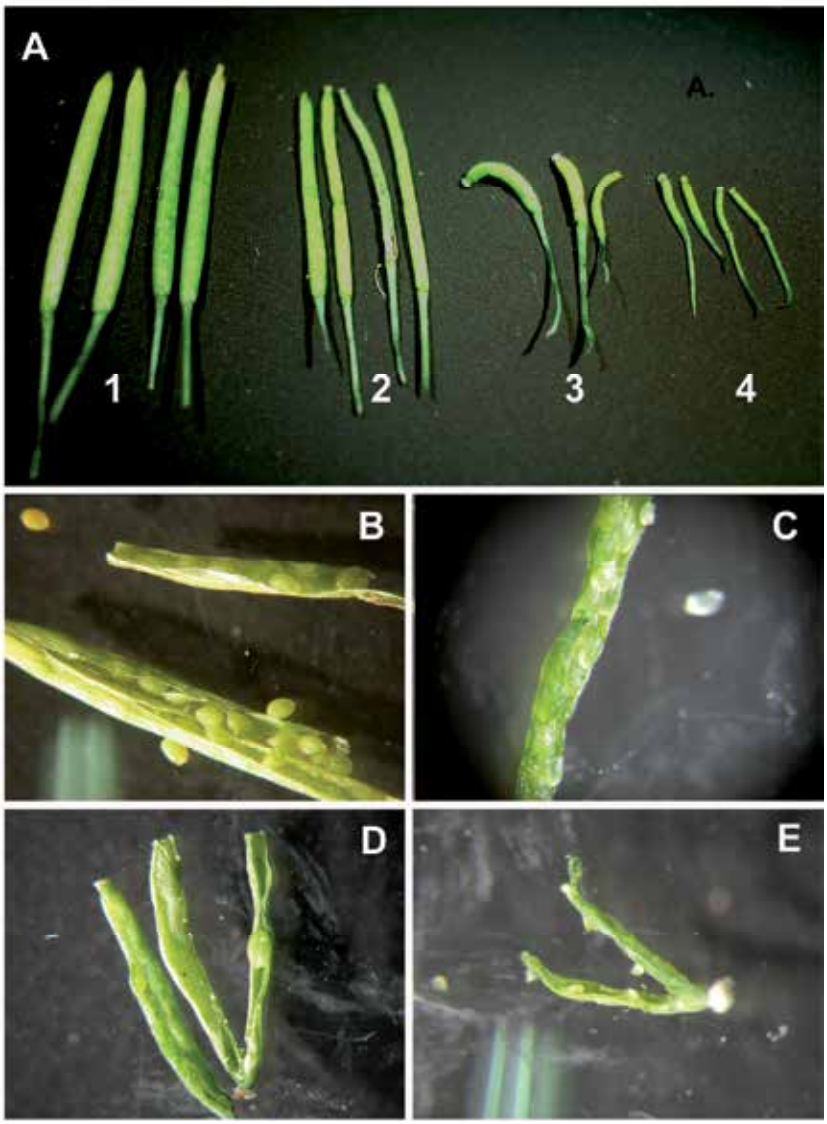

Fig. 3. Morphological classification of Arabidopsis siliques. A. Different silique types (types 1, 2, 3 and 4) classified. **B.** Dissection of type 1 silique; **C.** Dissection of type 2 silique; **D.** Dissection of type 3 silique; **E.** Dissection of type 4 silique (Adapted from Chen et al., 2008).

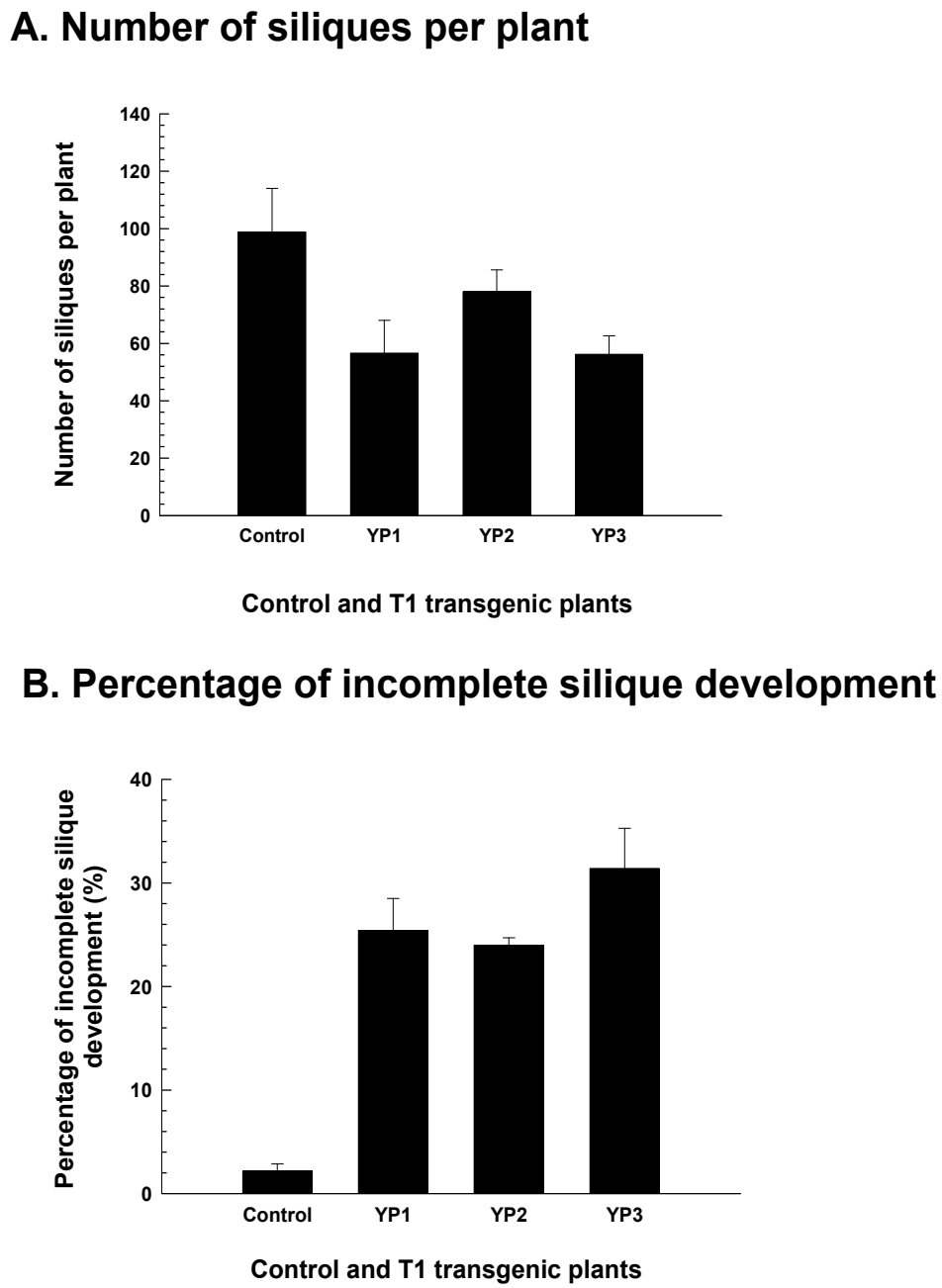

Fig. 4. Comparison of silique number per plant and incompletely-developed silique percentage among control and transgenic T1 plants ectopically expressing sweet potato *SPAE*. **A.** Comparison of silique number per plant. **B.** Comparison of incompletely-developed silique percentages. C and YP1/YP2/YP3 denote non-transformant control and transgenic *Arabidopsis* plants, respectively. The data are from the average of 5 plants per treatment and shown as mean ± S.E. (Adapted from Chen et al., 2008).

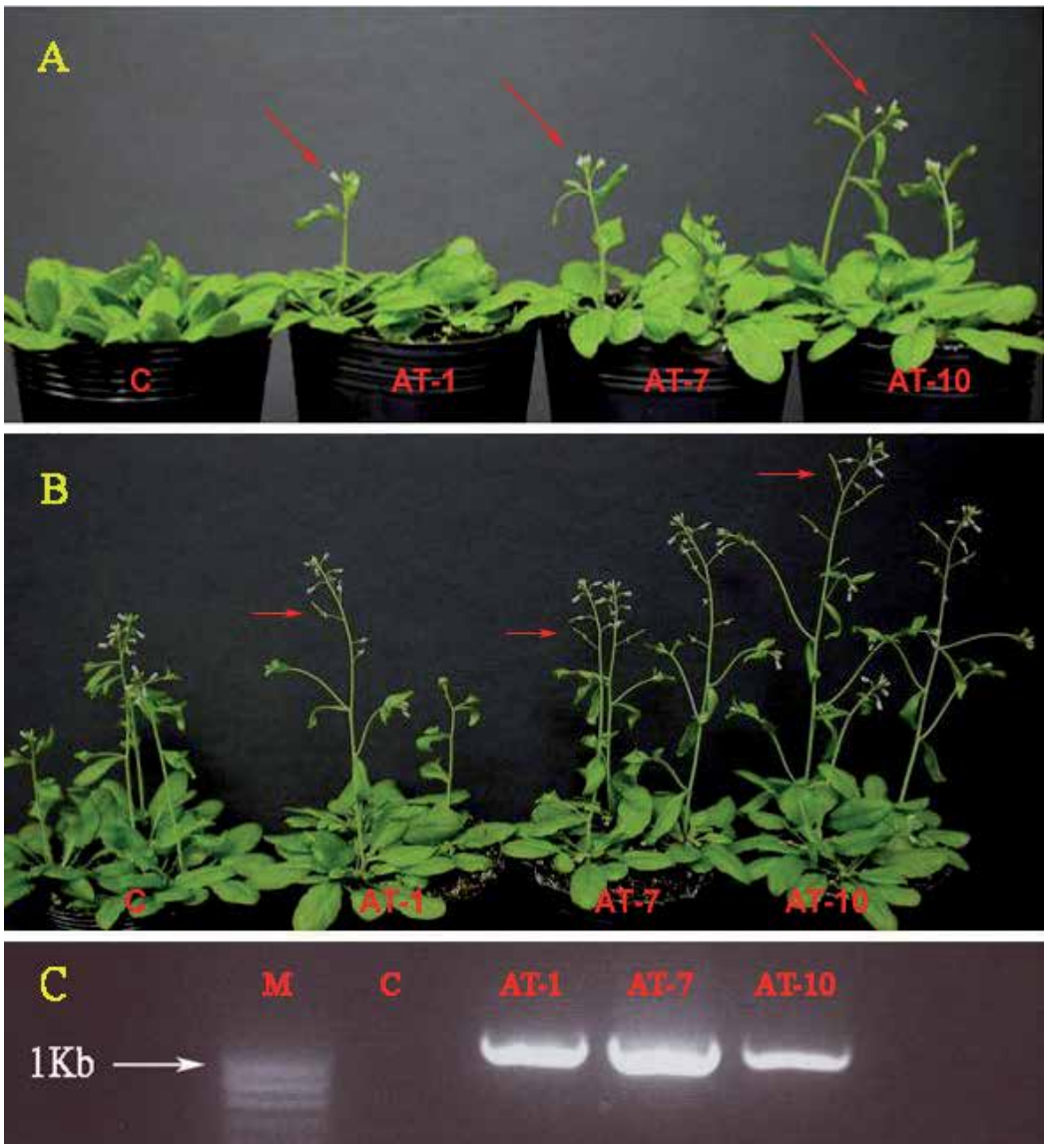

Fig. 5. Comparison of growth patterns among control and transgenic T1 Arabidopsis plants ectopically expressing sweet potato *SPCP2*. **A.** Transition from vegetative growth to flowering was observed and compared 30 days after seed germination. **B.** The appearance and size of inflorescences and siliques were observed and compared 35 days after seed germination. **C.** RT-PCR analysis of *SPCP2*. C and AT denote control and transgenic T1 *Arabidopsis* plants, respectively.

4.2 Expression of sweet potato papain-like cysteine protease *SPCP2* altered seed and silique development and enhanced stress tolerance in transgenic *Arabidopsis*

Transgenic *Arabidopsis* plants were isolated and identified with floral dip transformation method (Clough & Bent, 1998). Genomic PCR and RT-PCR analysis confirmed that the presence and expression of sweet potato papain-like cysteine protease *SPCP2* in transgenic *Arabidopsis* plants (Chen et al., 2010). Transgenic *Arabidopsis* plants also exhibited slightly earlier transition from vegetative to reproductive growth (Fig. 5). The reasons and mechanisms are not clear. However, an imbalance in the relative availability of carbohydrate and nitrogen in the shoot apical meristem (Raper et al., 1988; Rideout et al., 1992), the change of endogenous ascorbic acid content (Barth et al., 2006), and possible non-specific degradation and removal of flowering repressor(s) by ectopic SPCP2 expression are suggested.

Expression of sweet potato SPCP2 in transgenic *Arabidopsis* plants also caused elevated number of incompletely-developed silique (Fig. 6), and reduced average fresh weight per seed and lower germination percentage (Chen et al., 2010). The reasons for the altered phenotypic characteristics in transgenic *Arabidopsis* by ectopic *SPCP2* gene expression are not clear. However, SPCP2 exhibited high amino acid sequence identities with plant papain-like cysteine proteases, such as *Phaseolus vulgaris* CP2, *Vicia sativa* CPR2, and *Vigna mungo* SH-EP. These papain-like cysteine proteases together with vacuolar processing enzymes have been implicated in association with the degradation and mobilization of globulin storage proteins during seed germination and seedling growth in *Phaseolus vulgaris* (Senyuk et al., 1998), *Vigna mungo* (Okamoto et al., 1999), and *Vicia sativa* (Schlereth et al., 2000; Schlereth et al., 2001). These reports provide a possible explanation for the altered phenotypic characteristics observed in transgenic *Arabidopsis* plants, and suggest that sweet potato SPCP2 may have an enzymatic function similar to papain-like cysteine proteases, including *Vigna mungo* SH-EP and *Vicia sativa* CPR2 for protein degradation and nutrient recycling during leaf senescence.

Expression of sweet potato *SPCP2* in transgenic *Arabidopsis* plants exhibited higher salt and drought stress tolerance (Fig. 7), and contained higher relative water content than control (Fig. 8). The reasons for the altered stress responses in transgenic *Arabidopsis* by ectopic SPCP2 gene expression are not clear. However, SPCP2 exhibited high amino acid sequence identities with plant cysteine proteases, such as *Arabidopsis* RD19 and broccoli Bocp4. *Arabidopsis* RD19 was a drought-inducible cysteine protease (Koizumi et al., 1993), and belonged to osmotic stress-responsive genes (Xiong et al., 2002). Under osmotic stress such as drought, high salinity (NaCl or PEG) and ABA treatments, RD19 mRNA transcript was significantly enhanced compared to untreated control (Xiong et al., 2002). Broccoli Bocp4 exhibited high sequence identity to dehydration-responsive *Arabidopsis* RD19, and was also significantly induced in broccoli florets, which were kept in air (dry situation) but not in water or 2% sucrose solution 12 h post harvest (Coupe et al., 2003). Sweet potato cysteine protease *SPCP2* was also inducible by salt and drought stresses in detached leaves (Fig. 9), and its ectopic expression in transgenic *Arabidopsis* caused higher salt and drought resistances (Figs. 7 and 8). Our results agree with these reports and suggest a possible role of sweet potato cysteine protease *SPCP2* in osmotic stress regulation and salt/drought stress tolerance.

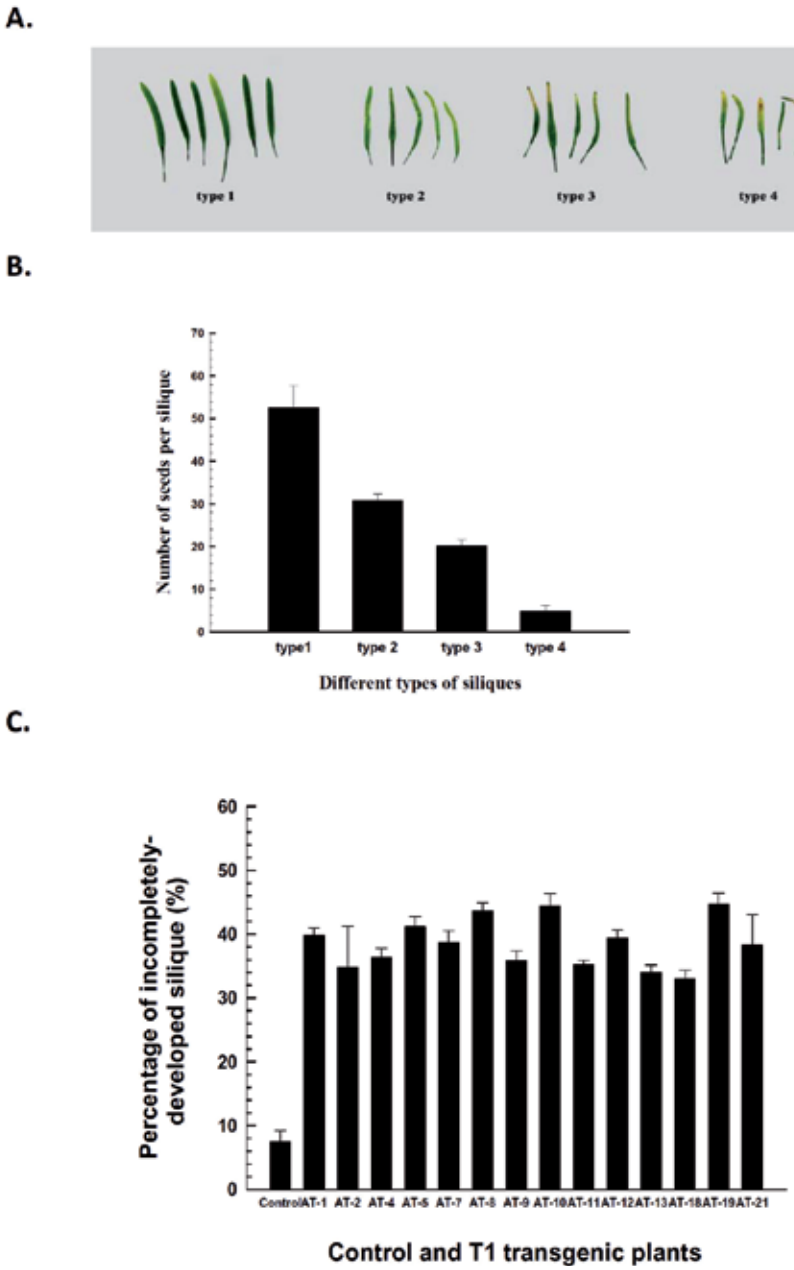

Fig. 6. Comparison of incompletely-developed silique percentages among control and transgenic T1 plants ectopically expressing sweet potato *SPCP2*. **A.** The appearance and size of different silique types (types 1, 2, 3 and 4) were observed and compared 35 days after seed germination. **B.** The average seed number of different silique type. **C.** Comparison of incompletely-developed silique percentage among control and transgenic T1 plants. C and AT denote control and transgenic T1 *Arabidopsis* plants,respectively (Adapted from Chen et al., 2010).

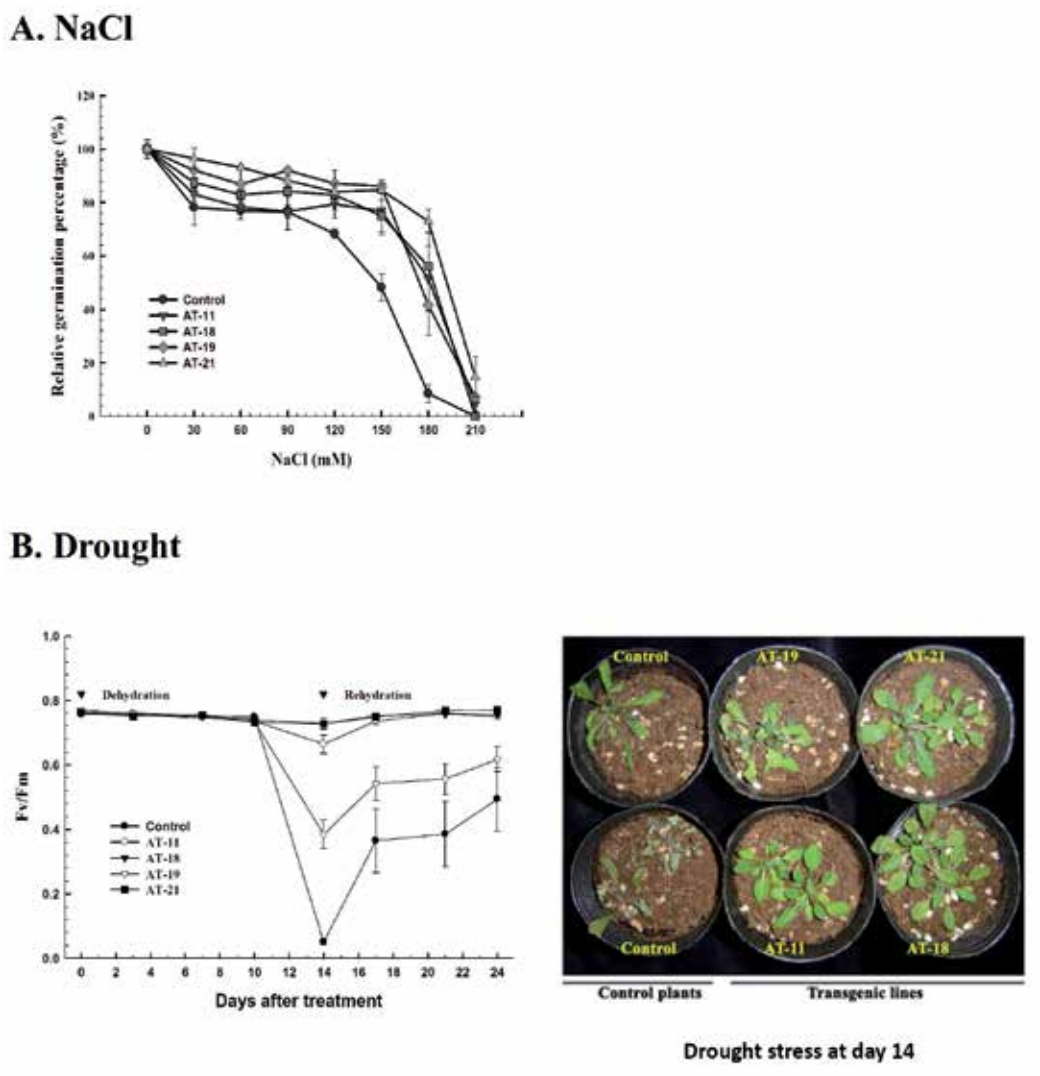

Fig. 7. Comparison of salt and drought stress tolerance among control and transgenic T1 *Arabidopsis* plants ectopically expressing sweet potato *SPCP2*. **A.** Salt. For salt treatment, seeds were germinated on half strength MS medium plus 3% sucrose and different concentrations of NaCl for ca. 2 weeks, and the relative germination percentages were recorded and compared. **B.** Drought. For drought treatment, upper panel of B is the photochemical Fv/Fm comparison among control and transgenic T1 *Arabidopsis* plants during dehydration and rehydration treatment. Lower panel of B is the morphological comparison among control and transgenic T1 *Arabidopsis* plant at day 14 after drought treatment. The data were the average of total 5 petri dishes for A or 5 seedlings per transgenic line for B, and shown as mean ± S.E. Control and AT-11/AT-18/AT-19/AT-21 denote wild type and transgenic T1 *Arabidopsis* plants, respectively. ▲ indicates the time points of dehydration and rehydration (Adapted from Chen et al., 2010).

5. Correlation of cysteine protease expression with storage protein degradation in sweet potato storage root during sprouting

In sweet potato storage root, trypsin protease inhibitors (TIs) are the main storage proteins and composed of a multiple gene family. It has been implicated that cysteine proteases are likely associated with the degradation of storage root trypsin inhibitors during sprouting (Huang et al., 2005). Therefore, expression and correlation of sweet potato asparaginyl endopeptidase SPAE and papain-like cysteine protease SPCP2 with the degradation and mobilization of the two major storage root trypsin inhibitor bands during sprouting were studied. The sprouts appeared and were visible within the first week of incubation of storage root at room temperature, whereas, degradation of trypsin inhibitors became significant in the later incubation. RT-PCR analysis of *SPAE* and *SPCP2* also demonstrated that their gene expression was significantly higher in the sprout and flesh of sprouting storage root than that of non-sprouting storage root (Fig. 10), and correlated well with the time course of degradation of the two major trypsin inhibitor bands (unpublished data). These data suggest that the asparaginyl endopeptidase SPAE and papain-like cysteine protease SPCP2 may play roles in association with storage root major trypsin inhibitor degradation during sprouting.

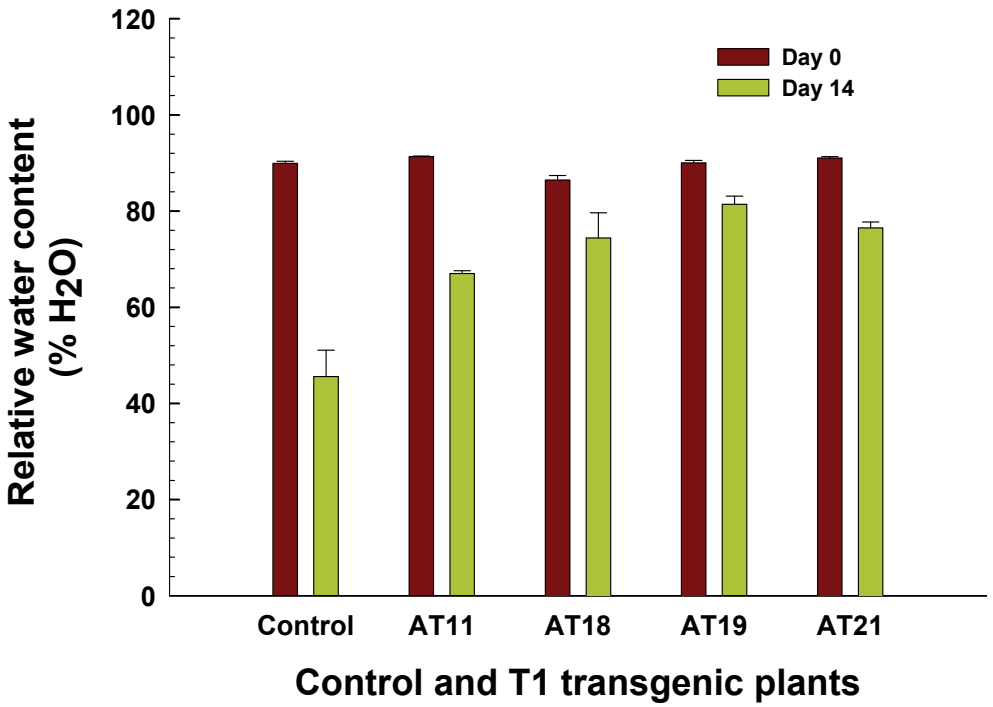

Fig. 8. Comparison of the relative water content (H2O%) between control and transgenic T1 *Arabidopsis* plants ectopically expressing sweet potato *SPCP2* at day 14 after drought treatment. The data were the average of total 5 seedlings per transgenic plants, and shown as mean ± S.E. Control and AT-11/AT-18/AT-19/AT-21 denote wild type and transgenic T1 *Arabidopsis* plants, respectively.

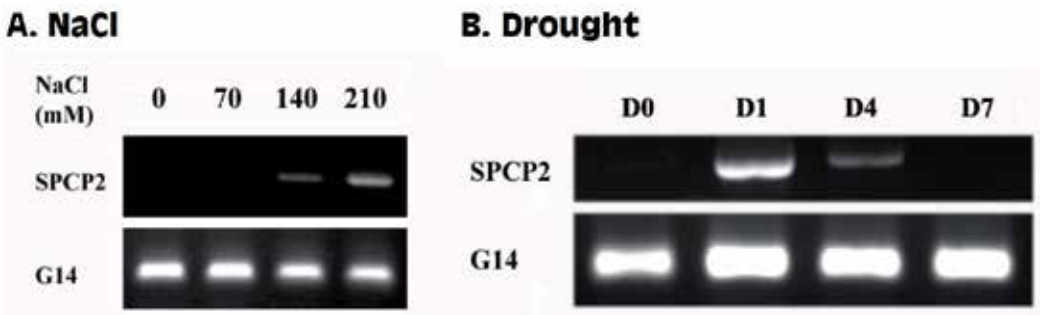

Fig. 9. Induction of sweet potato papain-like cysteine protease SPCP2 gene expression by salt and drought treatments. **A.** Salt treatment. Sweet potato detached leaves were treated with different salt concentrations (0, 70, 140 and 210 mM, respectively,) for 9 days and collected individually for RT-PCR analysis. **B.** Drought treatment. Detached sweet potato leaves were placed on dry paper tower in the dark for 0, 1, 4 and 7 days, and then collected individually for RT-PCR analysis. Sweet potato *G14* encoded a constitutively expressed metallothionein-like protein and was used as a control.

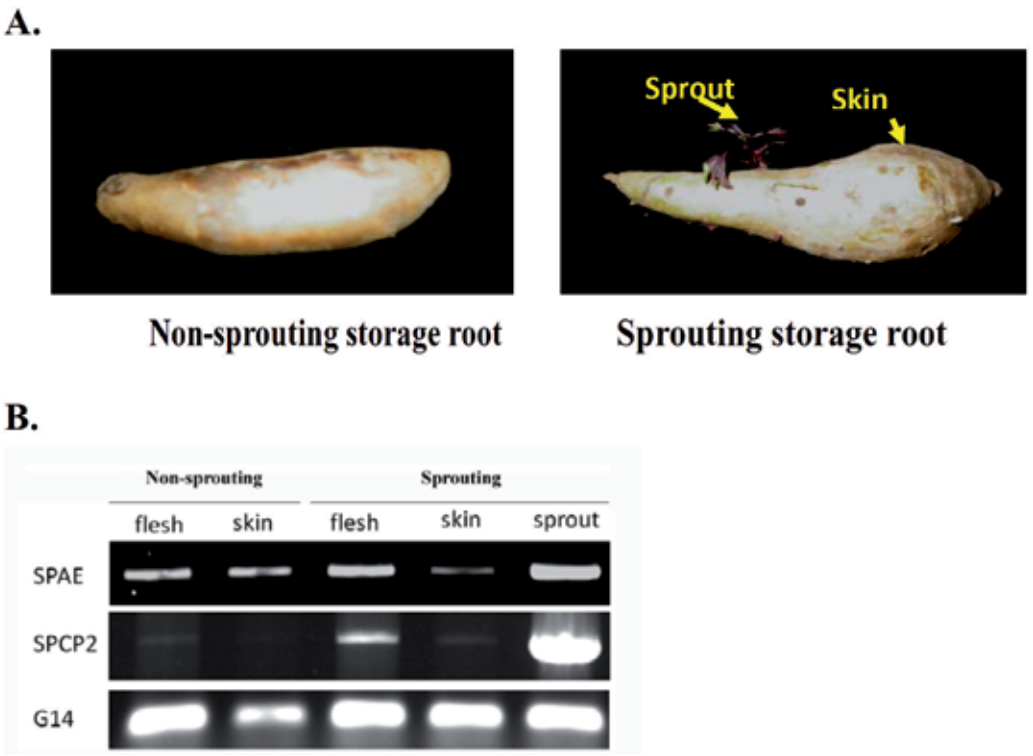

Fig. 10. Expression patterns of sweet potato asparaginyl endopeptidase SPAE and papain-like cysteine protease *SPCP2* in non-sprouting and sprouting st The orage roots. **A.** Storage root morphology. **B.** RT-PCR analysis of *SPAE* and *SPCP2*. Sweet potato *G14* encoded a constitutively expressed metallothionein-like protein and used as a control.

Sweet potato asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* cDNAs have been constructed and expressed in recombinant PET vector for fusion protein production and purification. Application of the purified fusion protein to sweet potato storage root or detached leaves will be performed in the future in order to study further whether they can promote (1) the degradation of storage root major trypsin inhibitors during sprouting, (2) protein degradation and recycling during leaf senescence, or (3) stress tolerance.

6. Conclusion

Sweet potato asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* are senescence-associated genes and significantly enhanced their expression in senescent leaves. Phylogenetic tree analysis shows that *SPAE* and *SPCP2* exhibit close association with vacuolar processing enzyme and papain-like cysteine protease, respectively, which are involved in seed globulin storage protein degradation. Ectopic expression of sweet potato *SPAE* and *SPCP2* in transgenic *Arabidopsis* plants also caused altered phenotypic characteristics, including abnormal seed and silique development, elevated incompletely-developed silique percentage, fewer silique numbers per plant, reduced seed germination percentage, and enhanced tolerance to drought and salt stresses. Based on these data, it can be concluded that sweet potato asparaginyl endopeptidase *SPAE* and papain-like cysteine protease *SPCP2* may play physiological roles in association with protein degradation and nutrient recycling during leaf senescence with enzymatic functions similar to seed globulin storage protein degradation and re-mobilization during germination and seedling growth.

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Part 3

Metabolomics

13

Transgenic Plants as a Tool for Plant Functional Genomics

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1. Introduction

Functional genomics aim to discover the biological function of particular genes and to uncover how sets of genes and their products work together. Transgenic plants are proving to be powerful tools to study various aspects of plant sciences. The emerging scientific revolution sparked by genomics based technologies is producing enormous amounts of DNA sequence information that, together with plant transformation methodology, is opening up new experimental opportunities for functional genomics analysis.

2. Plant functional genomics methods

The main methods of Plant Functional Genomics are as follows.

2.1 Functional annotations for genes

Gene function prediction is based on comparison of genomes and proteomes with searching homologies within different species to gene of interest with known functions from nucleotide and amino acid databases . Putative genes can be identified by scanning a genome for regions likely to encode proteins, based on characteristics such as long open reading frames, transcriptional initiation sequences, and polyadenylation sites. A sequence identified as a putative gene must be confirmed by further evidence, such as similarity to cDNA or EST sequences from the same organism, similarity of the predicted protein sequence to known proteins, association with promoter sequences, or evidence that mutating the sequence produces an observable phenotype.

2.2 Gene-targeted and site-directed mutagenesis. Reverse genetics methods (loss of function)

Using transgenic plant with insertion/deletion or site-specific mutations. Host gene is replaced with mutant allele. The most conventional approach to the analysis of gene function is loss-of-function mutagenesis by chemicals or fast neutrons that introduce random mutations or deletions in the genome (Ostergaard and Yanofsky 2004).

Transferred DNA (T-DNA) tagging or transposon tagging methods were developed to generate loss-of-function mutations because these tag sequences can be used to identify the genes disrupted by these elements (Sundaresan and Ramachandran 2001; Sussman et al. 1999). However, because many plant genes in *Arabidopsis*, rice, and other plants belong to gene families (Goff et al. 2002; Kaul et al. 2000), the characterization of gene functions by single-gene mutagenesis is not always possible. Many mutants generated by single-gene disruption do not show clear phenotypes because of genetic redundancy.

2.3 Overexpression of normal gene in transgenic plants (gain of function)

Gain-of-function approaches have been used as an alternative or complementary method to loss-of-function approaches as well as to confer new functions to plants. Gain-of-function is achieved by increasing gene expression levels through the random activation of endogenous genes by transcriptional enhancers or the expression of individual transgenes by transformation. Gain-of-function mutagenesis is based on the random insertion of transcriptional enhancers into the genome or the expression of transgenes under the control of a strong promoter (Matsui et al. 2006; Nakazawa et al. 2003; Weigel et al. 2000) . In this approach, phenotypes of gain-of-function mutants that overexpress a member of a gene family can be observed without interference from other family members, which allows the characterization of functionally redundant genes(T. Ito and Meyerowitz 2000; Nakazawa et al. 2001).

Alternatively it is possible to overexpress mutant forms of a gene that interfere with the (wildtype) genes function. Over expression of a mutant gene may result in high levels of a non-functional protein resulting in a dominant negative interaction with the wild type protein. In this case the mutant version will out compete for the wild type proteins partners resulting in a mutant phenotype.

The advantages of gain-of function approaches in comparison to loss-of-function for the characterization of gene functions include the abilities to (*a*) analyze individual gene family members, (*b*) characterize the function of genes from nonmodel plants using a heterologous expression system, and (*c*) identify genes that confer stress tolerance to plants that result from the introduction of transgenes.

The first gain-of-function approach in plants was the activation-tagging system (Kakimoto 1996). In this system, T-DNA that harbors strong promoter or enhancer elements is randomly integrated into the plant genome. The introduced promoter or enhancer elements activate genes near the site of insertion.

Other recently developed gain-of-function approaches include cDNA overexpression and open reading frame (ORF) overexpression systems. In these approaches, cDNAs from Cdna libraries, representative full-length cDNAs (fl-cDNAs), or ORFs are strongly expressed when they were cloned downstream of a strong promoter.

The production of a large population of gain-of-function mutants can accelerate the high-throughput screening of desired mutants and the characterization of gene functions.

In the activation-tagging method, plant genes are randomly activated to produce gain-of function mutants. In this strategy, the promoter or enhancer elements from the *cauliflower*

mosaic virus (*CaMV*) *35S* gene have been exploited (Odell et al. 1985). Genes near the insertion site are activated under the control of enhancer elements.

After the selection of mutants from the population of transformants, T-DNA insertion sites are determined to identify candidate genes.

Plasmid rescue, inverse PCR, or adapter PCR methods can be used to recover the genomic fragments near the T-DNA right and left border sequences (Spertini et al. 1999; Yamamoto et al. 2003). TAIL-PCR is also an efficient method to determine T-DNA insertion sites (Singer and Burke 2003)

Although many algorithms have been developed to predict the presence of transcriptional units within the genome, the accuracy of such predictions is still limited. Empirical information is required to correct annotation, and the main source of experimental information to achieve this is derived from RNA transcript analysis. Notable progress in *Arabidopsis* genome annotation has been made by the generation of expressed sequence tags, fl-cDNAs (Seki et al. 2002), and whole genome tiling array studies (Toyoda and Shinozaki 2005). The fl-cDNAs are also important as a resource for functional genomics, i.e., in the identification of gene function, because they contain all the information needed for the production of functional RNAs and proteins.

Approximately 240,000 *Arabidopsis* fl-cDNA clones have been generated (Sakurai et al. 2005; Seki et al. 2002) using the biotinylated CAP trapper method together with trehalose-thermoactivated reverse transcriptase (Carninci et al. 1996; Carninci et al. 1997; Carninci et al. 1998) . Large sets of fl-cDNA clones have also been produced from several plants such as rice (Kikuchi et al. 2003), wheat (Ogihara et al. 2004), poplar (Nanjo et al. 2007), soybean (Umezawa et al. 2008), barley (K. Sato et al. 2009), cassava (Sakurai et al. 2007), sitka spruce (Ralph et al. 2008), *Physcomitrella patens* (Nishiyama et al. 2003), and *Thellungiella halophila* (Taji et al. 2008).Well-characterized collections of cDNAs play an essential role in defining the function of genes and proteins in plants. The cDNA overexpression system is one of the approaches that use cDNA resources.

Progress in sequencing technology has revealed the genome sequences of many plant species that include *Arabidopsis*, rice, poplar, grape, papaya, and sorghum (2000; Jaillon et al. 2007; Ming et al. 2008; Paterson et al. 2009; Sasaki et al. 2002; Tuskan et al. 2006). A functional genomics approach is now required to clarify the function of genes in these plant species.

However, transgenic approaches for both forward and reverse genetic studies are not yet practical in many plants in which transformation methodology is inefficient or not available. A heterologous expression approach provides a solution for the high-throughput characterization of gene functions in these plant species.

One study used approximately 10,000 nonredundant fl-cDNA clones from the RIKEN *Arabidopsis* fl-cDNA collection (Seki et al. 2002). A representative of each flcDNA was mixed at approximately the same molar ratio to generate a cDNA mixture and then cloned into an expression vector under the control of the *CaMV 35S* promoter. This flcDNA expression library was used to transform *Arabidopsis* plants by *in planta* transformation. In these transgenic plants, fl-cDNAs are randomly expressed in individual *Arabidopsis* plants so that each plant carries one (or more) fl-cDNA(s).

The introduced fl-cDNAs can be cloned easily using vector-specific primers after the isolation of mutants. Thus, the cDNA that caused the mutant phenotype can be directly linked to a function.

The full-length cDNA over-expressing gene (FOX) hunting system is an alternative gain of-function approach that uses fl-cDNAs. The FOX hunting system was applied for the high-throughput analysis of rice genes by heterologous expression in *Arabidopsis* (Matsui et al. 2009). The efficient, rapid, and high-throughput transformation system developed in *Arabidopsis*, together with the short generation time and compact size of this plant, makes *Arabidopsis* an ideal host plant.

These advantages have enabled researchers to express heterologous genes in *Arabidopsis* to analyze their functions.

Whole genome sequencing makes it possible to predict the presence of genes in the genome. Of particular interest are the gene elements that encode proteins, called ORFs. Because ORFs can be distinguished from fl-cDNAs by their lack of 5_ and 3_ untranslated region (UTR) sequences, they can be considered a minimal unit of the gene that encodes information on the functional protein. The *Saccharomyces cerevisiae* ORFeome project was the first attempt to verify the genome annotation at the genomic scale and to clone all its predicted ORFs (Heyman et al. 1999). The ORF collection has been created for functional analysis in various organisms, e.g., RNAi approaches in *Caenorhabditis elegans* (Piano et al. 2005), cellular localization studies of YFP/GFP fusion proteins in *Schizosaccharomyces pombe* (Matsuyama et al. 2006), GFP fusion proteins in *Escherichia coli* (Kitagawa et al. 2005), and proteomics in human (Collins et al. 2004; Rual et al. 2004). These ORF clone collections can facilitate the large-scale analysis of individual genes.

2.4 Studying gene expression using DNA-RNA hybridization, gene silencing

Transgene expression in pair with reporter gene under control of inducible promoter allows to reveal temporal functional effects of gene expression and the compartmentalization of transgene products. The gene silencing techniques (also known as RNA-interference) allow to achieve temporary disrupting effects of gene expression (gene knockdown) . This procedure offer the possibility to explore gene expression more precise.

Because transgene-induced RNAi has been effective at silencing one or more genes in a wide range of plants, this technology also bears potential as a powerful functional genomics tool across the plant kingdom.

RNA-induced gene silencing (RNAi), was originally observed as unusual expression patterns of a transgene designed to induce overexpression of chalcone synthase in petunia plants (Napoli et al. 1990).

In the years following this observation, experiments in many model systems contributed to rapid advancements in understanding the underlying mechanisms, and RNA-mediated gene silencing processes came to be collectively known as RNA interference (RNAi). It is known that the 'triggers' for RNAi were small RNAs, 21–25 nts in length, that were processed from longer, double-stranded (ds) RNAs by endonuclease proteins referred to as dicers (Fire et al. 1998; Hamilton and Baulcombe 1999; Mello et al. 2001; Zamore et al. 2000).

These siRNAs cause direct degradation of mRNAs in a homology dependent manner and lead to post-transcriptional silencing of the silencing target. Other gene silencing methods are direct heterochromatin formation and DNA methylation at regulatory sequences for the target to be silenced, which alsoinduce transcriptional silencing of target loci in a homology dependent fashion (reviewed by ref. (Eamens et al. 2008). Now, it is understood that RNAi is an evolutionarily conserved mechanism for gene regulation that is critical for many examples of growth and development.

There are multiple pathways by which small RNA molecules can influence gene expression in plants, at both the transcriptional and post-transcriptional levels. These pathways vary in their sources of small RNAs and specific mechanisms of silencing (S. W. L. Chan 2008; Eamens et al. 2008; Verdel et al. 2009).

Because transgene-induced RNAi has been effective at silencing one or more genes in a wide range of plants, this technology also bears potential as a powerful functional genomics tool across the plant kingdom. A common strategy for functional genomics projects is to generate lines that are deficient for the activity of a subset of genes, and test the knock down lines for phenotypes to characterize the function of the knocked down gene.

In many cases, a single inverted repeat transgene can be designed to silence multiple, closely related genes (Springer et al. 2007).

To induce transcriptional silencing with a transgene, a typical strategy involves designing a construct such that a dsRNA is generated which bears homology to the promoter region of the intended silencing target (Mette et al. 2000). Herein, this method of silencing will be referred to as promoter directed RNA silencing.

To induce post-transcriptional silencing with a transgene, a portion of the coding region of the gene is typically introduced into an inverted repeat (IR) construct, and expression of that transgene will result in a dsRNA with homology to the coding region of the intended silencing target (McGinnis et al. 2005). This type of silencing is likely mediated by components of the trans-acting siRNA pathway in plants (reviewed by ref. Verdel et al. 2009). Herein, this method of silencing will be referred to as coding region directed RNA silencing.

2.5 Analysis of spatial and temporal expression of studied gene

Genomic studies tend to be done at the whole tissue/organ level due to the ease of collecting samples and/or the lack of tools necessary to isolate sufficient quantities of specific cell or tissue types. Recent studies, however, have shown that most transcriptional responses to environmental stimuli are cell-type specific (Dinneny 2008; Gifford et al. 2008). In addition, the many examples of ion-channels, hormone biosynthetic enzymes and signaling components with spatially complex expression patterns clearly illustrate the need to study all aspects of plant biology at high-spatial and temporal resolution to fully understand the plant–environment interaction.

The root of *Arabidopsis* provides an excellent system for generating and utilizing such tools due to the simple and stereotypical organization of tissues and cell types. Specific cell layers in the *Arabidopsis* root have been engineered to express green fluorescent protein (GFP). Fluorescence- activated cell sorting (FACS) can then be used to enrich for GFP-positive cells

(Birnbaum et al. 2003; Birnbaum et al. 2005). This method has been used to characterize the global transcriptional profiles of nearly all cell types in roots grown under standard conditions (Birnbaum et al. 2003; Brady et al. 2007) and to characterize transcriptional changes that occur in these cell types in response to salt stress, iron deprivation and nitrogen treatment (Dinneny et al. 2008; Gifford et al. 2008). A detailed description of these studies can be found in the following reviews of Dinneny (2010) and Iyer-Pascuzzi and co-workers (2009).

Genetically-encoded fluorophores offer a vast tool kit to study *in vivo* molecular events such as protein localization and gene expression. Fluorescent proteins have also been engineered to act as biosensors, which either emit fluorescence in response to a specific biological stimulus or undergo a change in intrinsic fluorescence intensity (Frommer et al. 2009).

Transgene expression is usually driven by a constitutive promoter. Thus, high expression levels in inappropriate tissue or developmental contexts might occur. This misexpression can cause the ectopic expression of endogenous genes and might result in a phenotype that is not related to the authentic functions of the transgene. In some cases, this misexpression can lead to the incorrect functional annotation of genes. Tissue-specific expression can provide information on intracellular events in each tissue. Replacing the *CaMV 35S* promoter with tissue-specific promoters is another way to analyze gene function in certain tissues.

Two component systems have been developed for conditional gene activation or silencing (Brand et al. 2006). They combine an activator locus that codes for an artificial transcription factor expressed in restricted tissues at precise developmental times.

The activation or the ectopic expression of developmentally controlled transcription factors sometimes causes an embryonic or seedling lethal phenotype, making it difficult to analyze the function of the gene . Thus, controlled gene expression by an inducible system might be an efficient approach to identify these genes (Zuo et al. 2002).

2.6 Microarrays

Microarrays allow the identification of candidate genes involved in a given process based on variation between transcript levels for different conditions and shared expression patterns with genes of known function. With appropriate controls and repeated experiments, significant data are obtained on gene expression profiles under various conditions (including stresses) or in various organs. Because of the large quantity of data produced by these techniques and the desire to find biologically meaningful patterns, bioinformatics is crucial to analyze functional genomics data. However, the DNA microarray and bioinformatics data are not sufficient for determining correct expression profiles due to limited accuracy of the obtained data. Next stage of investigations explores the properties and functions of selected genes. In this case, a transgenic plant construction is one of the most informative techniques.

2.7 Next generation sequencing

Previously, DNA sequencing was performed almost exclusively by the Sanger method, which has excellent accuracy and reasonable read length but very low throughput. Sanger sequencing was used to obtain the first sequence of the human genome in 2001 (Lander et al. 2001; Venter et al. 2001). Shortly thereafter, the second complete individual genome (James

D. Watson) was sequenced using next-generation technology, which marked the first human genome sequenced with new Next Generation Sequencing (NGS) technology (Wheeler et al. 2008). A common strategy for NGS is to use DNA synthesis or ligation process to read through many different DNA templates in parallel (Fuller et al. 2009). Therefore, NGS reads DNA templates in a highly parallel manner to generate massive amounts of sequencing data but, as mentioned above, the read length for each DNA template is relatively short (35–500 bp) compared to traditional Sanger sequencing (1000–1200 bp). NGS technologies have increased the speed and throughput capacities of DNA sequencing and, as a result, dramatically reduced overall sequencing costs (Metzker 2010).

Current NGS approaches can be classified into three major categories:

1. DNA-Seq. Genome-based sequencing yielding genomic deletions and rearrangements, copy-number variations (CNV) of smaller regions or elements, and single-nucleotide polymorphisms (SNPs).
2. RNA-Seq. RNA-Sequencing, yielding genome-wide and quantitative information about transcribed regions (exons, and subsequently transcripts).
3. Chromatin-immunoprecipitation (ChIP)-Seq. a) transcription factor (TF)-based ChIP, yielding genome-wide information about the physical binding sites of individual TFs to within a few hundred base pairs. b) Epigenetic ChIP (DNA methylation and/or histone modifications), yielding information about modifications and the accessibility of genomic regions to TFs and other factors.

The inclusion of NGS-based transcriptome sequencing for ChIP of transcription factor binding and epigenetic analyses (usually based on DNA methylation or histone modification ChIP) completes the picture with unprecedented resolution enabling the detection of even subtle differences such as alternative splicing of individual exons.

Next-generation sequencing technologies have found broad applicability in functional genomics research. Their applications in the field have included gene expression profiling, genome annotation, small non-coding RNA (ncRNA) discovery and profiling, and detection of aberrant transcription, which are areas that have been previously dominated by microarrays. Thus, functional genomics and systems biology approaches will benefit from the enormous data density intrinsic to NGS applications, which will beyond doubt play an important role both in definition as well as verification of mathematical models of biological systems such as a cell or a tissue.

As mentioned above the inventory of methods used to study gene product functions *in vivo* (i.e. in a living organism) includes gene silencing, induced mutagenesis, reporter gene strategy, microarrays, and some others. However, there are some limitations inherent to this type of approach. First of all, physiologically essential genes cannot be switched off, and the induced mutagenesis can lead to concomitant mutations. The use of microarrays can lead to misinterpretation of the results since changes in transcription are not always accompanied by changes in protein level (Mittler et al. 1998). Moreover, the transcription level fails to reflect post-translation modifications of protein products which often occur *in vivo*. It is also worth to mention that when an enzyme possesses many isoforms, it is difficult to measure the activity of each of them *in vivo* (Slakeski et al. 1990). In view of the above-mentioned limitations, development of novel models for functional genetics which will aid to overcome these difficulties is deemed very much desirable.

One of such models may be the approach that is developed in our laboratories that employ transgenic plants that constitutively express bacterial genes, which code enzymes that are functionally homologous to plant enzymes. Such an approach was proposed and used in our laboratory since mid-1980s (Piruzian et al. 1983; Piruzian and Andrianov 1986). It involves several stages: search a cloning of a gene of interest, sequencing, sequence modification (if needed, e.g. when codon usage in the gene is different from that in the model organism), gene transfer into the model organism, and studies of biochemical and phenotypic changes that entail expression of the foreign gene. Such an approach is feasible owing to the similarity of metabolic pathways and gene networks that regulate the activities of pro- and eukaryotic organisms under normal conditions and under exposure to various biotic and abiotic stresses. In addition, the use of bacterial genes helps to avoid many problems that arise during cloning, modifications and expression of eukaryotic genes in plants, whereas the constitutive nature of bacterial gene expression allows revealing "hot spots" of action of the homologous plant enzymes.

3. Usage of the methods of functional genomics for studying fundamental and applied aspects of plant life

3.1 Biotic stress tolerance

Activation tagging has been used for the isolation of mutants with resistance to biotic stress. For example, *CDR1-D* is a mutant that is resistant to sprayed suspensions of virulent *Pseudomonas syringae pathovar tomato* (*Pst*) (Xia et al. 2004). *CDR1* encodes an extracellular aspartic protease, which is a member of a large family of aspartic proteases in *Arabidopsis*. CDR1 functions in the production of a systemic signal that induces basal defenses. Another mutant, *FMO1–3D*, showed enhanced resistance to virulent *Pst* DC3000 (Koch et al. 2006). This phenotype is the result of the overexpression of a gene that encodes a class 3 FMO protein.

Recently we have proposed the model for studying the role of plant dioxygenases. Phenolic compounds serve as antioxidants and protect plants from active oxygen species. The content of phenolic compounds changes as plants grow and get mature and in response to biotic and abiotic influences, and these changes are achieved through modulation of enzymatic activities involved in their synthesis and degradation. Enzymes that take part in oxidation of aromatic compounds include dioxygenases (Tsoi et al. 1988). These enzymes oxidize phenolic compounds by breaking the aromatic ring, and thus enable subsequent biodegradation of phenols. There is evidence that plant dioxygenase (coded for by the *lls* gene of maize) may participate in the hypersensitive response of the plant to a pathogen attack (Lawton and Maleck 1998). For a study of the role played by dioxygenase in plants we have chosen the bacterial gene *nah*C (Y14173) of *Ps. putida,* coding for 1,2-dehydronaphtalene dioxygenase. Our choice was due to the fact that this enzyme possesses broad substrate specificity and can also use pyrocatechin as substrate (Tsoi et al. 1988), thus allowing to model a maximum number of dioxygenase isozymes. The expression of bacterial 1,2-dehydronaphthalene dioxygenase (coded by the *nah*C gene) in tobacco plants resulted in marked phenotypic and morphologic changes: chlorosis of the leaves, development of necrotic spots, delayed rooting and growth, and early flowering (Piruzian et al. 2002). Data on expression of bacterial 1,2-dihydroxynaphtalene dioxygenases in plants have not been reported in the literature. The necrotic spots on leaves of transgenic plants could have resulted from accumulation of phenolic substances. The above-mentioned

phenotype and morphology changes suggested that the expression of bacterial dioxygenase resulted in alteration of the level of phenolic compounds in the transgenic plant cells. Measurements of phenolic acid content indicate that normal metabolism of phenolic compounds is disturbed in the plants, and the disturbance apparently results in induction of a stress response and appearance of the necrotic spots. In our opinion, such transgenic plants are a promising model for the study of mechanism of genome functioning under normal conditions and under stress, as well for the study of functions of phenolic compounds.

3.2 Abiotic stress tolerance

Environmental stresses are the major factors adversely affecting plant growth and development as well as productivity. Of the various abiotic stresses, drought and osmotic stress cause considerable agronomic problems by limiting crop yield and distribution world-wide (Chaves and Oliveira 2004).

Drought and osmotic stress induce a range of alterations at the molecular, biochemical, and cellular levels in plants, including stomatal closure, repression of photosynthesis, accumulation of osmolytes, and the inducible expression of genes involved in stress tolerance (Shinozaki and Yamaguchi-Shinozaki 2007).

The accumulation of proline by plants is a common physiological indicator and occurs under various abiotic stresses. There is an increasing body of evidence supporting the role of proline as a compatible osmolyte that maintains cellular osmotic adjustment and stabilizes the structure of proteins and membrane integrity (Verbruggen and Hermans 2008). Overexpression of different genes has been shown to significantly enhance proline levels in transgenic rice and improve their tolerance to environmental stresses (Ito et al. 2006; Liu et al. 2007; Pasquali et al. 2008; Xiang et al. 2007; Xu et al. 2008; Chen et al. 2009).

The transference of a single gene encoding a specific stress protein does not always result in sufficient expression to produce useful tolerance, because multiple and complex pathways are involved in controlling plant drought responses (Bohnert et al. 1995) and because modification of a single enzyme in a biochemical pathway is usually contrasted by a tendency of plant cells to restore homeostasis (Djilianov et al. 2002). Targeting multiple steps in a pathway may often modify metabolite fluxes in a more predictable manner. Another promising approach is therefore to engineer the overexpression of genes encoding stress inducible transcription factors.

There is increasingly more experimental support for the manipulation of the expression of stress-related transcription factor genes as a powerful tool in the engineering of stress-tolerant transgenic crops. This would, in turn, lead to the up-regulation of a series of stress-related genes under their control in transgenic plants (P. K. Agarwal et al. 2006).For example, the overexpression of transcription factor genes, such as ZFP252, SNAC1, OsNAC6, OsDREB1A, and HvCBF4, could enhance rice tolerance to different environmental stresses (Nakashima et al. 2007; Oh et al. 2007; Xiong et al. 2006; Xu et al. 2008; Yamaguchi-Shinozaki et al. 2006).

Following the application of microarray technology, several hundred stress induced genes, mainly in the model plant *Arabidopsis thaliana*, have been identified as candidates for manipulation (Shinozaki and Yamaguchi-Shinozaki 2007) and have been classified into three

groups (Bhatnagar-Mathur et al. 2008): (a) genes encoding proteins with a known enzymatic or structural function. Examples include enzymes for synthesis of osmoprotective compounds, late embryogenesis abundant (LEA) proteins, osmotins, chaperons, channels involved in water movements through cell membranes, ubiquitins, proteases involved in protein turnover, and detoxifying enzymes; (b) genes with as yet unknown functions; and (c) regulatory genes, such as those coding for kinases, phosphatases and transcription factors.

Mutants with abiotic stress tolerance have been isolated by activation tagging and include the *edt1* mutant recently identified under drought conditions (Ahad et al. 2003; Ahad and Nick 2007; Pereira et al. 2004; Yu et al. 2008). This mutant showed a drought tolerant phenotype and reduced stomatal density. The enhanced drought tolerance of *edt1* was associated with an increase in the expression of the gene that encodes the transcription factor *HDG11*. The overexpression of *ArabidopsisHDG11* in tobacco can also confer drought tolerance and reduced leaf stomatal density (Zhang 2003).

FOX lines that consist of 43 stress-inducible transcription factors were constructed to elucidate stress-related gene function (Fujita et al. 2007). The T1 generation was screened for salt-stress-resistant lines and led to the identification of salt-tolerant lines. Among them, four lines harbored the same transgene, *AtbZIP60*, which encodes a basic domain/leucine zipper class transcription factor. The overexpression of *AtZIP60* leads to the upregulation of stress related genes, which suggests an important role for this transcription factor in stress-responsive signal transduction.

Transcription factors play an important role in plant development and stress responses. The *Arabidopsis* genome encodes more than 1,500 transcription factors. gain-of-function mutagenesis is an ideal approach to uncover the function of transcription factors (J. Z. Zhang 2003).

Weiste and colleagues (Weiste et al. 2007) generated an ORF collection composed of members of the ERF transcription factor family. They constructed a destination vector to enable ectopic expression driven by the*CaMV35S* promoter and included a HA-tag sequence to reveal transgene-specific expression. Using this library, they generated transgenic *Arabidopsis* plants that overexpress HA-tagged ORFs of the ERF transcription factor family. This approach yielded eight plants that show enhanced tolerance to oxidative stress resulting from the overexpression of the same ERF.

Typically a gene coding for a transcription factor in *Arabidopsis* is isolated, characterized and shown to improve drought response when overexpressed. The gene is then transferred to a crop plant where it often confers the same drought-tolerant phenotype. The HARDY (*HRD)* gene, coding for an AP2/ERF-like transcription factor (Pereira et al. 2007) is an example of this approach. *Arabidopsis* plants with a gain-of-function mutation in the *HRD* gene (hrd-D mutants) are drought resistant, salt-tolerant, and overexpress abiotic stress marker genes. Overexpression of the same gene in rice significantly improves water use efficiency both under well-watered conditions (50–100% increase) and under drought (50% increase). These plants also show enhanced photosynthetic assimilation and reduced transpiration (Pereira et al. 2007). *HRD* gene overexpression conserves drought tolerance in both dicots and monocots.

In other cases a gene coding for a transcription factor is isolated and characterized in *Arabidopsis*, but its orthologue gene in the crop plant of interest is identified and made to

overexpress. For example Nelson et al. (Nelson et al. 2007) showed that overexpression of the *Arabidopsis* CAAT box-binding transcription factor AtNF-YB1 confers improved performance in *Arabidopsis* under drought conditions. They next overexpressed the orthologue of AtNF-YB1 (called ZmNF-YB2) in maize and found that, under simulated drought conditions, the altered maize plants produced up to 50% more than unmodified plants (Nelson et al. 2007).

A high-throughput gain-of-function approach has been applied to isolate salt stress tolerance genes using cDNAs of *Thellungiella halophila* (Du et al. 2008). *Thellungiella halophila* is a type of salt cress similar to *Arabidopsis* that can grow under high salt conditions. The cDNA library was prepared after salt stress treatment. Approximately 125,000 transgenic *Arabidopsis* that express *Thellungiella halophila* cDNAs under *CaMV 35S* promoter were generated. Novel salt stress tolerance genes were isolated from this mutant collection.

Ethylene response factor (ERF) genes have been successfully introduced into rice, generating transgenic rice with enhanced tolerance to biotic and abiotic stresses. For example, the tobacco OPBP1 (an AP2/ERF transcription factor) can enhance salt tolerance and disease resistance of transgenic rice (Chen and Guo 2008); ectopic expression of the *Arabidopsis HARDY* gene in rice improves water use efficiency and the ratio of biomass (Pereira et al. 2007); overexpression of rice DREB transcription factor (*OsDREB1F)* increases salt, drought, and low temperature tolerance in rice (Chu et al. 2008). Overexpression of transcription factor *Sub1A-1* in a submergence-intolerant *Oryza sativa* ssp. japonica conferred transgenic plants with enhanced submergence tolerance (Ronald et al. 2006). The ethylene response factors SNORKEL1 and SNORKEL2 encoding ERFs trigger internode elongation and allow rice to adapt to deep water (Ashikari et al. 2009).

By overexpressing a Athsp101 protein, Katiyar-Agarwal and associates (2003) generated a heat-tolerant transgenic rice (cv. Pusa basmati 1) line. This group showed that almost all the transgenic plants recovered after severe heat stress of 45–50^{0}C and exhibited vigorous growth during the subsequent recovery at 28^{0}C, while the untransformed plants could not recover to a similar extent.

In our experiments with salt stress tolerance, we have selected a mutant of *E. coli* able to grow on a medium with a high salt content. The cells of the mutant strain have a high content of proline, and it has been shown that this was due to a mutation in N-terminal region of γ-glutamyl kinase encoded by the *pro*B gene. The mutation, which consists of single amino acid substitution (leucine is replaced by glycine) caused a conformational change in the regulatory region of the protein, made the enzyme less sensitive to the feedback inhibition by proline. We designated the gene coding for the mutant γ-glutamyl kinase as proB_{osm} (Neumyvakin et al. 1990, 1991). *E. coli* genes *pro*B_{osm} and *pro*A have been transferred into tobacco plants, each under control of a strong constitutive promoter CaMV 35S, which contains a duplicated enhancer sequence, or the P_{mas} promoter which induces gene expression predominantly in roots (Sokhansandzh et al. 1997). The rationale for using a mutant form of the prokaryotic proteins which determine a strictly define phenotype, osmotolerance, was that the phenotype of the plant model will be easy to assay. The expression of the osmotolerance phenotype in the model eukaryotic organism would be indicative that the prokaryotic protein is an ortholog of the eukaryotic protein. Transgenic plants carrying the bacterial proline operon genes had a higher resistance to the toxic

proline analogue (L-azetidin-2-carboxic acid) and to the high salt stress (were capable of rooting at NaCl concentrations in the medium over 350 мМ) (Sokhansandzh et al. 1997). Thus, our results demonstrate usefulness of the proposed model and the possibility of simulating the activity of a bi-functional plant enzyme with two bacterial enzymes.

In *Arabidopsis*, knockout or silencing of *HSP101* caused loss of the acquired thermotolerance, whereas the overexpression of *HSP101* in transgenic plants improved tolerance to high temperature stress (Gurley 2000; Hong and Vierling 2000). Agarwal and co-workers (2003) provided evidence that *AtHSP101* and *OsHSP101* impart thermoprotection to yeast cells by dissolution of heat-induced protein aggregates. High-temperature-tolerant rice plants have also been produced by overexpressing a rice small heat-shock protein sHSP17.7 (Sato et al. 2004). Oxidative stress may accompany heat stress by the formation of ROS (Foolad et al. 2007). More recently, Qi and associates (2011) have reported that *mtHsp70* over-expression suppresses programmed cell death (PCD) by maintaining mitochondrial membrane potential and preventing ROS signal amplification in rice protoplasts.

Koh and co-workers (2007) reported that knockout (KO) mutants of rice *OsGSK1*, an orthologue of *Arabidopsis* BIN2, showed enhanced tolerance to several abiotic stresses including high temperature. In comparison to non-transgenic plants, the wilting ratios for knock out mutants were as much as 26% lower after heat (45^0C) stress. Feng and associates (2007) raised transgenic rice plants overexpressing rice sedoheptulose-1,7-bisphosphatase *SBPase*. They showed that overexpression of *SBPase* resulted in enhanced tolerance of growth and photosynthesis to high temperatures in transgenic rice plants.

Huang and co-workers (2008b) generated transgenic tobacco expressing rice A20/AN1- type zinc finger protein gene (*ZFP177*). Compared to wild type tobacco, the transgenic seedlings showed higher tolerance to temperature stress.

Major efforts have been made to identify genes that are associated with drought stress in a number of plant species (Gong et al. 2010; Huang et al. 2008a; Manavalan et al. 2009; Tran and Mochida 2010; Zheng et al. 2010). In rice, identification of drought-responsive genes has been carried out by means of expression profiling studies such as microarrays, expressed sequence tags (ESTs), RNA gel blot analyses and qRT-PCR (Rabbani et al. 2003; Rabello et al. 2008; Ramachandran et al. 2008; Reddy et al. 2007; Zhou et al. 2007). As a result, hundreds of genes that were induced or suppressed by drought stress have been identified. A number of these genes have been analyzed in detail, resulting in their characters as regulatory genes, such as transcription factor (TF) and protein kinase encoding genes, whose products regulate other stress-responsive genes. Some of the identified stress-responsive genes are functional genes which encode metabolic components, such as late embryogenesis abundant (LEA) proteins and osmoprotectant-synthesizing enzymes, important for stress tolerance (Yang et al. 2010).

Recently, Yang and associates (2010) classified drought-responsive genes into three groups based on their biological functions: transcriptional regulation, post-transcriptional RNA or protein phosphorylation, and osmoprotectant metabolism or molecular chaperons. However, among the genes that are affected by drought many genes have unknown functions. Efforts will be continued to determine the functions of the unknown drought-responsive genes.

Aquaporins, which are water channel proteins that translocate water across cell membranes, have been demonstrated for their roles in various physiological processes including stomatal closure (Li et al. 2008).

The rice plasma membrane intrinsic proteins (OsPIP) proteins are subfamilies of aquaporins and are divided into two subgroups, OsPIP1 and OsPIP2. Several members of OsPIP1 and OsPIP2 subfamilies were responsive to drought and salt stresses. Transgenic *Arabidopsis* overexpressing OsPIP2–2 showed enhanced tolerance to salt and drought stresses (Guo et al. 2006).

Transgenic rice overexpressing *Datura stramonium* S-adenosylmethionine decarboxylase (adc) gene showed increased tolerance to drought due to an increase in polyamine content (Capell et al. 2004), suggesting that OsAdc1 may be a potential candidate for development of enhanced drought-tolerant rice cultivars.

In some cases however, constitutive expression of a gene normally only induced by stress, has negative effects – so-called pleiotropic effects (Chan et al. 2002; Kasuga et al. 1999; Nakashima et al. 2007) – on growth and development when stress is not present. One solution is to use inducible (rather than constitutive) promoters that allow expression of a transgene only when it is required, while it is silenced otherwise. For example constitutive expression in *Arabidopsis* of *DREB1/CBF3*, a gene coding for a transcription factor induced by osmotic stress, confers tolerance to stress, but causes severe growth retardation under normal growth conditions (Kasuga et al. 1999). However, if this gene is expressed under the control of an osmotic stress-inducible promoter like RD29A no growth retardation occurs, and the plant is highly resistant to several stress conditions (Kasuga et al. 1999).Similarly,In tomato, overexpression of the *Arabidopsis CBF1* gene, encoding a transcription factor belonging to AP2/ERF family, confers increased drought, cold and oxidative stress tolerance compared to wild-type plants, but plant growth is severely affected (Chan et al. 2002). By contrast, when the same gene was placed under the control of a synthetic promoter derived from the barley *HVA22* gene, it was expressed mainly under abiotic stresses, so that the plant had the same tolerance characteristics towards stresses, but plant growth under normal conditions was not affected (Lee et al. 2003).

An ideal stress-inducible promoter would be completely silenced under normal conditions, but induced by stress in a fairly short time (a few hours) after stress onset. The promoter of the *Arabidopsis AtMYB41* gene, which is not expressed in any tissues under standard growth conditions but is highly induced in response to drought, salt and abscisic acid (Cominelli et al. 2008), may therefore be a very useful promoter.

3.3 Increase of productivity

The development of unique transgenic plants provides an applied angle, in making available highly nutritious "speciality crops," and also adds to the genetic resources that can be used to develop insightful knowledge base about genetic, biochemical, and physiological regulation of various metabolic pathways and functional metabolites. The transgenic tomatoes that accumulate higher polyamines, Spd and Spm, during ripening are a kind of a "gain of function" genotype, and we are using them to address the questions on the role of polyamines in fruit metabolism, in particular, their crosstalks with other functional molecules to enable higher nutritional quality of vegetables and fruits.

In this case, a fruit ripening-specific promoter was used to drive the expression of yeast *SAM decarboxylase* gene, with the result that the introduced gene was not active during the early growth and development of the plant but became active along with the normal ripening process of the fruit (Mehta et al. 2002).

An analysis of the principal, soluble constituents of wild-type and Spd/Spm-accumulating transgenic tomato, generated using high-resolution NMR spectroscopic methods, showed that the same metabolites were present in wild-type/azygous control tomatoes as in the transgenic tomato fruit. However, the latter conspicuously revealed differential metabolite content as compared to the controls (Mattoo et al. 2006). The red transgenic fruit were characterized by higher accumulation of the amino acids glutamine and asparagine; micronutrient choline; the organic acids citrate, fumarate, and malate; and an unidentified compound A. Compared to the control, wild-type fruit, the levels of valine, aspartic acid, sucrose, and glucose in the transgenic red fruit were reduced. These changes reflected specific alteration of metabolism, since the levels of isoleucine, glutamic acid, aminobutyric acid, phenylalanine, and fructose remained similar in the nontransgenic and transgenic fruits. Consequently, the transgenic red fruit have significantly higher fructose/glucose and acid [citrate+malate]/sugar [glucose+fructose+sucrose] ratios (Mattoo et al. 2006), consistent with higher fruit juice and nutritional quality reported in the 2 transgenics (Mehta et al. 2002), attributes favorably considered as higher quality in tomato breeding programs.

3.4 Xenobiotic tolerance

We have created transgenic plants expressing a mutant, glyphosate-resistant EPSP synthase from *E. coli* (Piruzian et al. 1988). The rationale for using a mutant form of the prokaryotic ortholog protein which determines a strictly define phenotype, herbicide resistance, was that the phenotype of the plant model will be easy to assay. The expression of the glyphosate tolerance phenotype in the model eukaryotic organism would be indicative that the prokaryotic protein is an ortholog of the eukaryotic protein. First we isolated an *E. coli* strain with resistance to glyphosate. The mutation was localized in locus aroA and was shown to result in a replacement of Ala with Pro in the bacterial EPSP synthase (Piruzian et al. 1988). We constructed expression vectors for plant transformation and obtained tobacco plants that tolerated a five-fold higher glyphosate concentration than inhibited the growth of control plants (Mett et al. 1991; Piruzian et al. 2000). Thus it was shown that with the use of transgenic plants expressing the mutant EPSP synthase, a bacterial enzyme insensitive to glyphosate could be active in the plant instead of the plant enzyme which was sensitive to the herbicide (Mett et al. 1991). This experiment also demonstrated a principal possibility of modeling plant enzymatic activity by using bacterial enzymes.

3.5 Studying processes of plant physiology

One of the best examples of the use of the activation-tagging system to identify genes involved in plant development is the isolation of *YUCCA* genes, which encode flavin mono-oxygenase (FMO) proteins involved in auxin biosynthesis. Six loci, which encode proteins with a role in auxin biosynthesis, have been identified in *Arabidopsis* by using activation-tagging technology (Pereira et al. 2002; Zhao et al. 2001). The *YUCCA* family consists of 11 members in the *Arabidopsis* genome (Cheng et al. 2006). All activation-tagged mutants of *YUCCA* genes showed phenotypes that were characteristic of auxin-overproducing mutants

(Boerjan et al. 1995; Delarue et al. 1998). Double, triple, and quadruple loss-of-function mutants of *YUCCA* genes showed deleterious developmental disorders, although no single mutants displayed visible phenotypes (Cheng et al. 2006). This functional redundancy might lead to difficulties in the isolation of mutants related to auxin biosynthesis by the loss-of-function approach. Thus, gain-of-function mutagenesis is a powerful tool to elucidate the function of genes that compose a gene family.

The *pap1-D Arabidopsis* mutant generated by activation tagging is an intense purple color caused by the overproduction of phenylpropanoid derivatives, such as anthocyanins (Borevitz et al. 2000). The *PAP1* gene encodes a member of the R2, R3 MYB transcription factor family that comprises more than 100 members in *Arabidopsis* (Paz-Ares et al. 1998; Weisshaar et al. 1998). The heterologous expression of *Arabidopsis PAP1* can enhance the accumulation of anthocyanins in tobacco plants. The activation-tagging method has also been employed to identify a transcriptional regulator of secondary metabolites in tomato (Mathews et al. 2003). The overexpression of *ANT1*, which encodes a MYB-type transcription factor, caused an intense purple color in many vegetative tissues throughout development and purple spotted fruit on the epidermis and pericarp in tomato.

LeClere and Bartel (2001) generated *Arabidopsis* lines that overexpresses random cDNAs driven by the *CaMV 35S* promoter. They generated more than 30,000 *Arabidopsis* transgenic plants and isolated a mutant that showed a pale green phenotype caused by the overexpression of a truncated cDNA that encodes chloroplast ferredoxin-NADP+ reductase (FNR). This phenotype was caused by the cosuppression of endogenous genes by transgene overexpression, which led to dominant loss-of-function phenotypes.

Using *Arabidopsis* FOX lines, Okazaki and colleagues isolated six mutants that contained an increased number of chloroplasts in their leaves (2009). The overproduction of plastid division (PDV) proteins that regulate the rate of chloroplast division in *Arabidopsis* leads to an increase in the number, but a decrease in the size, of chloroplasts.

Another *Arabidopsis* FOX line that carries the cDNA, which encodes the cytokinin responsive transcription factor 2 (CRF2), also showed an increased number of chloroplasts.

Thus, the FOX hunting system is capable of the highthroughput characterization of gene functions.

A high-efficiency transformation method has been developed in rice. This makes rice an ideal host plant for the FOX hunting system (Ichikawa et al. 2007). More than 28,000 rice fl-cDNAs have been generated (Kikuchi et al. 2003). Approximately 12,000 rice lines have been generated in which 13,980 independent fl-cDNAs were overexpressed under the control of the ubiquitin promoter. Among several phenotypes in the T0 generation, three dwarf lines that carry the same novel *gibberellin 2-oxidase* (*GA2ox*) gene were isolated.

The ORF overexpression approach can also be used to investigate the function of putative genes identified by computer-based means. Small secreted peptides, less than 150 amino acids long, were predicted to identify genes involved in plant development in *Arabidopsis* (Hara et al. 2007). Plants that overexpress 153 predicted genes that encode these small secreted peptides were generated, and this approach led to the identification of the *Epidermal Patterning Factor 1* (*EPF1*) gene. *EPF1* was expressed in stomatal cells, and precursors may be involved in the control of stomatal patterning through the regulation of asymmetric cell division.

The T-DNA vector pER16, which contains the estradiol-inducible promoter, was used for the conditional activation of nearby genes by the addition of estradiol. This system was used to identify the gene *PGA6*, which encodes WUSCHEL (WUS), a homeodomain protein involved in the regulation of stem cell fate in *Arabidopsis* shoot and floral meristems (Mayer et al. 1998).

Recently we have proposed a new strategy for creating experimental models for plant functional genomics. It is based on the expression in transgenic plants of genes from thermophilic bacteria encoding functional analogues of plant proteins with high specific activity and thermal stability. We have validated this strategy by comparing physiological, biochemical and molecular properties of control tobacco plants and transgenic plants expressing genes of β-glucanases with different substrate specificity. We demonstrate that the expression of bacterial β-1,3–1,4-glucanase gene exerts no significant influence on tobacco plant metabolism, while the expression of bacterial β-1,3-glucanase affects plant metabolism only at early stages of growth and development. By contrast, the expression of bacterial β-1,4-glucanase has a significant effect on transgenic tobacco plant metabolism, namely, it affects plant morphology, the thickness of the primary cell wall, phytohormonal status, and the relative sugar content. We propose a hypothesis of β-glucanase action as an important factor of genetic regulation of metabolic processes in plants.

It should also be mentioned that many plant enzymes have numerous isozymes, and for this reason the activity assays as well as functional studies of particular isozymes *in vivo* are difficult (del Campillo 1999; Libertini et al. 2004). It should be noted that it was the use of thermostable heterologous proteins that enabled us to obtain these results. Overexpression of a homologous plant gene or a gene from a related species, apart from the additional difficulties of cloning plant genes with their exon–intron structure, could result in technical problems in detecting and assessing the activity of these proteins in transgenic plants. Thus, the use of thermostable bacterial proteins–functional analogs of plant beta-glucanase is not only adequate but also a more convenient method when compared to over-expression of a plant gene from its own genome or that of a related species.

The next our work was the construction of experimental models for studying the role of isopentyl transferases in phytohormone synthesis and plant differentiation.

It has been supposed that phytohormones, cytokinins in particular, are largely responsible for the viability of plants following exposure to abiotic stress and to pathogens. Therefore, an employment of genes whose expression alters the phytohormone balance for studying plant metabolism is deemed especially promising. One of such enzymes is isopentenyl transferase, a key enzyme of the cytokinin biosynthesis pathway. As a functional bacterial analogue of this enzyme, we have used isopentyl transferase (coded for by the T-*cyt* or *ipt* gene), the key enzyme of cytokinin synthesis from the T region of the *Agrobacterium tumefaciens* Ti plasmid. The product of the *ipt* gene is involved in crown gall formation in plants. In accordance with our strategy, we cloned the *ipt* gene and introduced it into the tobacco genome (Iusibov et al. 1989). Regenerated shoots at first did not form roots and were devoid of apical domination, a fact that was also reported by others. As reported by Zhang and co-workers, (1996), the expression of isopentyl transferase in transgenic tobacco plants may be controlled by auxins. We have therefore used exogenous auxin and this allowed us to obtain normal transgenic plants having an intermediate level of cytokinin in comparison with normal transgenic plants and the crown gall tissue (Makarova et al. 1997).

On the whole, expression of the agrobacterial gene leads to cytokinin overproduction (a two-fold excess of total cytokinins), decrease of the abscisic acid level, elevated level of chlorogenic acid, and disturbed morphogenesis and regeneration processes in the plants (Makarova et al. 1997; Yusibov et al. 1991). The altered hormone balance naturally affected such a vitally important process as photosynthesis. In particular, we have shown that elevated cytokinin affects the expression of some plant genes. For example, we found that plants transgenic for the *ipt* gene had a higher level of mRNA of the chloroplast gene of the ribulose biphosphate carboxylase (RBC) smaller subunit (*rbc*L) (Yusibov et al. 1991). Thus, the expression of bacterial isopentyl transferase causes significant metabolic changes in the transgenic plants. These include altered hormone balance (cytokinins, abscisic acid), altered expression of some chloroplast genes (the RBC smaller subunit) involved in photosynthesis, and altered morphology of the plants.

To study the role of enzymes related to hydrocarbon metabolism in plants we have chosen the gene *xyl*A *of E. coli*. This gene codes for xylose (glucose) isomerase (P00944) (EC 5.3.1.5) which converts xylose into xylulose and *vice versa* as well as fructose into glucose and *vice versa*. *E. coli' s* enzyme is thermostable (Piruzian et al. 1989), a property which ensures an easy assay of the enzyme in plants. The *xyl*A gene under control of the 35S CaMV promoter was transferred to tobacco plants using the *A. tumefaciens* vector system, and it was shown that an active enzyme is produced in the transgenic plants (Goldenkova et al. 2002). The plants had larger leaves, grew faster and had stronger roots than the controls. The expression of bacterial xylose (glucose) isomerase induces morphological changes in transgenic plants that correlate with changes in the expression of chloroplast genes involved in photosynthesis and maintaining the phytohormone balance. Thus transgenic plants of the XylA type represent a promising model system for studying photosynthesis as a function of phytohormone activity.

4. Conclusion

In plant functional genomics most approaches have introduced genes with a constitutive or inducible promoters, resulting in gene overexpression in transgenic plants. In some cases, however, it has been conferred by gene down-regulation by RNA interference, co-suppression or loss-of-function mutants. Each approach has advantages and disadvantages in different aspects of high-throughput characterization of gene functions. The first aspect is the basic construction strategy to produce a large population of mutant lines. The second aspect is whether mutants generated in each system can cover the vast numbers and wide variety of genes. Identification of all gene functions is the final goal of functional analysis at a genome level, and the production of mutants for this purpose. The final aspect is the enhancement of endogenous gene expression with tissue specificity. Highthroughput functional genomics is helping to shift the focus from the characterization of individual gene functions to a more systems-based holistic or synthetic approach to understand the genetic mechanisms that underlay gene regulation and complex signaling networks.

5. Acknowledgment

This work was supported by grant from Russian Academy of Sciences ("Biodiversity program"), and grant Russian Foundation for Basic Research (grant # 10-04-01195-Б).

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Transgenic Plants as Gene-Discovery Tools

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1. Introduction

Mutation study is an important strategy to dissect gene functions. Classical chemical or irradiation mutagenesis is one of the most powerful screen approaches to uncover genes involved in certain genetic pathways. However, the tedious works to hunt down the gene corresponding to a mutant allele by map-base cloning make an intrinsic limitation of this approach. Loss-of-function mutations resulting from Transferred DNA (T-DNA) insertion or Mobile Genetic Elements (MGE) insertion have overcome this shortcoming. Transgenic approaches are also useful to circumvent the difficulties in the study of gene function because of genetic redundancy or lethality. Gain-of-function mutations achieved by activated expression of endogenous genes by transcription enhancer or by specific gene over-expression through transformation have also revealed function of many plant genes. This review will describe the major loss or gain-of-function mutagenesis approaches by T-DNA vector transformation or endogenous MGE and those available insertion mutant resources which have greatly facilitated the researchers to extensively identify gene functions in the past few years.

2. Loss-of-function mutations

The most conventional tool for the functional analysis of all genes in a certain organism is to generate indexed loss-of-function mutagenesis on a whole genome scale. For example, the creation of gene-indexed loss-of-function mutations for all genes has been achieved decades ago in the unicellular budding yeast *Saccharomyces cerevisiae* by gene replacement via homologous recombination (Ross-Macdonald et al. 1999; Winzeler et al. 1999; Giaever et al. 2002). However, genome wide gene disruptions with confirmed index information are not so easy for the multi-cellular eukaryotes. Loss-of-function can be achieved by chemicals

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such as ethyl methannesulfonate (EMS) or by high energy irradiation such as fast neutrons to introduce random mutations (Ostergaard and Yanofsky 2004). These methods are easy to generate large scaled mutagenesis, but the mutation site identifications by traditional forward genetic method need tedious work. Although popular to individual research groups who focus on certain aspect of their interested field, such kinds of methods are not convenient for systematic research of gene functions on a genome wide scale.

Loss-of-function mutations achieved by T-DNA insertion or MGE insertion provide practicable methods to identify the genes disrupted by these transfer elements because their insertion sites can be explored by PCR base methods such as tail-PCR, plasmid rescue, inverse PCR or adapter PCR (Liu et al. 1995; Liu and Whittier 1995; Spertini et al. 1999; Yamamoto et al. 2003). *Arabidopsis* is currently the only multi-cellular organism reported possible to achieve saturation of mutation with accurate index for each genes because *Arabidopsis* owns several crucial advantages than other species: genome with small size, easily to be transformed, self-pollination, short life cycle and bulk storage of seeds. Rice is the second better learned species that has been widely used for establishing systematic insertion mutant libraries because rice is one of the most important crops and easy to be transformed by *Agrobacterium* mediated transformation. So this review will introduce the basic strategies of the major transgenic approaches using *Arabidopsis* and rice as model plants and summary the emerged transgenic plant resources which have become the most powerful gene discovery tools in the plant kingdom.

2.1 Saturated T-DNA insertion

The ultimate goal of genome research is to characterize the function of all the genes. As the major step towards this goal, the genome sequencing projects of several plant species were already completed. However, functional genomics studies are still in progress to deduce the functions of all the sequenced genes. Saturation mutagenesis by T-DNA insertion is one of the most successful approaches for analysis of systematic gene functions in the past decade years benefitting from the continuous improvement of transgenic techniques (Parinov and Sundaresan 2000). The *Agrobacterium* vacuum infiltration method for *Arabidopsis* transformation was developed to avoid the complex transgenic process of tissue culture based method including introduction of DNA by particle bombardment or *Agrobacterium* and plant regeneration (Bechtold 2003). This method was further modified and the transgenic *Arabidopsis* can be easy achieved by simply dip the floral tissue into a solution containing sucrose, surfactant Silwet L-77 and *Agrobacterium tumefaciens* carrying target genes (Clough and Bent 1998). The basic scheme for the generation of T-DNA insertion mutants by flora dip method for *Arabidopsis* is shown in Figure 1. *Agrobacterium* harbouring the binary vector was used to transform *Arabidopsis* by floral dip method. The T-DNA region between left border (LB) and right border (RB) of the binary vector was randomly transformed into the genome of host plant. The transgenic plants can be screened by selection marker depends on the T-DNA vector used (For example: *NPTII* is the selection marker of pROK2 T-DNA vector which is used to generate Salk insertion mutant lines). The flanking sequence at both sides of insertion can be further sequenced by PCR based method.Taken the advantage of flora dip method, a vast number of T-DNA insertion lines have been generated which represent almost saturated insertions into *Arabidopsis* genes in past ten years.

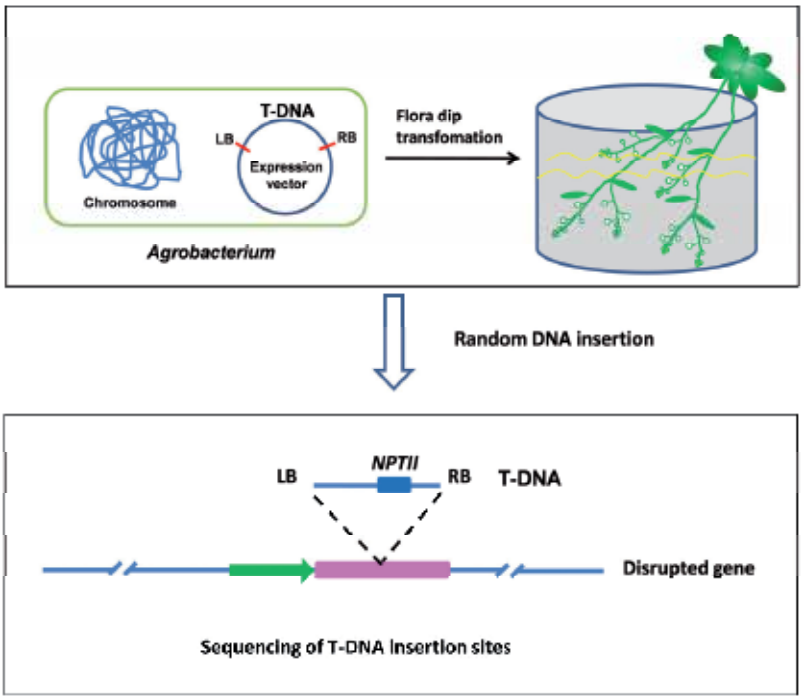

Fig. 1. A basic scheme for the T-DNA insertion method by flora dip in *Arabidopsis*.

2.2 The enhancer trap system

Besides to disrupt the gene functions by direct insertion for large-scale discovery of gene function, T-DNA can also be utilized to identify novel regulatory elements. As a further development of T-DNA random insertion strategy, the enhancer trap system is established by random integration of a report gene cassette as the T-DNA into the genome. This reporter cassette includes a minimum or truncated promoter which is not able to drive the expression of the report gene unless activated by the endogenous regulatory sequence close to the integration site (Figure 2). Therefore, the expression of reporter gene implies the existence of enhancer element close to the insertion site. Because the T-DNA cassette of enhancer trap system can both generate gene mutations and report the presence of enhance element around the insertion site, the enhancer trap system was widely applied in the bacterium, Drosophila, *Arabidopsis*, moss and rice to unveil gene functions and identify regulator elements (Casadaban and Cohen 1979; Sundaresan et al. 1995; Bellen 1999; Campisi et al. 1999; Hiwatashi et al. 2001; Zhang et al. 2006).

As a successful example, the Rice Mutant Database (RMD) is established with the enhancer trap system and maintained by National Center of Plant Gene Research (Wuhan) at Huazhong Agricultural University. The enhancer trap T-DNA fragment used by RMD carries three critical components as indicated in Figure 2:

a. -48 CaMV minimum promoter. This truncated promoter is unable to drive gene expression unless there is a transcription enhancer element close to it.

b. A recombinant DNA sequence encoding an artificial transcriptional activator by the combining of GAL4 and VP16. GAL4 is a DNA bind domain which specifically binds to the Upstream Activator Sequence (UAS) and V16 is a transcriptional activator domain derived from a herpesvirus protein (Utley et al. 1998) which is able to activate the expression of gene adjacent to the UAS.
c. The *β-Glucuronidase* (*GUS*) report gene downstream of the 6 tandems UAS (6xUAS).

The enhancer trap T-DNA including these three components is randomly integrated into host genome by *Agrobacterium*-mediated transformation. If the integration site is by chance neighbour to a host transcriptional enhancer element, the -48 CaMV minimum promoter will drive the expression of the GAL4/VP16 recombinant transcriptional activator which will then bind to 6xUAS and activate the expression of the GUS report gene. Some of the enhancer elements regulate spatial- or temporal gene expression and thus the transgenic lines with the T-DNA inserted near these enhancer element show expression of the report gene in a tissue specific pattern. Besides causing gene mutations and providing an efficient approach for identification of the transcriptional enhancer, enhancer trap T-DNA can create certain pattern lines which are useful to ectopic express target genes in certain tissues simply by cross the pattern lines with the target lines which are transformed by the target genes driven by 6xUAS. Figure 2 shows the basic scheme for the enhance trap system: The T-DNA of enhance trap vector includes -48 CaMV promoter, *GAL4/VP64* transcription activator and *GUS* reporter under control of 6xUAS. The transgenic lines were obtained by *Agrobacterium* mediated transformation of rice callus. The enhance trap T-DNA was randomly integrated into the rice genome and the enhancer nearby the insertion site activates the expression of *GAL4/VP64* which further promote the expression of *GUS* report gene in the Pattern line. The Target line was created through the transformation of host plant with T-DNA containing 6xUAS :: *Target gene*. By crossing Target line with Pattern line, the target gene will express in the same pattern as that of the *GUS* report gene due to the trans-activation of 6xUAS regulator by GAL4/VP64 transcription activator.

2.3 Mobile Genetic Elements (MGE) insertion

In addition to T-DNA insertion lines, MGE insertion is also a popular approach to generate large number of mutations. MGEs which can move around within the genome include several kinds of mobile DNA elements such as transposon or retrotransposon. Transposons describe the DNA which can be cut away from one site and paste to other place within the genome. Retrotransposon however, make themselves a copy and then paste to other position within the genome.

Several transposable elements identified in maize have been used to obtain large population of insertions in genes for functional genomics studies. For example, the maize transposable element *Activator* (*Ac*) first identified by McClintock (Mc 1950) is a kind of transposon widely used for creating MGE insertions. *Ac* element can insert themselves into genes and cause insertion mutations to create a recessive allele. The mutations caused this way are unstable because the *Ac* element can be excised from the inserted gene by the transposase which is coded by Ac element itself. *Dissociation* (*Ds*) element is usually stable because they are incapable of excising itself from the inserted gene unless with the help of *Ac* element.

Researchers combine these two mobile elements and named it as the *Ac/Ds* system to generate mutant populations. Generally, the individual *Ds* parental lines and *Ac* parental lines are created by transformation of *Ac* element and *Ds* element independently into the host organism. Then the two parental lines are crossed to induce the translocations of the *Ds* element in the next generation. For example as shown in Figure 3, *Ac* parent line and *Ds* parent line are created by transformation of host plants with the *Ac* element and *Ds* element respectively. By crossing *Ac* parent line and *Ds* parent line, *Ds* element is activated by *Ds* element to transfer from one position to another position within the genome which will create random disruption of gene functions in the following generations. Stable *Ds* insertion mutant lines can be created by genetic method to make *Ac* element segregated away with *Ds* element by combination of a positive selection marker on *Ds* element and a negative selection marker on *Ac* element (Sundaresan et al. 1995). Besides in maize, the *Ac* element has shown translocation activity in *Arabidopsis* (Fedoroff and Smith 1993). With the *AC/Ds* or other similar MGE systems, several research groups have generated mutant resources with a high proportion of single-copy transposon insertions (Sundaresan et al. 1995; Martienssen 1998; Tissier et al. 1999; Ito et al. 2002; Kuromori et al. 2004; Ito et al. 2005; Nishal et al. 2005)

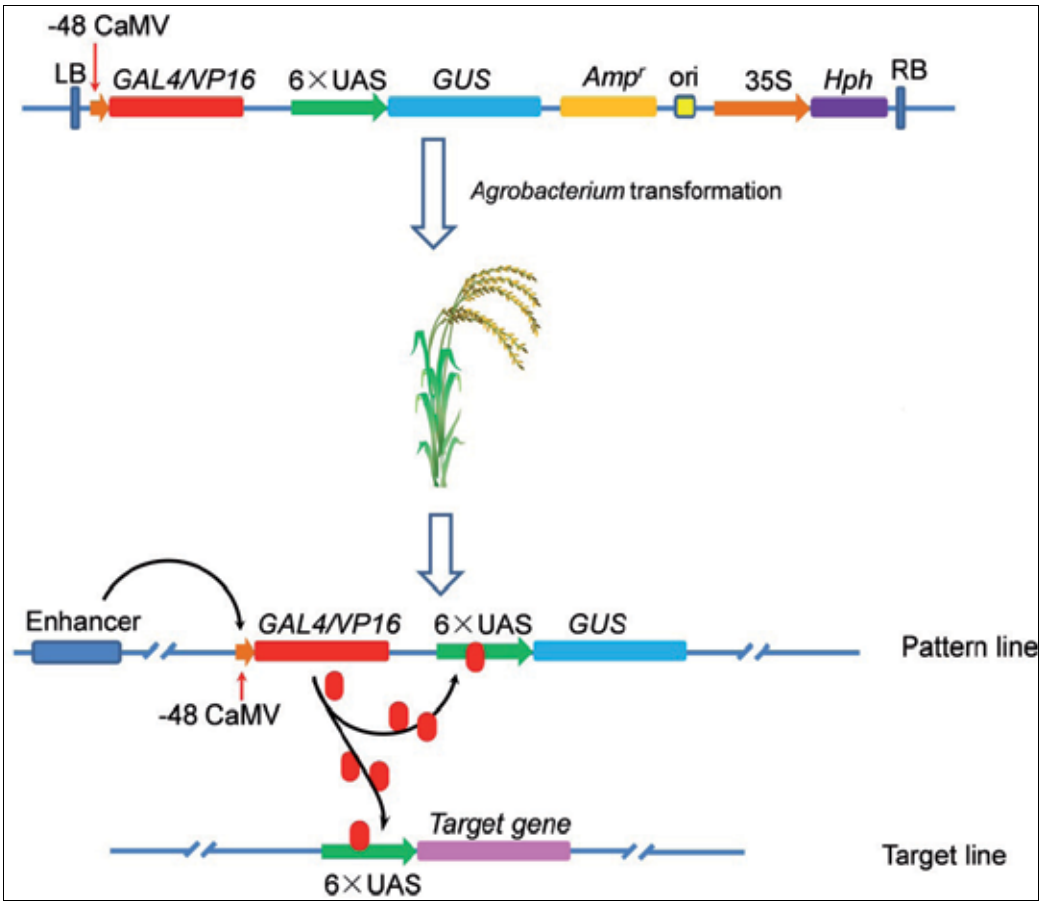

Fig. 2. Overview for the enhance trap system used by RMD.

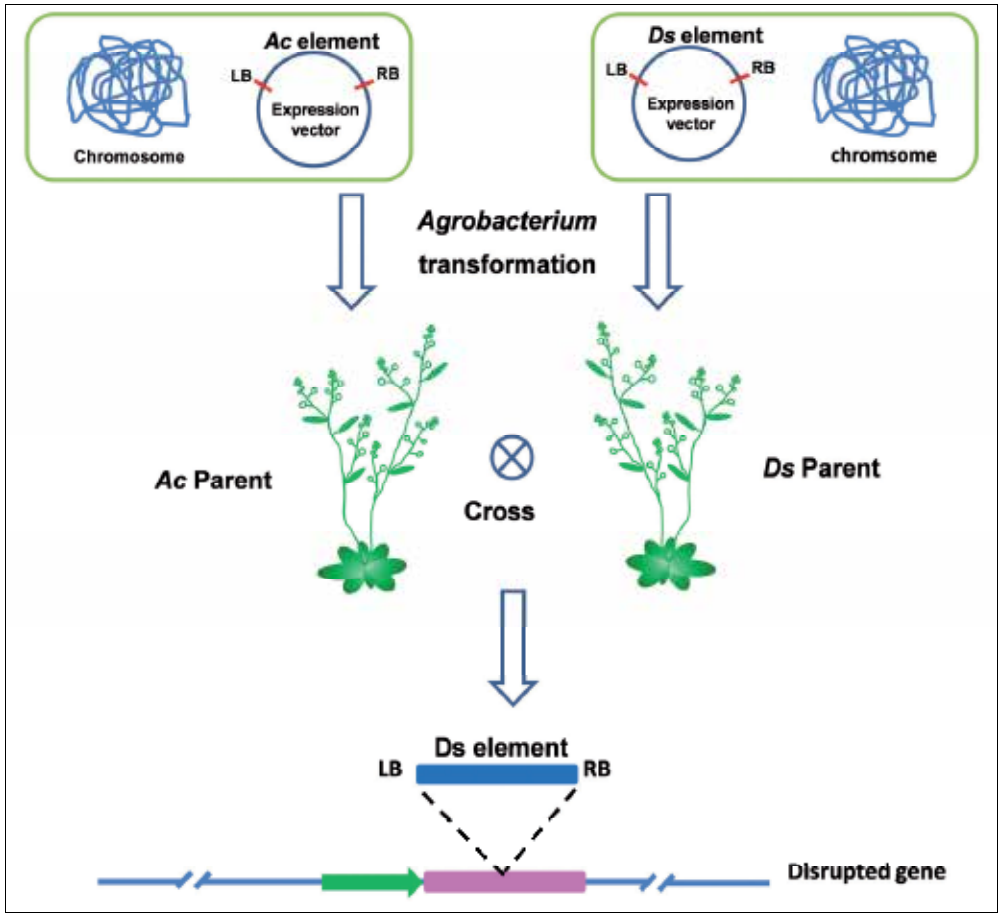

Fig. 3. Scheme for the generation of insertion mutant lines by *Ds* transposon.

Tos17 is one kind of copia-like retrotransposons in rice (Hirochika et al. 1996) ,which can duplicate and paste to elsewhere in the genome. *Tos17* owns several special features that make it suitable for engineering large scale insertion mutagenesis:

a. The copy number of *tos17* is quiet low, ranging from one to five among rice cultivars. For example, the genome of cv. Nipponbare, the selected cultivar for the IRGSP (International Rice Genome Sequencing Project)(Sasaki and Burr 2000), contains only two native copies of *tos17*.
b. Transposition of tos17 is inactive under normal conditions but only activated in the callus by tissue culture and then becoming stable again in the regenerated plants (Hirochika et al. 1996; Miyao et al. 2003).
c. The transposition site of *tos17* prefers gene-dense regions over centromeric heterochromatin regions with a three times higher insertion frequency in genic regions than in intergenic regions (Hirochika et al. 1996; Hirochika 2001; Miyao et al. 2003; Piffanelli et al. 2007).
d. Its size is just a little bit over 4kb and its insertion sequence is clearly known for flanking sequencing.

Tos17 is stably present in genome during the normal life cycle of rice. By the tissue culture of the rice callus, the transcription of *tos17* is activated and the reverse transcript DNA fragments are integrated into new places in the genome which creates disruption of genes. The original *tos17* and its duplications become silence again in the regenerated insertion mutant plants (Figure 4). Taking these advantages of *tos17*, Large-scale *tos17* T-DNA mutant library of Nipponbare has been established by tissue culture and stably preserved by normal generation (Miyao et al. 2003; Sallaud et al. 2004; Miyao et al. 2007; Piffanelli et al. 2007).

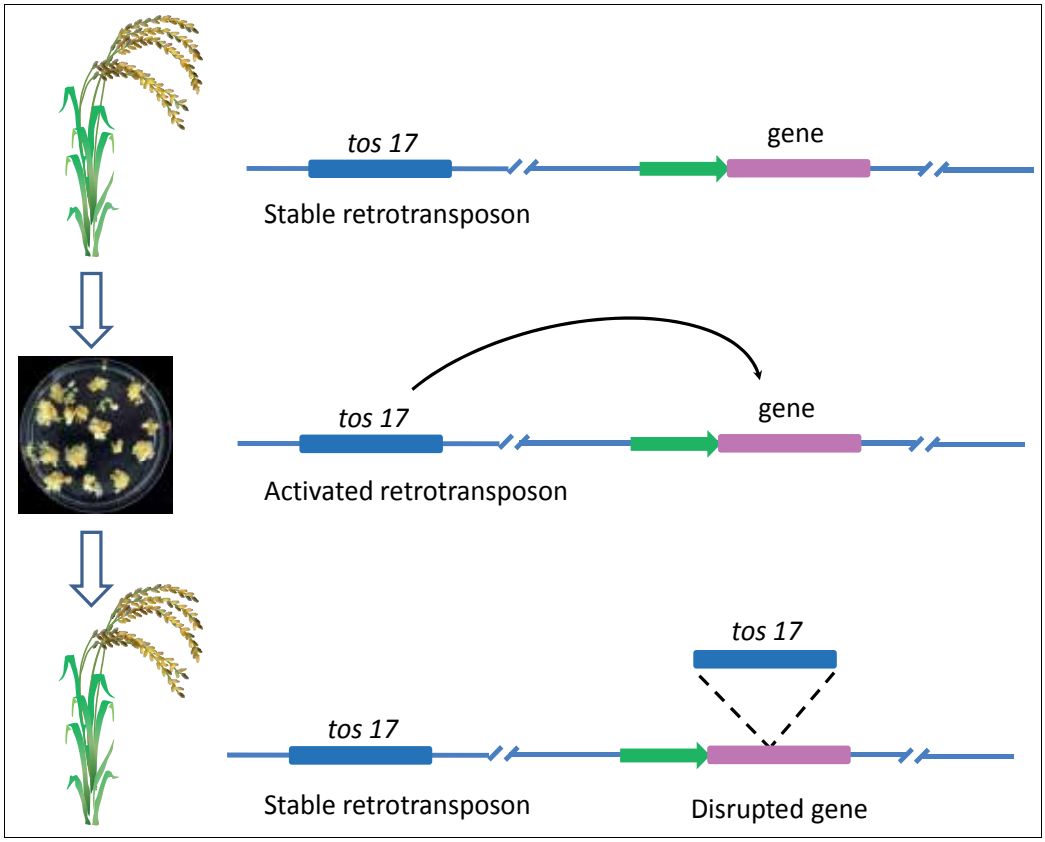

Fig. 4. Scheme for the generation of *tos17* insertion mutant lines by tissue culture.

3. Gain-of-function

Screen for loss-off-function mutations is a primary tool for dissecting a genetic pathway. However, because many genes belong to gene families, loss-of-function screens are not always possible to identify genes that act redundantly. In addition, some genes are critically required for the survival of plants. The homozygote mutants of these genes will not be available for entire function research because of embryonic or gametophytic lethality. As another option, gain-of-function technologies were developed to compensate the limitations of loss-of-function approaches or confer new function in transgenic plants, which is achieved through activation expression of endogenous genes by transcription enhancer which is randomly introduced into the genome or through ectopic gene over-expression driven by constitutive promoter.

3.1 Activation tagging

Activation tagging is a gain-of-function method that generates transgenic plants by T-DNA vectors with tetrameric cauliflower mosaic virus (CaMV) 35S enhancers which can lead to an enhancement expression of adjacent genes in the distance ranging between 0.4 to 3.6kb from the insertion site (Weigel et al. 2000). Differently from the action of the complete CaMV 35S promoter, CaMV 35S enhancers can activate both the upstream and downstream gene transcription. In addition, it has been reported that in at least one case, rather than led to constitutive ectopic expression, CaMV 35S enhancers elevate transcriptional activity based on the native gene expression pattern (Weigel et al. 2000).

Activation tagging technique was firstly developed by Walden and colleagues in decades years ago (Hayashi et al., 1992). Since then, several large scale activation tagging mutant resources have been generated and activation tagging method was widely used to isolate new genes. As an early example, the activation-tagging technique was used in tissue culture to identify cytokinin-independent mutants in *Arabidopsis* and *CKI1* gene whose overexpression can bypass the requirement for cytokinin in the regeneration of shoots was identified (Kakimoto, 1996). Based on the original activation tagging vectors, Weigel and colleagues (2000) developed new generation of vectors possessing resistance to the antibiotic kanamycin or herbicide glufosinate which is low toxic to humans or easy to select transgenic plants in soil in large scale. By screening a set of the transgenic lines, they identified 11 dominant mutants with obviously morphological phenotypes and 9 of them were confirmed due to the activation of adjunct genes by reproducing the phenotype in a new set of transgenic lines through overexpression of the adjunct candidate genes on both sides of insertion.

To accelerate the recapitulation process of phenotype resulted from the enhancer of T-DNA insertion, a new activation-tagging method has been developed using a pair of plasmids including pEnLOX and pCre. pEnLOX contains multimerized CaMV 35S transcriptional enhancers flanked by two *lox P* sites on both sides while pCre includes the *cre* gene which can remove the DNA sequence between two *lox P* sites (Pogorelko et al. 2008). the activation-tagging lines containing the pEnLOX were named the E-lines, and the helper lines containing pCre was named the C-lines. By crossing the E-lines with the C-lines, the CaMV 35S enhancers can be removed from the chromosome coming from E-lines and thus the reversion from mutant phenotypes to the wild-type phenotype may be detected in the next generation.

Activation tagging has also been applied to generate rice activation-tagging lines (Jeong et al. 2002; An et al. 2005; Jeong et al. 2006; Hsing et al. 2007; Wan et al. 2009). Based on the basic activation tagging technology, a dual function T-DNA vectors have been developed for both promoter trapping and CaMV 35S enhancers activation tagging(Jeong et al. 2002). By analysis of the gene expression in these rice activation tagging lines, the authors reported the activated the expression of genes located up to 10.7 kb from insertion site of the enhancers (Jeong et al. 2006). The activation tagging vector pSK1015 is used as an example in Figure 5. The T-DNA contains *BAR* gene as transgenic plant selection marker and 4×35 enhancers as activation element. The activation tagging T-DNA is integrated into the genome of host plants by *Agrobacterium* mediated transformation. The expressions of both side genes around the inserted T-DNA enhancer are elevated.

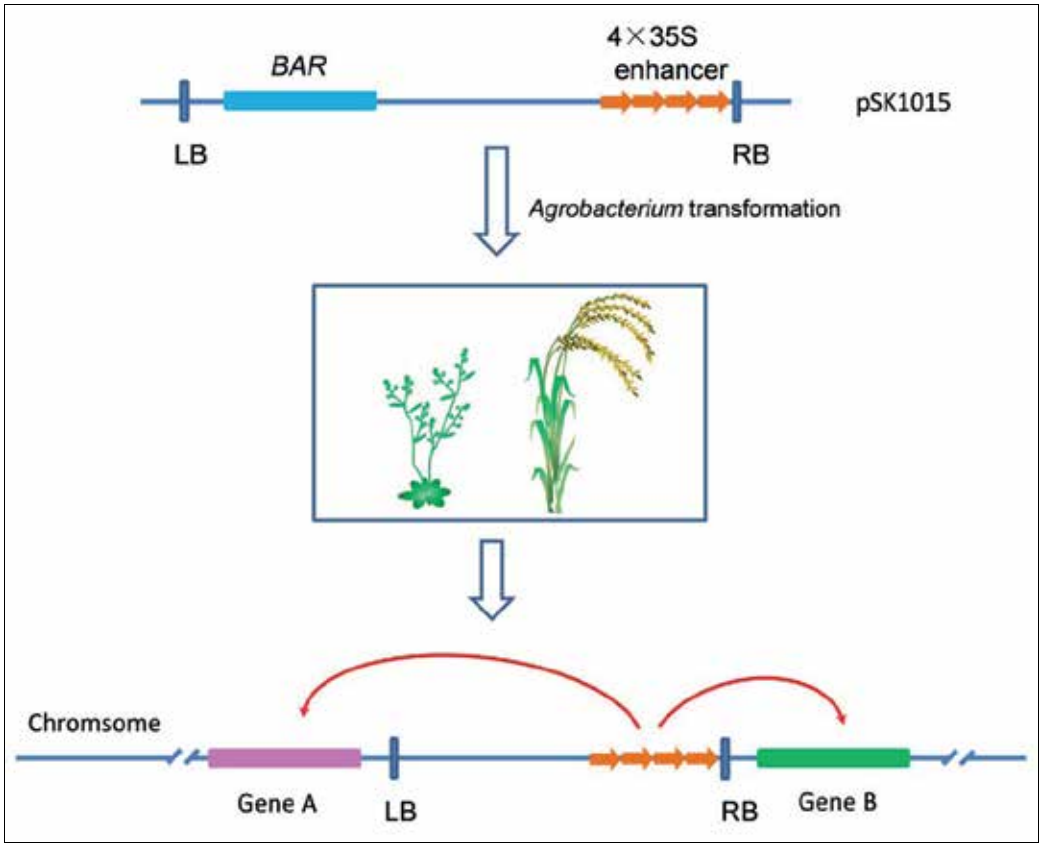

Fig. 5. Scheme for the generation of activation tagging transgenic plants.

3.2 Fox hunting system

FOX hunting system (full-length cDNA overexpressor gene hunting system) is used to ectopic expression of full-length cDNAs (fl-cDNA) in plants to generate systematic gain-of-function mutant populations (Figure 6). The most significant difference between FOX hunting system and other transgenic gene discovery methods is that FOX need to construct a large number of expression vectors containing as many as possible the independent fl-cDNAs, while other methods generally depend on one or a small number of expression vectors. Although the construction of numerous vectors seems labour-intensive, FOX hunting system shows unique benefits in discovery of new gene functions and potential utility of heterologous genes in improvement of agronomic traits.

Firstly, FOX hunting system can be flexibly used to systematically research of gene functions by generation of transgenic plants (also named FOX lines) expressed the fl-cDNA derived from the same species or heterologous host.

The first systematic gain-of-function transgenic population by FOX hunting system was produced by overexpression of *Arabidopsis* fl-cDNAs in *Arabidopsis* and these FOX lines showed various physiological and morphological phenotypes. Thus far, FOX approach has

been applied to generate several other FOX population using *Arabidopsis* or rice as model plants by the transformation of rice with rice fl-cDNA or the transformation of *Arabidopsis* with rice fl-cDNA. In addition, FOX hunting technology is an idea approach to systematically investigate the gene functions for those plants such as maize and wheat, the genome of which are too large or not easy to be sequenced.

Secondly, FOX hunting system is powerful to identify lead genes from relative or distant species to improve the traits of plants. It's interesting that the *Arabidopsis* FOX lines with ectopic expression of some rice genes that has no homolog gene in *Arabidopsis* also display abnormal phenotypes, which demonstrates that heterologous gene expression could introduce new function among different species. FOX approach has also been used to screen salt stress tolerance genes in salt cress (*Thellungiella halophila*). As a well know example of Monsanto's huge success by the similar approach, the gene (*BAR* or *PAT*) resistance to herbicide, firstly isolated from *Streptomyces* bacterial (Thompson et al. 1987), has been widely used to generate transgenic varieties of crops including canola, cotton, maize etc., for resistance to glufosinate which interferes with the biosynthesis of the amino acid glutamine and ammonia detoxification and causes cessation of photosynthesis. Therefore, this approach has the great potential to identify desirable genes to improve the agronomic trait of crop varieties. Figure 6 shows the basic scheme of FOX hunting system: Full length cDNAs were cloned into the FOX hunting over-expression vectors. Those individual vectors were transformed into host plants by mixed *Agrobacterium*. The full length cDNA over-expression cassettes were randomly inserted into the host genome and the ectopic expression of exogenous full length cDNAs can induce abnormal phenotypes.

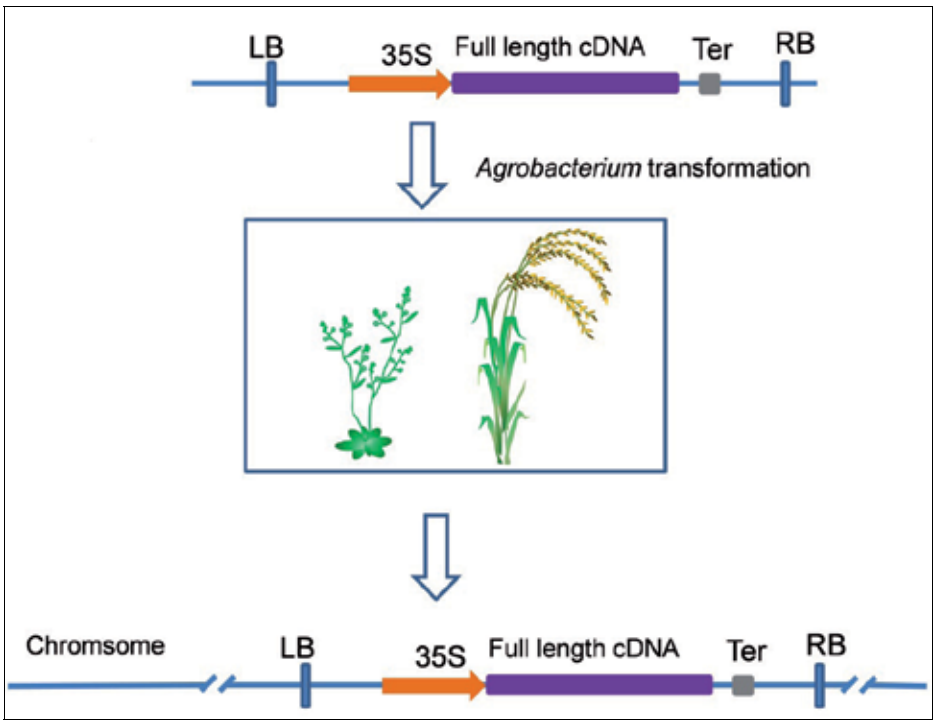

Fig. 6. Scheme for the generation of FOX hunting transgenic plants.

4. Summary of the transgenic resources

By reviewing the research progress in past few years, we are always impressed by that most of the basic scientific discoveries in plant field were using *Arabidopsis* or rice as the model plants. One of the significant reasons is that these two species are easiest to be transformed than other plants and thus the researchers can take the advantages of the public available transgenic resources to test their hypothesis in a relatively short period of time. In the following sections, we summarized the transgenic resources in Table 1 and described those representative ones for each of the methods. Some of the MGE insertion mutant lines are generated by endogenous transposon or retrotransposon rather than artificial transgene. Regarding those MGEs disrupt the gene functions in the similar way as T-DNA insertion and MGE mutant lines represent an important portion of genetics resources, we also introduce them briefly in this review for the interest of readers.

4.1 T-DNA insertion resources

SALK T-DNA insertion database is the most successful example in the history of transgenic resource so far. In 2003, over 225,000 *Arabidopsis* T-DNA insertion mutants have been generated by the SALK Institute, resulting in more than 88,000 insertions with precise locations determined in the whole genome of *Arabidopsis* (Alonso et al. 2003). Till recently, the numbers of T-DNA insert sites mapped to the genome of *Arabidopsis* by the Salk Institute have been over 150,000. Combined with the collections of other resources (SAIL, Wisc and GABI-KAT) around the world (Sessions et al. 2002; Rosso et al. 2003; Nishal et al. 2005; Li et al. 2007), totally 25,762 genes were identified with at least one T-DNA insertion mutation, which represent nearly 83% of the entire 31,128 protein-coding and non-coding RNA genes in the *Arabidopsis* genome (http://natural.salk.edu/geno/sum.txt). In addition, large-scale genotyping of those T-DNA insertion mutant lines has been ongoing for nearly 6 years to obtain as much as possible homozygous insertion mutants. As of today, 44,122 homozygote T-DNA lines, representing 24,476 individual genes have been sent to *Arabidopsis* Biological Resource Center (ABRC) for reproduction and distribution (http://signal.salk.edu/cgi-bin/homozygotes.cgi) (http://abrc.osu.edu/). The SALK Homozygote T-DNA Collection Project is nearly completed and this resource greatly facilitates the researchers to analyze the comprehensive phenotype and further understand the systematic gene functions at a genome wide level. Some other T-DNA insertion mutant resources are available for researchers to refer and utilize, which are listed in the Table 1.

4.2 Enhancer trap resources

The earlier Enhancer trap resource is reported in 1995 by Robert Martienssen and colleagues in the Cold Spring Harbor Laboratory (CSHL). They designed an ingenious strategy which combined the *Ac/Ds* MGE insertion method, Gene trap and Enhancer trap technology to generate a large DNA insertion population and 21,661 insertion events have been mapped to the genome of *Arabidopsis* (Sundaresan et al. 1995; Martienssen 1998). NASC established by Jim Haseloff and colleagues in the University of Cambridge contains 250 GAL4-GFP enhancer-trap lines which using GFP as report gene (Haseloff et al. 1997). Based on the similar strategy, the researchers in the Department of Biology, University of Pennsylvania established Enhancertraps database providing information of *Arabidopsis* lines transformed

with Jim Haseloff's GAL4 enhancer trap vector. This database currently release 510 enhancer trap transgenic lines that show specific expression pattern in multiple kinds of organs, tissues or cell type. RMD (Rice Mutant Database) is a successful enhancer trap resource of rice generated by Qifa Zhang and colleagues as describe in section 2.2. Since first released in 2006, approximate 132,193 T-DNA insertion lines generated by this enhancer trap system have been characterized with the flanking sequence of insertion, report genes expression or phenotype of transgenic mutation. RMD currently releases the comprehensive information of the large scaled transgenic lines including the flanking sequences of T-DNA insertion sites, seed availability, reporter-gene expression patterns, as well as mutant phenotypes, etc (http://rmd.ncpgr.cn/).

4.3 MGE insertion resources

Multiple MGEs identified in maize and some of them have been exploited to generate insertion mutant resources for maize as well as other plants such as *Arabidopsis*. For example, Exon Trapping Insert Consortium (EXOTIC) utilized both *Ac/Ds* system and *Enhancer/Suppressor-mutator* (*En/Spm*) system to create large insertion mutant populations and 23,537 insertion sites have been mapped to the genome of *Arabidopsis* (Tissier et al. 1999). Also using the *Ds* element, RIKEN BioResource Center (RIKEN BRC) built a resource containing 18,566 *Arabidopsis* mutants with insertion positions mapped to the genome. As an alternative, *tos17* retrotransposon characterized in rice is employed in mutational analysis of rice genome by several institutes such as National Institute of Agrobiological Sciences (NIAS)(Miyao et al. 2003) and Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)(Sallaud et al. 2004). Because other plant species are not easy to be transformed by direct trangene method, MGE insertion may become a more and more important strategy for the functional genomics studies of those plants other than *Arabidopsis* or rice.

4.4 Activation tagging resources

As a useful supplement of loss-of-function mutant resources mentioned above, activation tagging technique was developed and widely used to generate gain-of-function resources. Currently, there are 22,600 activation-tagging lines available in SALK and NASC (Weigel et al. 2000). RIKEN also established a large scale of activation-tagging lines with the number of 32,650 (Nakazawa et al. 2003). With the same method, 47,932 T-DNA tag lines in japonica rice were generated using activation-tagging vectors in POSTECH Rice T-DNA Insertion Sequence Database (RISD) (Jeong et al. 2006). Taiwan Rice Insertional Mutant (TRIM) created about 45,000 activation tagging lines using a T-DNA vector containing an enhancer octamer(Hsing et al. 2007). As a T-DNA insertion resource that using hosts plants other than *Arabidopsis* and rice, GmGenesDB in University of Missouri created 900 activation tagging lines of soybean.

4.5 Fox hunting resources

FOX hunting system was developed by a novel gain-of-function system that used normalized full-length cDNA introduced into host plants via large scaled transformation (Ichikawa et al. 2006). The first FOX hunting resource were established by transformation of

Arabidopsis with 10,000 *Arabidopsis* full length cDNA driven by 35S promoter, which contains more than 15,000 transgenic lines with 2.6 cDNA insertions on average in the genome. By a similar approach, they created 12,000 independent FOX hunting lines by transformation of rice with rice full length cDNA (Nakamura et al. 2007). The third FOX hunting system were created by transformation of *Arabidopsis* with the rice Full length cDNA for generating a heterologous gene resource which contain more than 23,000 independent *Arabidopsis* transgenic lines that expressed rice fl-cDNAs (Kondou et al. 2009).

5. Perspective

Take advantage of the dramatic improvements in transgenic efficiency, researchers are able to generate gain-of-function and loss-of-function transgenic resources with vast numbers of transformed *Arabidopsis* and rice plants. As a general procedure to utilize the loss-of-function resources, the individual researchers firstly order their interested insertion mutant lines by web based search of related database and then perform a PCR based genotyping to confirm the T-DNA insertion in their interested gene. Following a careful analysis of phenotype of the insertion homozygote lines, the researchers will confirm the abnormal phenotype of the mutants caused by T-DNA insertion via complementary experiment which is to transform the insertion mutant with a functional intact gene to rescue the phenotype. One of the common problems faced to the researches is that how should we do if the homozygote mutants show no obviously phenotype due to homolog gene redundancy or redundant genetic pathway to other gene with similar function. The first solution is to order all the redundant mutants and cross them to create double, triple or even quadruple mutant. If the redundant genes are correlated too close on the chromosome, it would be impossible to get the homozygote multiple mutants. Fortunately, other methods such as RNA interference, artificial micro RNA technology or TILLING (Targeting Induced Local Lesions in Genome) method are developed which can combine with the genetic cross method to create loss-of-function mutant of multiple gene loci (Schwab et al. 2006; Till et al. 2007; Warthmann et al. 2008). However, genetic pathway redundancies rather than homolog gene redundancies are not rare. In such case, the researchers will have neither homolog genes nor literature information to help them to figure out which gene loci should they use to create multiple mutant. New strategy may be developed to generate multiple site mutation resources for the researchers to screen obvious phenotype and further digest the complex genetic pathway.

To compensate the shortage of loss-of-function methods, gain-of-function transgenic can cause obvious phenotype even if the gene is redundant with other gene or the loss-of-function insertion is lethal. In addition, gain-of-function method has potential to discover useful genes which can be utilized to improve the agronomic traits of economic plants. For example, salt cress (*Thellungiella halophila*) is a very salt-tolerant species which are closely related to *Arabidopsis* (90-95% DNA sequence identity). In order to isolate salt stress tolerance genes, salt cress cDNAs under CaMV 35S promoter were transformed into *Arabidopsis* in a large scale and two genes ST6-66 and ST225 were discovered to improve the salt tolerance of *Arabidopsis* (Du et al. 2008). As another example described before, *Arabidopsis* FOX lines with ectopic over expression of rice specific genes in *Arabidopsis* also show abnormal phenotypes, further indicating that genes from various germplasms could

introduce novo functions. Actually, genes from distant species have already been successfully used to improve the agronomic traits of crops such as *BAR* gene used for weed control, or *CRY* gene (encoding Bt toxin in *Bacillus thuringiensis*) used for pests control (Thompson et al. 1987; Bravo et al. 2007). We speculate that gain-of-function methods will be further engaged to identify more useful genes to improve crop traits as an important goal of plant science.

	method	Resource	Host plant	Web site for the resource	Reference
Lose of Function	T-DNA insertion	SALK Institute, ABRC	*Arabidopsis*	http://signal.salk.edu/tabout.html	(Alonso et al. 2003)
		Syngenta, SAIL	*Arabidopsis*	N/A	(Sessions et al. 2002)
		WiscDslox	*Arabidopsis*	http://www.hort.wisc.edu	(Nishal et al. 2005)
		GABI	*Arabidopsis*	http://www.gabi-kat.de	(Rosso et al. 2003; Li et al. 2007)
		INRA, FLAGdb	*Arabidopsis*	http://urgv.evry.inra.fr/FLAGdb	(Samson et al. 2002)
		POSTECH	Rice	http://www.postech.ac.kr/life/pfg/risd/index.htm	(Jeon et al. 2000)
		SHIP	Rice	http://ship.plantsignal.cn/index.do	(Fu et al. 2009)
		TRIM	Rice	http://trim.sinica.edu.tw/	(Hsing et al. 2007)
		ZJU	Rice	http://www.genomics.zju.edu.cn/ricetdna.html	(Chen et al. 2003)
	Enhancer trap	CSHL	*Arabidopsis*	http://genetrap.cshl.org/	(Sundaresan et al. 1995; Martienssen 1998)
		NASC	*Arabidopsis*	http://arabidopsis.info/CollectionInfo?id=24	n/a
		University of Penn	*Arabidopsis*	http://enhancertraps.bio.upenn.edu/default.html	n/a
		RDM	Rice	http://rmd.ncpgr.cn	(Zhang et al. 2006)
	MGE insertion	EXOTIC	*Arabidopsis*	http://www.jic.bbsrc.ac.uk/science/cdb/exotic/index.htm	(Tissier et al. 1999)
		RIKEN	*Arabidopsis*	http://rarge.gsc.riken.go.jp/dsmutant/index.pl	(Ito et al. 2002; Kuromori et al. 2004; Ito et al. 2005)
		NIAS	Rice	http://www.dna.affrc.go.jp/database	(Miyao et al. 2003)
		OTL, CIRAD	Rice	http://urgi.versailles.inra.fr/OryzaTagLine/	(Sallaud et al. 2004)
		UCD	Rice	http://www-plb.ucdavis.edu/labs/sundar/Rice_Genomics.htm	(Kolesnik et al. 2004)

		CSIRO	Rice	http://www.pi.csiro.au/fgrttpub/	(Eamens et al. 2004)
		GSNU	Rice	N/A	(Kim et al. 2004)
		EU-OSTID	Rice	http://orygenesdb.cirad.fr	(van Enckevort et al. 2005)
		Maize GDB	Maize	http://www.maizegdb.org/rescuemu-phenotype.php	(Fernandes et al. 2004)
Gain of function	Activation tagging	SALK, NASC	*Arabidopsis*	http://arabidopsis.info/CollectionInfo?id=59	(Weigel et al. 2000)
		RIKEN	*Arabidopsis*	http://activation.psc.database.riken.jp	(Nakazawa et al. 2003)
		Plant Research International	*Arabidopsis*	N/A	(Marsch-Martinez et al. 2002)
		TAMARA	*Arabidopsis*	http://arabidopsis.info/CollectionInfo?id=71	(Schneider et al. 2005)
		NI Vaviliv Institute of General Genetics RAS	*Arabidopsis*	N/A	(Pogorelko et al. 2008)
		JIC activate line	*Arabidopsis*	http://arabidopsis.info/CollectionInfo?id=29	n/a
		RISD	Rice	http://www.postech.ac.kr/life/pfg/risd	(Jeong et al. 2006)
		TRIM	Rice	http://trim.sinica.edu.tw	(Hsing et al. 2007)
		GmGenesDB	soybean	http://digbio.missouri.edu/gmgenedb/index.php	(Mathieu et al. 2009)
	FOX hunting	RIKEN	*Arabidopsis*	http:// nazunafox.psc.database.riken.jp	(Ichikawa et al. 2006)
		RIKEN, NIAS,RIBS Okayama	*Arabidopsis*	http:// ricefox.psc.riken.jp	(Kondou et al. 2009)
		NIAS	Rice	N/A	(Nakamura et al. 2007)

Table 1. T-DNA or MGE insertion mutant resources (N/A, not available).

6. Acknowledgment

Research in the authors' laboratories is supported by the National Natural Science Foundation of China (Grant No. 31171352) and National Transgenic Crop Initiative (Grant No. 2010ZX08010-002).

7. References

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Transgenic Plants as Biofactories for the Production of Biopharmaceuticals: A Case Study of Human Placental Lactogen

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Spain

1. Introduction

Throughout human evolution, plants have provided us with food, fibers to produce clothes, and medicines to treat different kind of diseases. In fact, we could say that plants represent the "chemists" of the antiquity. Examples of important therapeutic molecules obtained from plants are morphine, atropine, ephedrine, codeine, and digitalin and so on (Farnsworth et al., 1985). Plants have always been a common source of medicaments, either in the form of traditional preparations or as pure active principles. For many people worldwide, natural plant-based remedies are used to treat both acute and chronic health problems. Indeed, about 40% of the drugs prescribed in the USA and Europe come from active compounds found in plants (Rates, 2001; Sivakumar, 2006). The chemical synthesis of these plant-derived compounds led to the production of pharmaceuticals at an industrial level, allowing the development of medicament industry. These active compounds from plants have been basically small molecules. Aspirin, one of the most famous medicaments, was developed as an analogous of the salicylic acid extracted from willow bark in the 19st century (Knäblein, 2005). Moreover, the development of more sophisticated extraction and purification procedures allowed the extraction of alkaloids (morphine) and other kind of molecules from plants and mammals (Liénard et al., 2007). However, chemical synthesis has some limitations to produce complex therapeutic molecules such as antibodies. So, many therapeutic molecules have to be isolated and purified from living material (e.g. from blood plasma), which involves a high risk of transmission of pathogens to the manufactured product (Engelhard, 2007).

The emergence of genetic engineering in the early 1970s has made possible a new way to produce pharmaceuticals outside their natural host. The term "biopharmaceutical" appears to be originated in the 1980s and is used to refer to therapeutic proteins produced by modern biotechnological techniques (Walsh, 2005a). Genetic transformation techniques have allowed the scientist to transform living organisms like bacteria, yeast, animal cells and plants into production "biofactories". These organisms are forced to produce customized therapeutics making possible the treatment of diseases like genetic disorders, AIDS, and diabetes. Unlike chemically produced therapeutics, biopharmaceuticals are our own molecules and hence more compatible with biological systems (Rai & Padh, 2001).

Biopharmaceuticals represent the fastest growing sector within pharmaceutical industry, with worldwide sales of $94 billion from the total market of $600 billion in 2007. In 2009, biopharmaceuticals recorded global sales of $99 billion ($61 billion for therapeutic proteins and $38 billion for monoclonal antibody (mAb)-based products (Walsh, 2010), and these numbers are expected to reach sales of $125 billion by the year 2015 (Xu et al., 2011). Regarding the most lucrative molecules, mAb-based products indicated for treating cancer are the bestsellers of biopharmaceuticals. The next most lucrative group is represented by insulin and insulin analogs, generating $13.3 billion in sales, followed by EPO-based products whose sales stands at $9.5 billion (Walsh, 2010).

The biopharmaceutical industry relies basically in non-human mammalian cell lines like CHO cells, and bacterial systems. In mammalian cell lines human proteins are correctly processed and modified, but the scalability is limited, maintenance of bioreactors is expensive, and there is a risk of contamination of the product with human pathogens (Daniell et al., 2001; Twyman et al., 2005). *Escherichia coli* was the pioneer expression system and in 1977 it was used for the successful expression of somatostatin (growth hormone-inhibiting hormone). *E. coli* is widely used because it is the most cost-effective production system, allowing the large-scale production of proteins (Rai & Padh, 2001). Insulin produced in *E. coli*, also known as "humulin", received the marketing authorization in 1982 which represented the beginning of the biopharmaceutical industry (Walsh, 2005b). However, the prokaryotic nature of this organism limits the complexity of the proteins that can be correctly processed, and the appearance of inclusion bodies increases the cost of the product (Boehm, 2007). Yeast-based systems are able to grow in well-defined simple media, in large-scale bioreactors and at high cell-densities (Gerngross, 2004). Therefore interferon was expressed in yeast (1981). However, the low product yields, inefficient protein secretion and hyperglycosylation of proteins (addition of large number of mannose residues) are common in problems in this system. Transgenic animals allow the scaling-up of protein production but they require long generation times, and have technical difficulties. Moreover, ethical aspects also limit this system (Hunter et al., 2005). Insect cell-based systems provide a eukaryotic expression system readily amenable to scale-up, but they are inefficient in proteolytic cleavage of proteins as well as for glycosylation (Rai & Padh, 2001). Nevertheless, β interferon and factor IX were expressed in insect cells (1983) and transgenic animals (1988), respectively (Desai et al., 2010).

Genetic engineering techniques have made it possible to produce therapeutic molecules using different host system. But what are the ideal characteristics that should satisfy an expression system to be considered a good system? It is important to (1) produce proteins with the correct conformation and biologically active, (2) allow good productivities, (3) have minor economic costs associated to manufacturing and purification, and (4) be recognized as safe system by the society.

Despite the variety of expression systems available for biopharmaceutical industry, none of them meets completely all the requirements for a production system to be able to produce any kind of therapeutic protein. There is no ideal or universal expression system. All of them have some advantages and some limitations, so the choice for the best system should be done by evaluating biological as well as economic aspects, and bearing in mind the purpose of the product of interest.

2. Transgenic plants as production platform for biopharmaceuticals

The total global market for biopharmaceuticals is projected to grow at between 7% and 15% annually over the next several years, with mAb-based approvals continuing to dominate (Walsh, 2010). Efforts on human genome project have identified new proteins with therapeutic potential and the growing number of candidates in clinical trials will be in the market in the next years. So it just keeps getting more apparent that the traditional systems used by the industry so far will not be able to meet the growing demand and will represent a bottleneck in bringing therapeutic proteins to the society. Pharmaceutical industry requires a production platform able to provide enough quantity of safe and high-quality products at the lowest cost. Transgenic plants are becoming one of the most interesting alternative systems for biopharmaceutical production as they offer many advantages over traditional systems.

One of the most important benefits of transgenic plants is the capacity to obtain high production volumes at relatively low cost, due to their flexibility in terms of scale-up (Sparrow et al., 2007). It has been estimated that the cost associated with the production of 300 kg of a secretory antibody in maize or tobacco plants is 0.5-2 million $ per year, while the cost increases to 6-7 million per year using transgenic goat or mammalian cell cultures (Gerlach et al., 2010). Transgenic plants allow the increase or decrease of cultivated area depending on the market demand. Moreover, the availability of harvesting, storage and transport infrastructures turns plants into a very efficient production system (Kusnadi et al., 1997).

As eukaryotic organisms, protein synthesis pathways are highly conserved among plants and animals, so plant cells are able to correctly fold and assemble proteins as well as to perform post-translational modifications required for protein activity and stability (Kamenarova et al., 2005). This is demonstrated by the ability of plant cells to produce various types of antibodies, such as IgGs and IgAs, which are complex proteins requiring the assembly of various polypeptide chains through disulphide bonds (Twyman et al., 2003). Apart from cultured mammalian cells, only plants are able to assembly the light and heavy chains of antibodies (Gomord et al., 2004). For this reason, amino acid sequences of human proteins expressed in plant cells are usually the same as native counterparts, which ensures the quality of products produced in plants (Schillberg & Twyman, 2007). Moreover, plant cells have a very high ratio of biologically active protein (92% of total protein in tobacco BY-2 cells) when compared to *E. coli* (12%) and *P. pastoris* (40%) (Boehm, 2007). Proteins produced in plant cells are less likely to be contaminated with human or animal pathogens and hence product safety can be guaranteed.

The diversity of plant host makes possible the production of oral vaccines in edible parts of the plant, eliminating the need of cold-chain and economic costs associated with purification processes, and thus being more amenable for developing countries. Moreover, tissues like seeds, tubers or fruits allow the storage and stability of biopharmaceuticals produced in plants (Desai et al., 2010).

One of the major challenges of plant systems, besides low protein expression level which will be discussed below is the inability of plants, as in other eukaryotic systems, to perfectly reproduce human-type glycosylation on biopharmaceuticals. Glycosylation is the most widespread post-translational modification with more than half of the human

biopharmaceuticals being glycoproteins. Glycosylation affects their function, plasma half-life and/or biological activity (Saint-Jore-Dupas et al., 2007). Main differences between plants and mammal cells are relative to modification of glycans in Golgi apparatus. Plants do not have sialic acid residues present in human glycoproteins, and add α (1,3)-fucose and β (1,2)-xylose residues which are responsible of immunogenic response in human therapy (Chen et al., 2005). However, there are some strategies available to "humanize" recombinant glycoprotein produced in plants. The first one is based on endoplasmatic reticulum (ER) retention of proteins in their biosynthetic pathway bypassing Golgi apparatus. This can be done through the addition on KDEL sequence in the recombinant protein (Gomord & Faye, 2004). The second one relies on inactivation or down-regulation of plant xylosyltransferase and fucosyltransferase using RNA interference technology. Alternatively it is possible to "humanize" glycoproteins co-expressing human glycosyltransferases (Gomord et al., 2004) or by *in vitro* galactosylation of recombinant proteins using mammalian enzymes (Bardor et al., 2003).

Another future challenge for plants is the concern about biosafety and regulatory issues. General public is very reluctant to transgenic crops, especially since the appearance of those resistant to pests or pesticides, and unfortunately transgenic plants for biopharmaceutical production must suffer the same rejection. The main biosafety problems are related to risks associated to human/animal health and to the environment, so it is very important to evaluate and manage carefully those risks through risk assessments (Peterson & Arntzen, 2004). There are many strategies available to avoid those problems: apart from delimitation of physic barriers and strict agricultural management (crop destruction, field cleaning, and crop rotation) to avoid mixing of modified and food/feed crops, the most obvious alternative is the use of non-food/feed crops (tobacco, *Arabidopsis thaliana*, *Phiscomitrella patens*, *Lemna* sp.), or the use of greenhouses/glasshouses where crops are confined and controlled. Biological containment provides a natural and additional barrier to gene flow. Production of biopharmaceuticals in self-pollinating species (rice, wheat, and pea) or engineering other species to make them cleistogamic (self-pollination before flower opening) is interesting for gene containment (Commandeur et al., 2003).

Biotechnological tools have allowed the development of different strategies for gene and protein containment. Chloroplast transformation technology is a very attractive approach as chloroplast genome is maternally inherited in most crops, and gene spread via pollen occurs at low frequency (Svab & Maliga, 2007). The use of male-sterile plants (Gils et al., 2008) or "terminator" technology (also known as GURTs, "Genetic use restriction technologies), which is based on a repressible system to produce plants with non-viable seeds unless the plants are exposed to specific activators (Lee & Natesan, 2006), have also been used to contain transgenes. Other strategies include organ- or tissue-specific expression of transgenes, ER-retention of proteins and inducible promoters (Obembe et al., 2010).

It is clear that taking into account the previously reported concerns regarding health and environmental risks, transgenic plants producing biopharmaceuticals must be regulated strictly, and in fact they are. However, there is a need for the harmonization of international regulation of plants producing biopharmaceuticals in European Union and USA (Gerlach et al., 2010) and to unify the criterions. Plants as production system for biopharmaceuticals comply not only with the strict regulatory requirements covering other GM crops, but also the regulations set out by agencies that oversee the production of pharmaceuticals (Sparrow et al., 2007).

Plants represent a very versatile and plastic expression system to produce biopharmaceutical proteins, because they offer a great variety of strategies. Depending on production needs, product quality, or compliance with certain legal requirements, we can choose between different plant-based systems without the need to use bacteria or animal cells.

The most used strategy for protein production is that based on whole plants. Most biopharmaceuticals are produced in nuclear transformed plants, which are obtained mainly by using soil pathogen *Agrobacterium tumefaciens,* and allow the stable expression of transgenes with the required post-translational modifications. Genetic transformation of chloroplast to obtain transplastomic plants constitutes a promising alternative strategy for protein production due to the important advantages over nuclear transformation. Proteins can achieve high expression levels due to high copy-number of transgene per cell. It has been reported an expression level of up to 31% of total soluble protein (TSP) for an animal vaccine produced in transplastomic tobacco plants (Molina et al., 2004). Chloroplast transformation is based on homologous recombination events so there is no gene silencing (Maliga, 2003). It is possible to integrate multiple genes in operons and, as previously mentioned, this system offers transgene containment through maternal inheritance (Bock & Khan, 2004). However, in the chloroplast it is not possible to carry out post-translational modifications present in the ER limiting the range of proteins that can be produced in this system. Against stable nuclear or chloroplast transformation, plants can be transiently transformed by agroinfiltration with *A. tumefaciens*, biolistic methods and viral vectors. This strategy is very useful for transformation construct verification and to test functionality of recombinant proteins (Fischer et al., 2004).

2.1 Plant cell cultures

As an alternative to whole plants, biopharmaceuticals can be produced using plant cell cultures. Plant cell culture is a very interesting and promising alternative system and has been used for almost two decades for protein production, as well as for secondary metabolite production.

Suspended cell cultures are derived from callus, which are unorganized and generally undifferentiated cell aggregates derived from plant tissues cultured in solid media supplemented with growth regulators. Calli are suspended in liquid media to form a homogeneous suspension. Transgenic suspension cultures can be obtained from wild type callus or suspensions transformed by *A. tumefaciens* or biolistic methods, depending on plant species, but also from transgenic plant tissue such as leaf or stem. With the second approach there is no need for further genetic manipulation like transformed tissue selection and line screening (Hellwig et al., 2004).

Since 1990, when Sijmons et al. reported the expression of human serum albumin in transgenic tobacco suspension cells, a diverse array of biopharmaceuticals have been produced using suspended cell cultures. Huang & McDonald (2009) reported an extensive list of recombinant proteins produced in this system, including human erythropoietin, human granulocyte-macrophage colony-stimulating factor (hGM-CSF), human interleukins, hepatitis B surface antigen, and many types of antibodies. Although productivity of plant cell cultures can vary considerably from 0.5 μg/L to 200 mg/L (Hellwig et al., 2004), many proteins have been produced at high yields (>10 mg/L) (Xu et

al., 2011). Most of biopharmaceutical proteins have been expressed in tobacco cells like well characterized BY-2 and NT-1 cell lines, because these host lines are fast-growing, well synchronized and susceptible to *Agrobacterium* mediated transformation (Nagata & Kumagai, 1999). Other host plants used include rice, an emerging host specie (Kim et al., 2008), sweet potato (Min et al., 2006), tomato (Kwon et al., 2003), and carrot (Shaaltiel et al., 2007).

Suspended cell cultures offer many advantages over whole plant systems, and perhaps one of the most important is that *in vitro* plant cultures, as grown in confined and sterile conditions, avoid the political resistance to release of genetically modified plants to the field. Moreover, confined culture eliminates problems related to weather conditions, soil quality, season, plagues, and contamination with agrochemicals and fertilizers that affect field grown plants (Weathers et al., 2010). This is an ideal characteristic for production of high purity biopharmaceuticals. Whole plant systems require longer period of time to be productive (sowing, growing, harvesting) while proteins could be manufactured in days or weeks on a time-scale compatible with that of market demands (Doran, 2000). Growth in bioreactors not only allows the reduction in production cycles, but also the scalability and the tight control of growth parameters (temperature, O_2 supply, agitation, pH), ensuring product quality and batch-to-batch consistency, as well as product traceability (Ma et al., 2005). These features make the system amenable to good manufacturing processes (GMP), facilitating compliance with regulatory and environmental requirements (Spök et al., 2008). Bioreactor technology provides a great variety of culture modes and bioreactor types to increase recombinant protein production. Large-scale culturing can be done in standard stirred-tank bioreactor, pneumatic bioreactor (bubble column or air-lift), wave bioreactor, membrane bioreactor, hollow fiber bioreactor or miniature bioreactor (Huang & McDonald, 2009). Regarding to operation modes, we can choose between batch culture, fed-batch culture, continuous and semi-continuous culture, and perfusion culture (Xu et al., 2011). Choosing a suitable bioreactor type and operation mode should include adequate oxygen mass transfer to cells, low shear stress to cells, and proper nutrient supply to cells and product removal from cells (Huang & McDonald, 2009).

The potential of suspended cells to secrete biologically active proteins into the culture medium has a big impact on downstream processing costs. Cells are grown in relative simple and synthetic protein free media which facilitates the recovery of proteins (Hellwig, 2004). Protein secretion is determined by the presence of signal peptide and protein size to pass through wall pores. Due to their undifferentiated nature, callus cells lack functional plasmodesmata, and as grown in suspended form, there is minimal cell-to-cell communication (Su, 2006). All those features can reduce post-transcriptional gene silencing (PTGS) because signal transmission between cells is avoided (Doran, 2000). In conclusion, plant cell suspension cultures integrate many of the advantages of whole plant systems with those of microorganisms and mammalian cell cultures.

In contrast to suspended cells, plant cells can also be immobilized for biopharmaceutical production. This system is based on the immobilization of cells using encapsulating gels, such as alginate, which protect cells against mechanical damages. Immobilization also facilitates the re-use of cells in continuous or semi-continuous culture, allowing higher inoculums than standard methodologies (James, 2001).

Differentiated organs such as hairy roots and shooty teratomas have also been developed for protein production. Hairy root cultures have been used for decades to produce secondary metabolites such as resveratrol (Medina-Bolivar et al., 2007). To generate hairy roots, wounded transgenic host plant is co-cultivated with *Agrobacterium rhizogenes* which transfers *rol* and *aux* genes which are responsible of root phenotype and induction (Sivakumar, 2006). Hairy root cultures, grown in hormone-free medium, are genetically stable over time and allow uniform expression of proteins at high level in relatively short periods of time (Franconi et al., 2010). Secretion of proteins is also allowed in root cultures which can be cultured in bioreactors or in hydroponic tanks from where they take water and nutrients while releasing proteins continuously (Knäblein, 2005). Shooty teratomas are generated from transgenic seedlings co-cultured with *A. tumefaciens* strain T37. After co-culture and Agrobacteria elimination, shoots are cultured in liquid medium (Sharp & Doran, 2001).

2.2 Emerging plant-based systems

In the last decade alternative novel expression systems have been developed for the production of biopharmaceuticals. We would like to highlight the potential of novel systems such as mosses, algae and aquatic plants which can be cultured in contained conditions in bioreactors.

Mosses as multicellular eukaryotic organisms can produce biologically active therapeutic proteins. *Physcomitrella patens* is the model organism which has been studied for a long time. It can be cultured during its complete lifecycle, but when cultured in liquid medium vegetative growth is favored (Franconi et al., 2010). The fact that *P. patens* is grown in small plant fragments, and not as protoplast, provides genetic stability avoiding somaclonal variation (Knäblein, 2005). The moss is photoautotrophic and only requires inorganic salts, water and CO_2 for growth, so it is easily cultured in stirred glass tanks or tubular photobioreactors. The most interesting feature of *P. patens* is its high frequency of homologous recombination which facilitates the precise knockout of genes (Decker & Reski, 2007). In this context, genes for α(1,3)-fucosyltransferase and β(1,2)-xylosyltransferase were disrupted by homologous recombination to obtain double knockout clones of *P. patens* with no allergenic N-glycans (Koprivova et al., 2004). The potential of this species for biopharmaceutical production gave rise to the company Greenovation in 1999. Since 2001, the company is developing the "bryotechnology", using double-knockout strain of *P. patens* cultured in photobioreactors. Currently this company is producing a variety of biopharmaceuticals such as growth factor (VEGF), serum proteins (HSA), peptide hormones (EPO), enzymes (phosphatase), vaccines and a wide range of oncology mAb´s in 100L tubular bioreactors and 200L disposable bag systems (www.greenovation.com).

Microalgae represent a diverse group of prokaryotic (cyanobacteria) and eukaryotic photosynthetic microorganisms that are found in marine and freshwater environments. Microalgae combine simple and inexpensive growth requirements and ability for post-transcriptional processing of proteins, with the rapid growth rate and potential for high-density culture (Walker et al., 2005). Most green algae are classified as generally regarded as safe (GRAS), making processing of expressed products more amenable to regulatory issues (Potvin & Zhang, 2010), and offering a safe platform for vaccine production. Microalgae can be grown in contained bioreactors in a matter of weeks from initial transformation event to

large-scale protein production, and as single cell type culture, there should be less variation in protein accumulation, making downstream processing more uniform (Specht et al., 2010). However, light utilization and distribution in the bioreactor represents a limiting factor for cell growth which requires careful design of bioreactors. In this regard, heterotrophic growth using glucose as carbon source should be advantageous for well established "dark" bioreactors allowing high cell density (Franconi et al., 2010). *Chlamydomonas reinhardtii* is the most successfully used microalgae, because it is genetically well characterized with all three genomes (the nuclear, chloroplast and mitochondrial) sequenced, and genetic transformation methods are well established (Rasala & Mayfield, 2011). Proteins such as human antibodies (Mayfield & Franlikn, 2005), human glutamic acid decarboxylase 65 (hGAD65) (Wang et al., 2008), domain 14 of fibronectin (14FN3), VEGF and HMGB1 (Rasala & Mayfield, 2011) have been produced in *C. reinhardtii*.

Finally we would like to pay attention to aquatic higher plants from *Lemnaceae* family such as those from *Lemna*, *Spirodela* and *Wolffia* genus. These edible plants are safe, fast-growing, and easy to grow and harvest species amenable to genetic transformation using *A. tumefaciens* or biolistic method (Weathers et al., 2010). *Lemna minor* has been used to produce mAb´s fused with an RNA interference construct targeting the expression of endogenous genes α(1,3)-fucosyltransferase and β(1,2)-xylosyltransferase to obtain an antibody without plant specific N-glycans (Cox et al., 2006). Many therapeutic proteins have been expressed in this system, mainly by Biolex Company and its subsidiary LemnaGene who produce interferon for hepatitis C treatment, plasmin for thrombosis treatment and anti-CD20 antibody optimized for the treatment of non-Hodgkin's B-cell lymphoma (www.biolex.com). At the present time, *Spirodela oligorrhiza*, with an expression level for GFP protein of 25% of TSP represents the best expressing system for nuclear transformation in higher plants (Franconi et al., 2010).

2.3 Factors influencing therapeutic protein production in plants

Many authors agree that one of the most important limitations of transgenic plants is their low expression level of recombinant proteins. Increasing the amount of recombinant protein is crucial for the system to be economically viable. The yield of recombinant proteins produced in plants depends on many factors: the intrinsic limitations of host plant, limitations imposed by transgene expression and protein stability, which are optimized by careful design of expression vector, and downstream processing. Finally, we would like to analyze the environmental factors affecting transgenic plants in open field.

2.3.1 Choice of host plant

The range of plant species amenable to genetic transformation is very wide so there is no an ideal host for molecular farming. There are many factors needed to be taken into account when choosing the host plant: from production factors such as infrastructure availability, storage and distribution cost, or plant productivity, to factors affecting environment and human health and food safety (Schillberg & Twyman, 2007).

Usually host plants are divided into food crops and non-food crops. Tobacco is the most used specie among non-food crops due to its easy of genetic transformation (both nuclear and chloroplast genomes), high biomass yield and seed production, and ability to produce many therapeutic proteins (antibodies, vaccines, cytokines, serum and blood proteins,

hormones). Its widespread use has led to the production of low-alkaloid cultivars suitable for oral delivery of vaccines (Tremblay et al., 2010). It has also been reported that the expression of biologically active GM-CSF in commercial sugarcane which is propagated vegetatively from stem pieces offering a "secure" platform for production of recombinant proteins (Wang et al., 2005). Food-crops include seed-crops, vegetables and fruits. Species such as cereals, rice, maize, soybean, or barley allow the expression of recombinant proteins in the seed which enables the long-term storage and the containment of proteins avoiding the exposure of non-target organisms. However seed-crops must go through a flowering cycle to produce seeds, so it is very important to control pollen transfer (Sparrow et al., 2007). Oilseed crops are useful for protein production because the fusion of recombinant proteins to the endogenous protein oleosin allow the easy recovery of recombinant proteins (Stoger et al., 2005). Vegetable crops include potato, worldwide cultivated specie with a very well-developed agricultural infrastructure. Tuber has been used mainly for oral vaccine production using tissue-specific promoter patatin. This vegetative organ offers stability to the recombinant protein. Carrot, alfalfa, lettuce and spinach have also been used for vaccine production because can be consumed raw, because can be grown again after leaf harvest, or because are very easy to scale-up (Sparrow et al., 2007). Fruits such as tomato and banana, more palatable than potatoes for raw consumption, are suitable for oral vaccines because of high biomass yield (tomatoes) and easy distribution (bananas) (Kamenarova et al., 2005).

2.3.2 Design of the expression vector

The expression vector is the vehicle to integrate the gene of interest into the plant genome. As stated before, the final yield of recombinant protein depends on many factors, and most of them can be addressed through inclusion of suitable regulatory sequences in the vector. In this context we can control factors that affect transcription, translation as well as protein accumulation. Figure 1 shows the schematic representation of a general expression vector showing potential regulatory sequences.

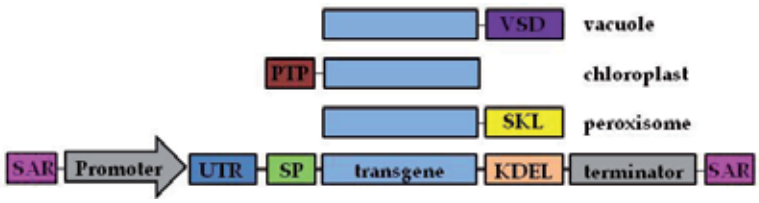

Fig. 1. Schematic diagram of plant expression vector showing the main regulatory sequences. SAR, scaffold attachment region; UTR, untranslated region; SP, signal peptide; KDEL, endoplasmatic retention signal; SKL, peroxisomes target sequence serine-lysine-leucine; PTP, plastid transit peptide; VSD, vacuole sorting determinant.

2.3.2.1 Promoter

This is one of the most important factors affecting transcription of genes because it is responsible of when, where and how does the gene transcribe. Promoters can be from plant or viral origin and usually are divided into constitutive, tissue-specific and inducible categories. Constitutive promoters drive the continuous expression of the transgene in all plant tissues. The best known and most used promoter is derived from the cauliflower mosaic virus (CaMV35S), a strong promoter mainly used for dicot species (Xu et al., 2011).

The duplication of an enhancer region located in the 5′ region of CaMV35S promoter allowed the development of the strongest version commonly known as double CaMV35S (2xCaMV35S) (Kay et al., 1987). For monocot species, rice actin gene or bean arcelin gene promoters are most used. The choice of promoter also depends on the nature of recombinant protein: when the protein of interest is toxic for the host plant, tissue-specific expression could be a helpful strategy (Desai et al., 2010). This type of promoter allows the control and restricted expression of transgenes to specific tissues such as leaf (small subunit of Rubisco), seeds (maize globulin-1, barley D-hordein, rice glutelin), and storage organs (tuber patatin). Inducible promoters allow not only the tight control of gene expression, but also the quantity of expression level. Induction can be regulated through chemical stimuli (steroid, sucrose, salt) and/or environmental factors (light, temperature, wounding). In this kind of promoters it is very important to specify between promoter and inductor, the rapid response upon induction, and the safety of the inducer for plant (Corrado & Karali, 2009).

2.3.2.2 5′ UTR sequences

The UTR sequences are located in the upstream region of ORF and are related to the efficiency of initiation of translation. In eukaryotes this process is thought to follow the scanning mechanism conducted by small subunit of ribosome from 5′cap of mRNA through the untranslated leader until the first start codon (AUG) is found (Kermode, 2006). Including 5′ UTR sequences from Alfalfa mosaic virus (AMV), Tobacco mosaic virus (TMV), *Chalcone synthase* (CHS), or *Alcohol dehydrogenase* (NtADH) have been successfully used to enhance the translation efficiency, allowing transgene levels 30 to 100-fold higher (Satoh et al., 2004; Schiermeyer et al., 2005).

2.3.2.3 Terminator sequences

Also known as polyadenylation signals, these sequences are very important for RNA stabilization because they are responsible for the correct processing of RNA after stop codon (Desai et al., 2010). The most commonly used terminator sequences are derived from the *A. tumefaciens nopaline synthase* gene which has been used successfully in both dicot and monocot plants.

2.3.2.4 Codon optimization of transgenes

Many of the transgenes introduced in plants for biopharmaceutical production come from humans. Codon usage between divergent species is often very different and will affect the expression level of recombinant proteins (Lessard et al., 2002). Based on available data of codon usage for a given host plant, transgene codons with the lowest usage frequency are modified by changing the nucleotide sequence without changing the amino acid sequence (Desai et al., 2010).

2.3.2.5 SAR/MAR sequences

The inclusion of sequences containing scaffold/matrix attachment regions (SAR/MAR) flanking the expression cassette has been used as a mechanism to increase transgene expression. SAR/MAR are AT-rich DNA sequences of 300-500 bp-long which interact with nuclear scaffolds organizing the structure of the genome (Allen et al., 2000). These sequences facilitate transcription of genes by changing the chromatin topology into less condensed regions (Kermode, 2006). With the use of SAR/MAR sequences it is possible to increase transgene expression levels through stabilization of the expression in progeny and reduction of expression variability (Ulker et al., 1999).

2.3.2.6 Subcellular targeting

Specific targeting of recombinant proteins to plant organelles not only guarantees the correct post-translational modifications required, but also allows the enhancement of protein stabilization, minimizing proteolytic degradation and facilitating downstream processing such as purification (Kermode, 2006). Eukaryotic preproteins synthesized with an N-terminal signal peptide are targeted to the secretory pathway. In the absence of signal peptide, recombinant protein is accumulated into the cytosol. In most cases this is not an appropriate compartment for recombinant proteins due to its hydrolytic activity and its negative redox potential which is unfavorable for protein folding (Benchabane et al., 2008).

In the case of proteins with signal peptide and no further targeting, expressed proteins are secreted from the ER to the apoplast. This compartment has been implicated in the production of proteolytic fragments in several transgenic plant systems due to proteases found in the secretory pathway between the ER and Golgi (Doran, 2006).

One of the most used compartments within plant cells for subcellular targeting is the ER. This organelle has many advantages for recombinant protein accumulation due to its specific characteristics. ER has very low hydrolytic activity and can tolerate unusually high accumulation of proteins without compromising plant development and reproduction because of its plasticity to become a reservoir of protein and oil bodies (Vitale & Pedrazzini, 2005). Recombinant proteins targeted to ER are protected from proteolytic degradation and correct folding and disulphide bond formation are allowed (Benchabane et al., 2008). Inclusion of KDEL/HDEL tetrapeptide in the C-terminal region of the protein ensures ER-retention. Targeting recombinant proteins into the ER has led to an increase in expression levels from 4.5 to 100-fold (Fiedler et al., 1997; Schouten et al., 1996; Torres et al., 1999).

Another suitable cell compartment for recombinant proteins accumulation is the vacuole. Important functions of this organelle include control of cell turgor, turnover of macromolecules, sequestration of toxic compounds, and finally, storage of high-energy compounds (Benchabane et al., 2008). From two types of vacuoles in plants, only protein storage vacuoles which are very abundant in seeds are suitable for recombinant proteins because of their mild environment (Stoger et al., 2005).

With chloroplast transformation, it is possible to target the recombinant protein to this organelle through the suitable targeting signals. The chloroplast-transit peptide of potato rbcS1 gene allowed the expression of human papillomavirus type 16 L1 (HPV-16 L1) protein into chloroplast of *Nicotiana benthamiana*, obtaining expression levels of up to 11% of TSP (Maclean et al., 2007). Moreover, chloroplast targeting can avoid the toxic effect of recombinant protein in the cytosol (Gils et al., 2005; Hühns et al., 2008).

It has also been reported that the subcellular targeting to mitochondria (Menassa et al., 2004) and chloroplast and peroxisomes at the same time, it is possible to accumulate the recombinant protein 160% of that in chloroplasts alone and 240% of that in peroxisomes alone (Hyunjong et al., 2006).

2.3.2.7 Protein/tag fusions

Expression of the recombinant protein attached to a protein with enhanced stability may prove to be useful to enhance fusion partner. In plants, several authors have reported the suitability of engineering fusion proteins to improve expression levels (Wirth et al., 2006; Xu

et al., 2010). Many fusion proteins can also serve as purification tags due to their specific binding features or easy of recovery, but those will be discussed in the next section.

2.3.3 Downstream processing

The downstream processing of recombinant proteins includes protein extraction, purification and characterization, a process that may represent 80% or more of total production costs (Twyman et al., 2003). Purification strategies may be based on standard chromatographic techniques, but affinity purification using His-tag, protein A, or glutathione S-transferase (GST) is an alternative approach widely used by researchers (Arnau et al., 2006). We have successfully used His-tag to purify human placental lactogen (hPL) from tobacco leaf tissue (Urreta et al., 2010).

Fusion of recombinant proteins to endogenous proteins such as oleosin or γ-zein is also very attractive for therapeutic protein production and is gaining more attention for commercial issues. Oleosin fusion platform has been developed by SemBioSys Inc. (www.sembiosys.com) and allows recombinant protein to be targeted to the oil bodies in rapeseed and safflower. The purification is based on separation of oil bodies by simple and inexpensive schemes (Schilberg & Twyman, 2007). Similarly, fusion of recombinant protein to γ-zein, a prolamin of maize, induces protein body formation and high accumulation of foreign protein within ER. Protein bodies are insoluble and easily purified by centrifugation (Vitale & Pedrazzini, 2005). An alternative emerging purification strategy relies on elastin like polypeptide (ELP) fusion to recombinant protein (Floss et al., 2009).

2.3.5 Environmental factors

Plants, as grown in the field or in the glasshouse, are subjected to environmental factors. Those factors influence their health condition, and subsequently, the quality and the quantity of recombinant protein. The stability of foreign protein in plants grown in the field is very important because it determines the homogeneity of the product. The most important environmental factors are light, temperature, soil (nutrients), water and insect attacks (Jamal et al., 2009). Most of these factors can be controlled to greater or lesser extent for plants cultivated in the glasshouse. However, when transgenic plants are grown in the field, light and temperature became the critical factors. Light is crucial for plants as energy source and temperature also affects plant growth and productivity being especially dangerous at high temperatures. The analysis of the influence of plant´s physiology on recombinant protein accumulation is crucial to plan the best strategy for plant harvesting (Conley et al., 2010).

Although protein production in plants has long been investigated, the most of the reported works are based on plants grown in glasshouses under controlled conditions. Arlen et al. (2007) reported that the field production of chloroplast derived interferon (IFN) from tobacco plants. They cultivated 0.26 acre containing 7369 plants and obtained 107.7 kg of biomass from a single harvest. This biomass contained approximately 87 g IFN (0.8 mg g^{-1}). It must be noted that tobacco plants can be harvested 4-6 times within a grow season from a 1 acre. They also tested light and temperature influence on glasshouse grown plants revealing the importance of leaf maturity and illumination, and most importantly, they observed higher IFN yield in those plants (1-3 mg g^{-1} fresh weigh).

3. Case study: Production of human placental lactogen in transgenic plants

Human placental lactogen (hPL), or chorionic somatomamotropin, is a 22 kDa peptidic hormone secreted by the placenta during pregnancy (Barrera-Saldaña et al. 1982). This protein is involved in the adaptation of islets of Langerhans to pregnancy through the regulation of beta (β) cell mass and function (Brelje et al. 1993). The capacity of hPL to improve β cell function, proliferation and survival *in vitro* and *in vivo* (Vasavada et al. 2000) makes possible its use as therapeutic protein for Langerhans islet transplantation to patients with type 1 diabetes. The transplantation of pancreatic tissue has become an interesting alternative treatment for diabetes, allowing the independence of patients to insulin injection. Unfortunately, the low availability of pancreatic tissue and great prevalence of the disease limits the potential of this treatment. Therefore, hPL together with other peptidic growth factors (HGF and PTHrP) belongs to the short list of proteins capable of improving the critical beta cell parameters, namely function, proliferation and survival, to improve tissue availability for islet transplantation (Fujinaka et al. 2007). Currently, the commercially available hPL protein is purified from human placenta which increases not only the risk of human pathogen propagation but also increases the cost of the protein in the market. This hormone has also been expressed in *E. coli* as inclusion bodies, which required the solubilization and refolding of the protein (Lan et al., 2006), making the process and the product more expensive. Due to the potential of hPL protein for type 1 diabetes treatment, we analyzed the suitability of potato plants as an alternative production system for hPL protein. In our laboratory, we thought that due to the potential of PL as a candidate for the treatment of type-1 diabetes, it would be very interesting to investigate alternative production strategies for the safe and easy production of this protein at low cost. Of course, plants represent for us the better production platform for all the reasons previously exposed and discussed, as well as for our experience on therapeutic protein production in plants.

We have successfully expressed hPL protein in *Nicotiana tabacum* cv. Xanthi plants reaching expression levels of up to 1% of TSP (Urreta et al., 2010). *In vitro* bioassays using the rat insulinoma (INS-1) cell line showed that recombinant protein was able to induce cell proliferation, demonstrating that plant cells can produce the biologically active hPL protein. Due to the difficulty to evaluate the relative performance of different crops for commercial production of therapeutic proteins which requires the production of the same protein in different host plant (Schilberg & Twyman, 2007), we attempted to produce hPL protein in *Solanum tuberosum*. We chose potato because it is easily transformed and widely used for molecular farming. Potato is an important crop worldwide and in our region, Álava (Spain). Indeed, our research institute has a long experience in the cultivation and improvement of this crop, being the reference germplasm bank in Spain.

To achieve our objective, the cDNA encoding h*PL* gene was obtained by RT-PCR from human placenta mRNA (Clontech) as previously described (Urreta et al., 2010). Briefly, the amplified sequence of 654-bp was ligated into pGJ2750 binary vector (kindly provided by Max Planck Institute, Köln, Germany) to obtain pNEKhPL1 expression vector where h*PL* gene is regulated by the promoter and terminator of CaMV 35S gene. The expression vector was introduced into *Agrobacterium tumefaciens* EHA105 strain and co-cultivated with *Solanum tuberosum* cv. Désirée leaf disks following the protocol described by Dietze et al. (1995). The regenerated shoots (Figure 2A) were subcultured in MS medium (Murashige & Skoog 1962) supplemented with kanamycin (100 mg L^{-1}) at 22ºC, under 120 µmol m^{-2} s^{-1} of photon flux and a 16-h photoperiod in a growth chamber. Most of the regenerated putative

transgenic plants had the same phenotype as the non transformed wild type plants (Figure 2, B-C). Integration of h*PL* and *neomycin phosphotransferase* (*npt*II) genes in the genome of regenerated plants was verified by polymerase chain reaction (PCR) using specific primers. All transgenic plants screened by PCR were positive (Figure 2, D-E), ensuring the correct integration of T-DNA in the genome of plants. Further molecular characterization of recombinant hPL protein was assessed by western blot. The proteins were separated on 12% polyacrylamide gels under reducing conditions and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked for 1 hour in PBS with 0.05% (v/v) Tween-20 (PBS-T) and 5% non-fat dry milk, and incubated with polyclonal anti-hPL antibody (RB9067, NeoMarkers, Fremont, CA, USA). Blots were incubated with Anti-Rabbit IgG secondary antibody conjugated with alkaline phosphatase (Sigma-Aldrich, St Louis, MO, USA). Antibody binding was detected with NBT/BCIP (Sigma). The recombinant protein was detected in leaf protein extracts of transgenic plants cultivated in the greenhouse, which showed the expected molecular weight of 22 kDa (Figure 2F) demonstrating that plant cells are able to process signal peptides from human origin.

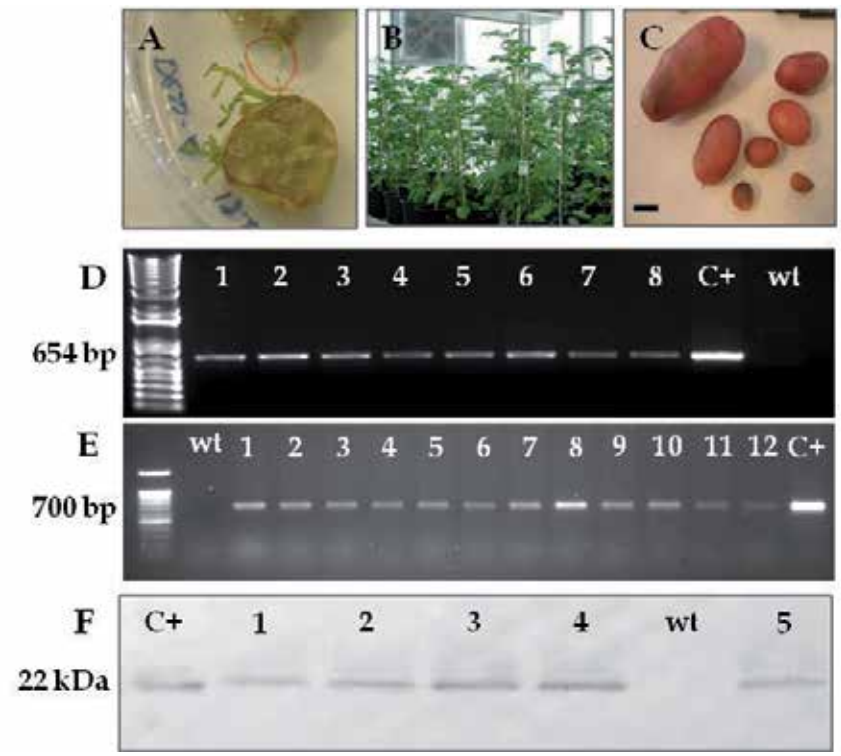

Fig. 2. Phenotypic and molecular characterization of transgenic potato plants. A, regenerated shoots obtained from leaf tissue co-cultured with *A. tumefaciens*. B, phenotype of potato plants cultivated in the glasshouse. C, tubers from transgenic lines (scale 1:2 cm). D and E, PCR amplification of h*PL* (D) and *npt*II (E) genes in the genome of regenerated plants. 1-12 independent lines; c+, pNEKhPL1 vector; wt, non transformed plant. F, western blot of transgenic plants cultivated in the glasshouse. C+, 10 ng of commercial hPL protein (NeoMarkers); 1-5, 20 μg of TSP from different transgenic lines.

The amount of hPL protein was determined by sandwich enzyme linked immunosorbent assay (ELISA) in leaf tissue of plants grown *in vitro* and cultivated in the greenhouse. Maxisorp 96-well microtiter plates were coated with polyclonal anti-hPL antibody (NeoMarkers) overnight at 4ºC. The wells were blocked with 1.5% horse serum in PBS-T and then samples as well as serial dilutions of commercially available hPL protein were added in PBS for 1 hour at 37ºC. Monoclonal anti-HPL antibody (MCA322, Serotec, Oxford, UK) and alkaline-phosphatase conjugated goat anti-mouse IgG antibody (Sigma) were coated subsequently to finally detect color development using 4-nitrophenyl phosphate (Sigma-Aldrich). Absorbance was measured at 405 nm in a microplate reader (Multiskan RC, Labsystems, Helsinki, Finland) and TSP content was determined by the Bio-Rad protein assay. The results showed that plants cultivated in the greenhouse had 2.4-fold higher expression levels than those grown *in vitro* (Table 1). The lower protein levels detected in plants grown *in vitro* could be due to the stressful conditions of culture conditions. It has been described that the artificial medium and the high air humidity and low gas exchange of this culture type could induce disturbances in plant development (Kadlecêk et al. 2001) which possibly limit the production of endogenous and foreign protein levels. Using the expression vector pNEKhPL1, the expression levels reached a maximum of 0.21% of TSP. These expression values are similar to those reported by other authors using vectors similar to pNEKhPL1 (Castañón et al., 2002; Kim et al., 2003; Mason et al., 1998; Ritcher et al., 2000).

Culture condition/tissue	Nº of plants analyzed	Expression level (mean value±s.e.)	Expression range
In vitro	24	0.25±0.03	0.03-0.63
Glasshouse	24	0.62±0.13	0.06-2.12
Tuber	16	1.34±0.13	0.69-1.87
callus	20	4.46±0.26	2.77-7.41

Table 1. Yield of the recombinant human placental lactogen protein expressed in potato plants grown under different conditions and in different organ/tissues. The expression levels were measured by enzyme-linked immunosorbent assay (ELISA) and are represented as recombinant protein per total soluble protein content (ng hPL μg^{-1} PTS).

Compared to the levels obtained in tobacco plants (Urreta et al. 2010), those reached in potato plants are much lower. However it must be taken into account that pNEKhPL1 expression vector is the simplest version of a plant expression vector; with no ER-retention signal, SAR sequence, nor enhanced 35S promoter included in pNEKhPL2 vector used in tobacco plants (Urreta et al., 2010). This difference in expression levels highlights the importance of the design of expression vector including regulatory sequences suitable to achieve our goals.

Regarding to the influence of host plant, in our laboratory we also transformed tobacco plants with pNEKhPL1 expression vector, obtaining a maximum expression level 2-fold higher than that obtained in potato (data not shown). So in the case of hPL production in plants, tobacco seems to be more suitable than potato with allowing higher expression levels in leaf tissue of plants cultivated in the greenhouse.

Because of the constitutive nature of CaMV35S promoter, we also analysed the expression level of recombinant hPL in tubers from 16 transgenic plants. We obtained elevated levels of recombinant protein in this tissue, with a mean value 2-fold higher than that of leaf tissue

(Table 1). The maximum hPL expression level was 0.18% of TSP, slightly lower than the maximum in leaf tissue. These results are in the range of expression levels reported by other Authors in potato tubers using CaMV35S promoter (Mason et al., 1998; Zhou et al., 2003; Bielmet et al., 2003). Although CaMV35S promoter is not tuber specific, the expression levels obtained are also similar to those reported by Mason et al. (1996) and Castañón et al. (2002) using tuber specific promoters such as patatin. It is also noteworthy that from the tubers tested, 4 of them did not show detectable levels of hPL.

In order to assess the suitability of hPL production in plant cell cultures we induced the formation of callus from leaf tissue of transgenic plants cultivated *in vitro* (Figure 3, A). Leaf explants were cultured in MS medium supplemented with casein hydrolysate (0.2% w/v), 2,4-D (5 mg L^{-1}) and kinetin (0.2 mg L^{-1}), and incubated at 23ºC in the dark. Friable callus developed in 3-4 weeks (Figure 3, B) which were analysed by western blot. Recombinant protein was detected at 22 kDa, as expected, but not in all lines tested (Figure 3, C).

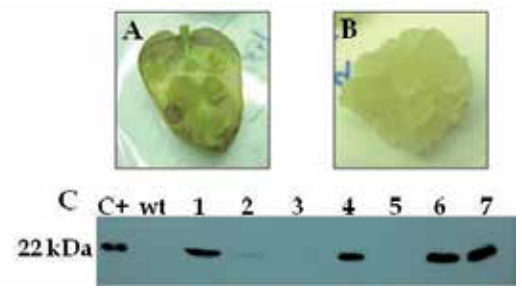

Fig. 3. Induction and characterization of callus tissue. A, induction of callus from leaf tissue cultured in MS medium supplemented with 2,4-D and kinetin. B, isolated callus line from leaf tissue and subcultured to fresh medium. C, western blot of callus protein extracts (10 µg). c+, commercial hPL protein (30 ng) (NeoMarkers); 1-7, transgenic lines; wt, callus induced from leaf explants of non transformed potato plants.

Although those calli were kanamycin-resistant, they did not express hPL protein. This could be as a result of promoter methylation during callus induction process as observed by other Authors (De Carvalho et al., 1992; Fojtova et al., 2003). The hPL protein levels were measured by ELISA. Results showed that a mean value 7-fold higher than that of leaf tissue of plants grown in the glasshouse, reaching a maximum level of 0.74% of TSP (Table 1). The expression of hPL protein in callus is favored because of the lower TSP content in this tissue. Data presented in this work represents a good starting point to further analyze the suitability of cell suspensions as an alternative plant-based expression system for hPL protein production. As previously discussed, plant cell cultures offer many advantages over whole plants for the production of human therapeutics, like rapid growth and easier purification strategies when the protein is secreted to the culture media. The expression vector pNEKhPL1 allows the targeting of hPL to the apoplast and hence to the culture medium. Moreover, using bioreactors we can avoid the release of genetically modified organisms to the field, and improve protein yields through the control of growth parameters allowing a homogeneous batch to batch production.

Finally, to further characterize the transgenic potato plants expressing the hPL protein, we analyzed the stability of the recombinant protein in the second generation (vegetative) of

four transgenic lines. Recombinant hPL levels were measured by ELISA, showing that one of the lines lost the expression of hPL and in the remaining lines the levels ranged from 0.2 to 0.38 ng μg^{-1} of TSP. These levels are higher than those of their counterpart grown *in vitro*, and similar to that of first generation cultivated in the glasshouse.

All the results obtained in this work suggest that although hPL protein is correctly produced in potato leaf tissue, tubers accumulate higher mean protein levels, allowing the storage of the protein. Further investigation will be necessary to analyze the stability of the protein in long term storage and the influence of temperature conditions needed to maintain its integrity. Although potato plants show lower expression levels than tobacco plants transformed with the same expression vector, expression levels in callus tissue opens the possibility for the production of hPL protein using plant cell cultures.

4. Conclusion

Transgenic plants represent an attractive production platform for therapeutic proteins due to all of the previously mentioned features. However, the commercial production of plant-made proteins is still limited, as compared to other expression systems currently available. Twenty two years after the first antibody produced in plants, the great research efforts of public and private institutions have lead to hundreds of plant-made protein publications and patents. Plant-based systems have demonstrated their suitability for protein production, ensuring their capacity for correct production of many types of therapeutic proteins and their great versatility to allow the production of proteins in different conditions. Many companies are exploiting plant systems for protein production (SemBiosys, Protalix, Dow Agrosicences, Méristem Therapeutics) but the pharmaceutical industry still shows reluctance to integrate plant systems in their production strategy. The lower protein levels obtained in plant systems can be greatly improved through the careful design of expression vectors and the choice of host plant. On the other hand, there is no biosafety and/or regulatory issue that can´t be solved. We hope that all the efforts made until now in the research field will be rewarded with a greater number of therapeutic proteins produced in plants in the market, for the benefit of society.

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16

Arabinogalactan Proteins in *Arabidopsis thaliana* Pollen Development

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1. Introduction

Pollen ontogeny is an attractive model to study cell division and differentiation. The progression from proliferating microspores to terminally differentiated pollen is characterized by large-scale repression of early program genes and the activation of a unique late gene-expression program in mature pollen.

Among the genes, or gene families that conform to the transition from a sporophytic type of development to a gametophytic program are the arabinogalactan protein (AGP) genes. AGPs are a class of plant proteoglycans, virtually present in all plant cells and in all plant species, from Algae to Angiosperms. They are predominantly located at the periphery of cells, i.e. on the plasma membrane and in the apoplast. Such ubiquitous presence insinuates that AGPs are vital components of the plant cell. Indeed, many studies have implicated AGPs in important biological phenomena, such as cell expansion, cell division, cell death, seed germination, pollen tube growth and guidance, resistance to infection, etc. (Seifert & Roberts, 2007). Evidences implicating AGPs in sexual reproduction have been obtained in our group, for several plant species (Coimbra & Duarte, 2003; Coimbra & Salema 1997; Coimbra et al., 2005) but how these molecules exert their function or how they interact with other cell components is yet to be defined. Only then the fragmentary knowledge that we have today about the function of these proteins may become pieces of a puzzle.

Following studies of pollen and pistil development in *Arabidopsis* with anti-AGP monoclonal antibodies (Coimbra et al., 2007; Pereira et al., 2006), it became clear that some AGPs may be suitable as molecular markers for gametophytic cell differentiation. Despite the tissue-specific carbohydrate epitopes of AGPs, these investigations do not allow the study of single AGP gene products. Therefore, a reverse genetics approach was undertaken to try to identify particular phenotypic traits attributable to certain AGPs, namely AGP6 and AGP11 that we had shown earlier to be pollen-specific (Pereira et al., 2006).

2. Arabinogalactan protein structure

2.1 Protein core

AGPs are complex macromolecules composed of a highly glycosylated protein core whose total mass may amount to only 5% or less of the total mass of the molecule (Fig. 1; Bacic et al., 2000; Nothnagel, 1997; Serpe & Nothnagel, 1999).

The nascent polypeptide chains of AGPs follow the cell's secretory pathway, and therefore contain an N-terminal signal sequence which targets the protein to the endoplasmic reticulum (ER). Most AGPs either contain or are predicted to contain, a C-terminal glycosylphosphatidylinositol (GPI) anchor addition sequence that is excised upon transfer of the protein to a pre-formed anchor present in the ER membrane. The mature protein core is rich in Pro/Hyp, Ala, Ser and Thr, which compose specific repetitive sequence modules. The pattern of these modules presumably constitutes the code that accounts for the characteristic glycan chains present in AGPs (Kieliszewski & Shpak, 2001; Schultz et al., 2004; Showalter et al., 2010). The repetitive amino acid modules (such as AP, TP, SP, or combinations of these) are typically scattered throughout the sequence of the mature protein. It is thus not likely that point mutations should affect the function of AGPs. Even if a mutation disrupts a glycosylation site, a number of others remain.

The properties of the protein backbone define subclasses of AGPs (Schultz et al., 2004). "Classical" AGPs typically contain the central Pro/Hyp-rich domain sandwiched between the N-terminal signal peptide and the C-terminal GPI addition sequence; the lysine-rich AGPs are like "classical" AGPs but contain a Lys-rich module, arabinogalactan (AG) peptides contain small mature protein cores, typically under 30 amino acid residues, the fasciclin-like AGPs (FLAs) may be considered chimeric AGPs because they contain typical glycosylation AGP modules and fasciclin-like domains (Johnson et al., 2003; Schultz et al., 2004; Showalter et al., 2010). Other atypical or chimeric AGPs, such as AGP31, have also been described (Showalter et al., 2010).

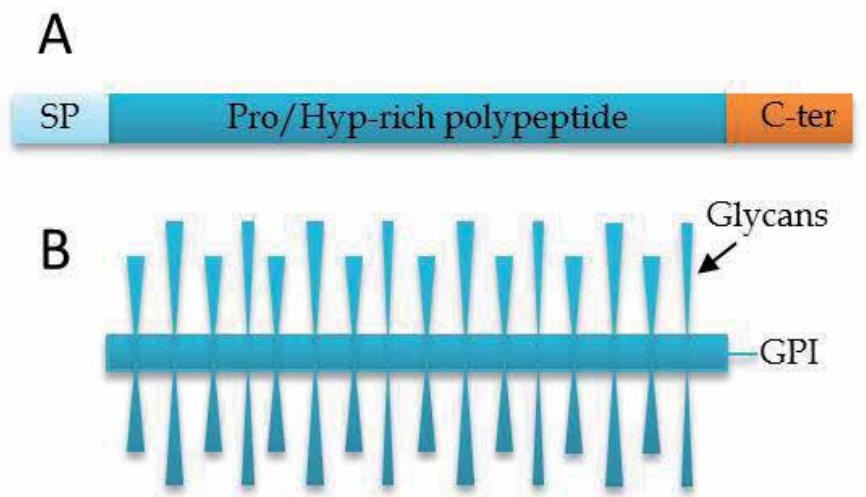

Fig. 1. Highly schematic representation of the molecular structure of a "classical" AGP. (A) Immature polypeptide, and (B) Mature proteoglycan with attached GPI anchor. SP, N-terminal signal peptide; C-ter, C-terminal GPI anchor addition sequence.

2.2 Glycan

AGPs belong to a wider superfamily of plant proteins, the hydroxyproline-rich glycoproteins (HRGPs) which also comprise the extensins and the proline-rich proteins. This classification is mainly based on the sugar content and glycosylation patterns (Kieliszewski

& Shpak, 2001; Shpak et al., 1999) of the molecules, the AGPs being the most heavily glycosylated of all.

AGP glycans are polyssacharide chains *O*-glycosidically linked to Hyp residues. Polysacharide chains are type II arabinogalactan chains, which consist of a main chain of β-(1→3)-galactopyranose units variously substituted at C6 with oligosacharide or polysacharide chains rich in Gal and Ara but may also contain other monosacharide residues such as Rha and GlcA (see Ellis et al., 2010 for a recent review).

One of the main tools to study the biology of AGPs has been the use of monoclonal antibodies that bind to AGP-specific sugar epitopes. From those studies we know that different AGP epitopes are only present in specific cell types or tissues, or in particular developmental stages. The genetic regulation of this diversity, both in space and in time, is further complicated by the regulation of the necessary battery of glycosyl transferase activities needed to the synthesis of complex sugar chains.

2.3 GPI anchor

The GPI anchor is a post-translational modification of eukaryotic proteins. Newly synthesized proteins that contain a C-terminal GPI-addition signal sequence become attached to a preassembled GPI anchor present in the membrane of the ER, concomitant with the cleavage of the signal sequence. As a result of this modification, GPI-anchored proteins (GAPs) become tethered to the outer layer of the plasma membrane facing the extracellular environment.

Many AGPs were either experimentally shown to be GPI-anchored (about twenty AGPs in *Arabidopsis*; Borner et al., 2003; Elortza et al., 2003, 2006; Lalanne et al., 2004; Schultz et al., 2004) or, based on amino acid sequence analysis, predicted to be GPI-anchored. In *Arabidopsis*, only a few AGPs are not predicted to contain a GPI anchor.

GPI anchors are themselves complex structures, having a highly conserved core composed of ethanolamine-PO_4-6Manα1–2Manα1–6Manα1–4GlcNα1–6*myo*-inositol-1-PO_4-lipid. As opposed to the majority of known GPI anchors, whose lipid part is diacylglycerol-based, the inositolphospholipid part of the only two plant GPI anchors characterized to date is an inositolphosphoceramide (Oxley & Bacic, 1999; Svetek et al., 1999).

Despite the high biological investment in such structures, involving more than twenty dedicated gene products for its biosynthesis, the role of the GPI anchor, or its contribution to the protein biological function is not evident, and remains unsolved for most, if not all, studied examples of GAPs. Moreover, during transit through the secretory system, GPI anchors may be subjected to lipid and/or carbohydrate side-chain remodeling, resulting in a number of GPI structural variants, which may be present in the same organism (Ferguson et al., 2008), and which undoubtedly raises the level of complexity of the biology of GAPs.

Despite all the unanswered questions, specific and commonly referred properties of GAPs mediated by the GPI are their likely association with lipid rafts (Borner et al., 2005), cellular polar sorting (particularly in animal and yeast cells; Legler et al., 2005), and controlled release to the extracellular matrix through the specific action of phospholipases. Indeed, stress conditions such as salinity, cold, drought, heat, wounding, and pathogen attack, are

known to activate phospholipase D (PLD), or phospholipase C (PLC) pathways (Testerink & Munnik, 2011), but the actual release of GAPs as a direct consequence of environmental or physiological stimuli is yet to be demonstrated.

Nevertheless, the combination of AGP characteristics, namely cell and/or tissue localization, possible release from the plasma membrane upon stimuli, and complex sugar content that makes AGPs, or specific fragments of AGPs, as likely candidates to perform signaling functions in plants.

3. Pollen development

In flowering plants, development of the haploid male gametophytes (pollen grains) occurs inside a specialized structure called the anther. Successful pollen development, and thus sexual reproduction, requires the correct development of the anther wall layers, and an increased growth of the four locules with fusion into two pollen sacs, and relies on the provision of nutrients and other materials from a specialized secretory tissue, the tapetum. The importance of the tapetum for pollen development is highlighted by findings that the majority of male-sterility mutants involve injuries that affect the expression of tapetum-specific genes. The gene products are released from the tapetal cells and transferred to the pollen surface in wild-type plants. Although there have been many excellent ultrastructural studies of tapetal and pollen development, the underlying biochemical processes have, until recently, remained unclear. This situation is now changing rapidly with the advent of the genome sequence and tools to allow the analysis of gene function (Wilson & Zang, 2009).

3.1 Microsporogenesis

In *Arabidopsis*, at the beginning of anther development, under the protoderm layer, groups of cells develop to give rise to a primary parietal layer and to a sporogenous layer (Owen & Makaroff, 1995). The first will divide to originate the different anther wall layers, and the sporogenous tissue will give rise to the microsporocyte cells. At the pre-meiotic stage of microsporogenesis, the five wall layers of the anther are well differentiated; the microsporocytes have thin cell walls and are successively surrounded by the tapetum, median layer, endothecium and epidermis (Fig. 2).

At the beginning of meiosis, microsporocytes are connected with the tapetal cells by plasmodesmata and it is at this moment that callose deposition begins. During meiosis, the microsporocytes are interconnected by cytomictic channels and their cytoplasm starts to dedifferentiate, which is probably related with the transition from a type of sporophytic gene expression to one of gametophytic gene expression. This transition relates to the change from a diploid to a haploid generation. At this time callose deposition continues, resulting in thick callose walls surrounding the microsporocytes. It has already been assumed that this callose wall can be the trigger to initiate the gametophytic type of development (McCormick, 1993). This physical isolation is important to activate such dramatic changes in development. Following meiosis and cytokinesis, the four haploid microspores are arranged in tetrads encased by a thick callose wall, within the callose wall a microspore-produced cell wall, the primexine is present (Coimbra et al., 2007).

At the end of meiosis, the external walls of the tetrads are dissolved to release individual microspores, by a mixture of enzymes containing endoglucanases and exoglucanases

secreted by the tapetum (callase). Alterations in the timing of this event, or failure to express β-1,3-glucanases, leads to abnormal disruption of the callose walls, which has been shown to be a primary cause of male sterility in cytoplasmic male-sterile lines of several species, including Petunia (Izhar & Frankel, 1971).

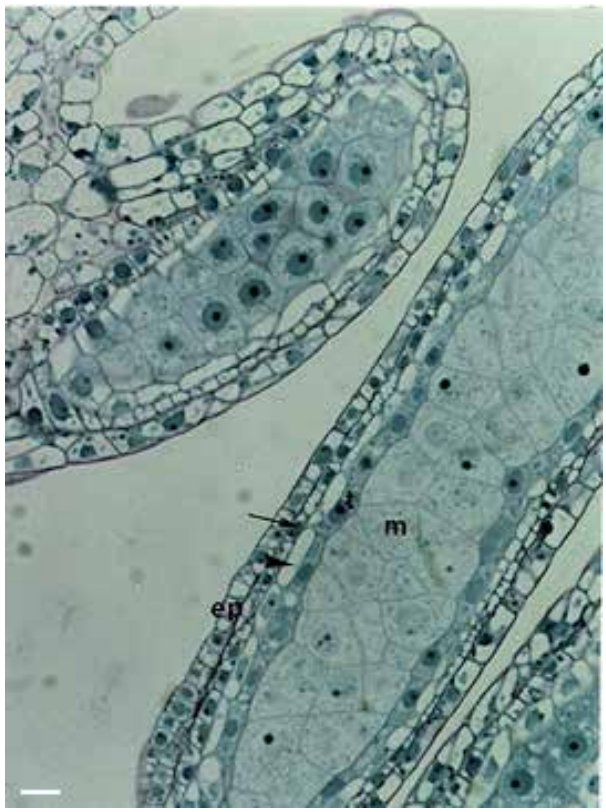

Fig. 2. Light microscopy image of *Amaranthus hypochondriacus* anthers, showing well differentiated microsporocytes (m) and the undifferentiated anther wall layers; from the outside the epidermis (ep), the endothecium (arrow), the middle layer (arrow head) and the tapetum (t) surrounding the microsporocyte cells. Bar 10 μm.

The coordinated development of the microsporocytes and the surrounding cell layers might involve cell-to-cell interactions and localized signaling. From studies with the *SPOROCYTELESS (SPL*) mutants, it was suggested that microsporocyte formation and anther wall development might be coupled. In these mutants, neither microsporocytes nor anther walls are formed and because the microsporocytes and the haploid spores of the tetrads become isolated by callose deposition it is likely that the signaling occurs early during the formation of microsporocytes. The expression of the *SPL* gene in the microsporocytes and its absence from the parietal cells suggests that SPL functions within the microsporocytes to regulate expression of a subset of genes required for microsporocyte formation. Therefore, the authors suggested that the microsporocytes promote, through signal exchange or cell-to-cell interactions, the differentiation and growth of the parietal cell layers, and consequently anther wall development (Yang et al., 1999).

3.2 Microgametogenesis

Microgametogenesis starts with the release of the microspores from the tetrads and leads through a simple cell lineage, to the formation of the mature pollen grains. The ultrastructural description of this developmental stage shows high exocytic activity in tapetum cells, the elaboration of the intine wall by microspores and the deposition of sporopolenin, one of the most complex polymers of plants, which is the outer pollen wall constituent. This outer wall, the exine, is initiated with the formation of the primexine layer at the tetrad stage of development (Owen & Makaroff, 1995).

Microspores start to build a roundish shape due to the formation of a central vacuole, which originates from the fusion and enlargement of small vacuoles. In response to this, the microspore nucleus shifts to an eccentric position against the microspore wall. The first mitosis of the microspore is asymmetric and originates a large and transcriptionally active vegetative cell, and a small generative cell with condensed chromatin that will divide again and originate the two male gametes (Tanaka, 1997) (Fig. 3).

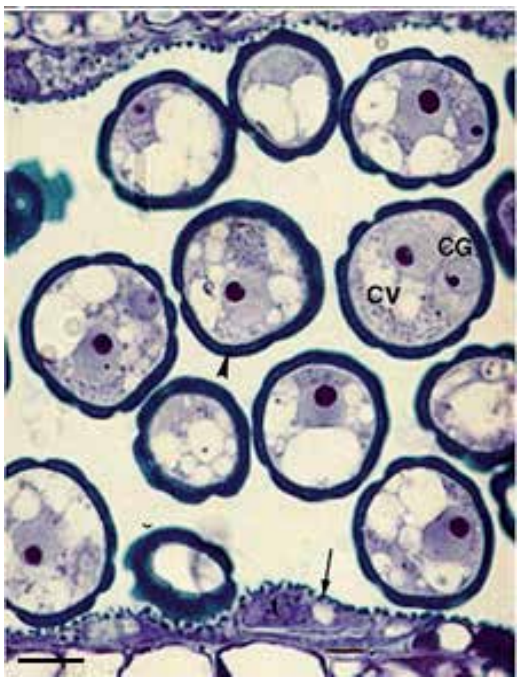

Fig. 3. Anther of *Amaranthus hypochondriacus* showing bicellular pollen grains with a large vegetative cell (CV) and a small and generative cell (CG) in an asymmetric position. The exine wall (arrow head) is being built by the pollen grain and by the sporopolenin depositions from tapetal cells (arrow). Bar 5 μm

The vegetative cell cytoplasm shows strong metabolic alterations related to the asymmetric cell division, one of the most striking events of cell differentiation occurring during the plant life cycle. A single mitotic division will give rise to two completely different cells in size, function and gene activity (Fig. 4). This asymmetric division is vital for generative cell differentiation. Microgametogenesis is quite regular in flowering plants, being the

distinction between the timing of the second mitosis the biggest difference; in the majority of Angiosperms, pollen is shed in a bicellular condition, and the generative cell divides inside the pollen tube; whereas in tricellular pollen species, like *Arabidopsis*, the generative cell divides inside the anther. Among the first gametophytic cell markers to be identified is the tomato pollen-specific LAT52 promoter that drives expression specifically in the vegetative cell after pollen mitosis I (Eady et al., 1995), and the lily generative cell-specific H3 histones (gcH2A, gcH3) (Xu et al., 1999). The cytoskeleton plays a central role in determining both nuclear migration and the eccentric division plane. After mitosis, the generative cell migration is a uniquely specialized cell-cell support, which creates a "cell within a cell". The generative cell then assumes a spindle shape and DNA replication takes place, whereas the vegetative nucleus remains arrested in G1 (Fig. 5E). At the end of microgametogenesis, the exine wall will be completely formed, with depositions secreted by tapetal cell (Fig. 3) and the tapetal cells will finally degenerate, depositing their contents in the exine sculptures of the pollen grains, forming the pollen coat.

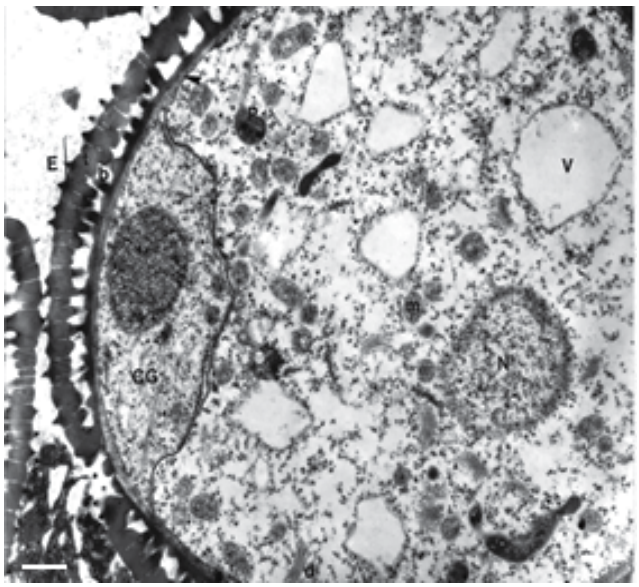

Fig. 4. Electron micrograph of a young bicellular pollen grain of *Amaranthus hypochondriacus*. The generative cell (CG) just formed is still in a spindle shape adjacent to the pollen wall. E- exine (b- bacullae, v– vacuole, m- mitochondria, n- nucleus).

All the steps of this complex process rely on a complex network of signaling events, which probably involve molecules of different kinds (Preuss, 2002). Pollen ontogeny is an attractive model to study cell division and differentiation. In this genomic era, new technologies begin to unravel the roles of specific genes involved in male gametogenesis (Honys et al., 2006; Quan et al., 2008; Toller et al., 2008; Twell 2011).

3.3 Pollen tube growth

Pollen tube growth begins with its emergence from the vegetative cell of the pollen grain after adhesion to the stigma surface. This process depends on the hydration of the pollen

grain which is accomplished after recognition. A strictly apical cell growth process maintains the pollen tube cytoplasm and its cargo, the sperm cells, in the most proximal region of the tube as it elongates through the female sporophytic tissues. Callose plugs are laid down at regular intervals behind the growing tip, and the region adjacent to the plug becomes vacuolated. The extreme end of the growing tip consists of a highly dynamic clear zone that contains vesicles and cell wall precursors. Activity in this zone involves continual biosynthesis of cell wall and plasma membrane and turnover of cytoskeletal components as the tube elongates.

In recent years, substantial progress has been made toward understanding tip growth mechanisms (Feijó et al., 1995). Pollen tube reorientation can occur in several minutes, so this might exclude the involvement of newly synthesized gene products. The existence of a tip-focused gradient of cytosolic free calcium has been shown to be maintained by an asymmetric activity of calcium channels and to be essential for growth (Malhó et al., 1995). Polymerization of the actin cytoskeleton is also essential, and tip localized F-actin is thought to mediate the membrane trafficking of secretory vesicles to the apex.

Mutants have been described in which pollen tubes are unable to locate the ovules, as a result of altered interactions with these structures (Higashiyama et al., 2003; Hülskamp et al., 1995). There has been controversy over whether a diffusible signal attracts the pollen tube or whether the female tissues define its path, but recent genetic and physiological data for several plant species, has showed that the female gametophyte produces at least one diffusible signal, which is derived from the two synergids cells (Higashiyama et al., 2003).

4. Arabinogalactan proteins in *Arabidopsis* pollen development

4.1 Immunolocalization of glycan epitopes

Specific monoclonal antibodies (mAbs) that bind to structurally complex carbohydrate epitopes of AGPs have been very useful in revealing the developmental dynamics of the AGP glycan moiety and represent a diagnostic tool for AGPs. Frequently used anti-AGP mAbs are JIM8, JIM13, JIM14, LM2, and MAC207. Accumulated information, obtained by the extensive use of anti-AGP mAbs by the scientific community, shows that AGPs are finely regulated and differentially expressed during pollen development, namely during sexual plant reproduction.

Given the importance of *Arabidopsis* as a model plant, a detailed map of AGP sugar epitopes in different flower parts and at different stages of development was obtained and clearly showed differences in the pattern of distribution of specific AGP sugar epitopes, during *Arabidopsis* anther development. These differences are apparent both in sporophytic and in gametophytic tissues, and it became evident that AGP-specific epitopes can work as markers for certain cell or tissue types, in very precise stages of sporogenesis and gametogenesis.

In the premeiotic stage of microsporogenesis, the epitopes recognized by the mAbs JIM8 and JIM13 are specifically and intensely localized in the tapetum cells and in microsporocytes (Fig. 5A). The selective labelling of the microsporocyte walls is quite

important at this stage of development when microsporocytes start a dedifferentiation program related with the transition from a sporophytic gene expression to a type of gametophytic gene expression. This transition relates to the change of generations, from diploid to haploid. As soon as prophase starts, the callose deposition also starts, resulting in thick callose walls surrounding the microsporocytes (Fig. 5C). After meiosis and cytokinesis of the four haploid microspores, the tetrads are completely encased by a thick callose wall. Within the callose wall a microspore-produced cell wall, the primexine, is present. It is interesting to notice that the first existent separation wall in microsporocytes is still present at this stage and still labelled by mAbs JIM8 and JIM13. This labelling pattern can be associated to the signals that must be produce for the efficient release of callase, by the tapetum cells, or a type of developmental time specificity related to the gametophytic development (Fig. 5D).

The presence of AGPs in the tapetum clearly shows that this tissue synthesizes and secretes these molecules (Fig. 5D). The interaction of the sporophytic tapetal cells and the gametophytic differentiation of meiocytes into microspores, are present in the synchronism of callase release from the tapetum ER, as well as from the sporopolenin precursors released into the anther locule. AGPs are strong candidates for cell differentiation signals at this stage of development. It can also be correlated the presence of AGPs with tissues that are set out to programmed cell death. In this study it was observed that the stage at which programmed cell death is triggered, the end of the tetrad stage, is associated with the stronger presence of AGPs recognized by mAbs JIM8 and JIM13.

The labelling with mAbs JIM8 and JIM13 is also strong in the microspores outer surface, which is the site where the intine wall will be built, indicating some association of this important developmental stage with AGP synthesis (Fig. 5D).

During pollen development, it is well documented the strong metabolic alterations in the vegetative cell cytoplasm related to the asymmetric cell division, one of the most striking events of cell differentiation occurring during the plant life cycle. JIM8 and JIM13 specifically label the generative and gametic cells, but not the gametophytic cell. This labelling may function as a molecular marker for cell development and may also be related to the signals necessary to direct these cells inside the pollen tube into their targets, in the embryo sac (Fig. 5E). Moreover, after the second pollen mitosis, the two resulting sperm cells that are inside the pollen grain or inside the pollen tube are still strongly labelled by these two mAbs. The specific labelling of the generative cell was also reported for oilseed rape (Pennell et al., 1991), for *Nicotiana tabacum* (Li et al., 1995) and for *Brassica campestris* male gametes (Southworth & Kwiatkowski, 1996). JIM8 and JIM13 do not label the pollen tube wall, which instead is labelled by MAC207 and LM2 (Pereira et al., 2006). These two mAbs are probably related to epitopes in structural AGPs present in several types of plant cell walls.

Antibodies MAC207 and LM2 showed similar binding patterns, both defining extended cell populations in different tissues (Figs. 5B and 5F), as opposed to JIM8 and JIM13 which seemed to define single tissues or single cell types and that can be suitable as molecular markers for pollen development in *Arabidopsis*.

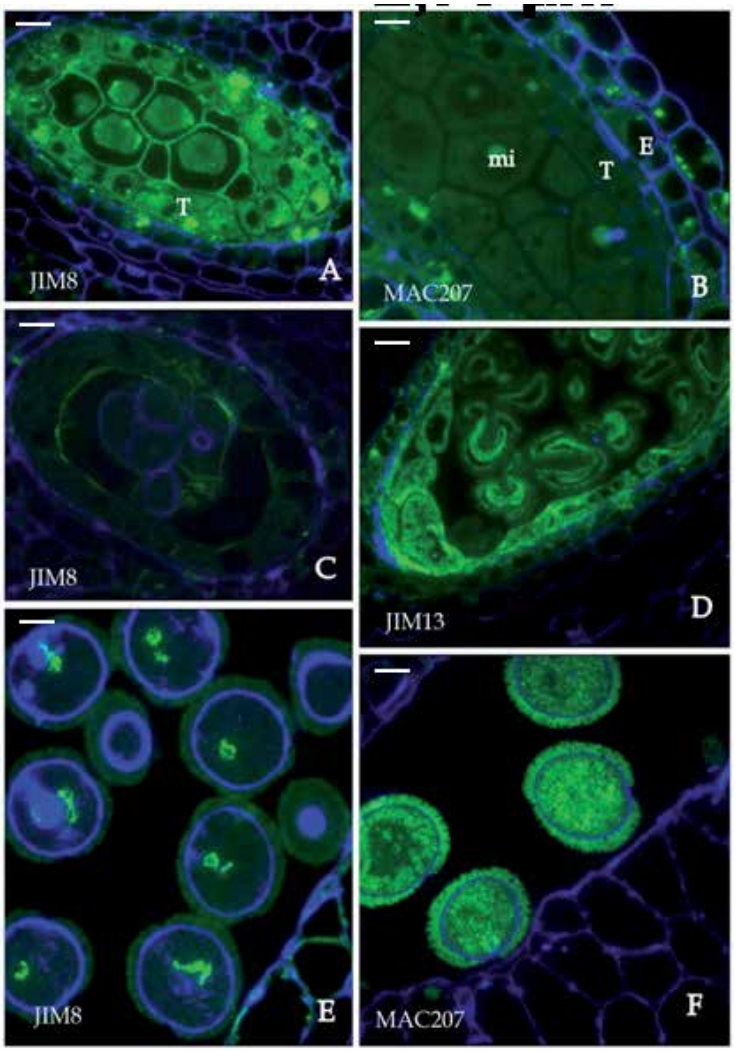

Fig. 5. Fluorescence microscopy of *Arabidopsis* anthers at different stages of pollen development labelled with monoclonal antibodies specific for arabinogalactan proteins (JIM8, JIM13 and MAC207) with FITC-conjugated secondary antibody. A) JIM8 labelling at the stage before meiosis. Tapetal cells and microsporocytes are labelled but there is a remarkable labelling of the wall that surrounds the microsporocytes that are dedifferentiating. B) Same stage of development labelled by MAC207 showing a diffuse labelling in the endothecium and epidermis cells. C) Tetrads of haploid microspores encased by a thick callose wall, the mAb JIM8 is labelling the initial wall of the microsporocytes. D) Microspores just released from tetrads. AGP epitopes recognized by JIM13 are present in tapetal cells and also in the endothecium, which is now developing. This mAb also binds to the microspores cytoplasm and outer surface where the intine wall will eventually develop. E) Tricellular pollen showing specifically the labelling of the two male gametes with JIM8. F) Same stage as in E showing a unspecific labelling with MAC207. T – Tapetum; mi – microsporocytes, E – endothecium.

In order to study the adhesion of pollen tubes via cell wall molecules, the cell wall characteristics of in vitro-grown *Arabidopsis* pollen tubes were investigated using a combination of immunocytochemical and biochemical techniques. Results showed a well-defined localization of cell wall epitopes. Low esterified homogalacturonan epitopes were found mostly in the pollen tube wall back from the tip. Xyloglucan and arabinan from rhamnogalacturonan I epitopes were detected along the entire tube within the two wall layers and the outer wall layer, respectively. In contrast, highly esterified homogalacturonan and arabinogalactan protein epitopes were found associated predominantly with the tip region. This work demonstrated that the *Arabidopsis* pollen tube wall has its own characteristics compared with other cell types in the *Arabidopsis* sporophyte which are probably related to the pollen tube specific growth dynamics (Dardelle et al., 2010).

4.2 AGP tagging

The use of tags engineered into specific AGP gene products is a particularly pertinent approach to study individual AGPs since alternative approaches, such as the use of specific anti-peptide antibodies or even basic analytical tools such as SDS-PAGE may convey challenging difficulties imposed by the massive presence of glycan chains of indeterminate composition and size. Several studies have been published during recent years that include experiments with AGP tagging (Levitin et al., 2008; Li et al., 2010; Sun et al., 2004a, 2004b, 2005; Yang & Showalter, 2007; Van Hengel & Roberts 2003; Zhao et al., 2002). The DNA constructs that underlie those experiments have been used for intracellular localization, expression characterization or purification.

For intracellular localization studies, DNA constructions should contain: i) a functional signal peptide sequence to direct the fusion protein to the ER; ii) the reporter gene, in general green fluorescent protein (GFP) gene has been used; and iii) the mature AGP sequence together with the GPI addition sequence for proper anchoring to the plasma membrane. Constructions such as these have been placed under the influence of the 35S Cauliflower Mosaic Virus (CaMV) promoter for constitutive overexpression studies (phenotype analysis) and to study the consequences of deleting the GPI addition sequence or the Lys-rich domain in Lys-rich AGPs (Li et al., 2010; Sun et al., 2004a, 2004b, 2005; Yang & Showalter, 2007; Zhao et al., 2002). DNA constructions placed under the control of AGP endogenous promoters instead of the 35S CaMV promoter are presently under analysis in our laboratory (unpublished results).

Van Hengel & Roberts (2003) used a *c-myc* tag placed at the C-terminus of a DNA construction containing the complete coding sequence of AGP30 (35S::AGP30-*c-myc*) and used it for electroblot analysis of plant extracts. Indeed, for those few *Arabidopsis* AGPs that may not have a GPI anchor, the tag may be placed at the C-terminus with reduced risk of becoming separated from the remaining polypeptide.

For cell or tissue expression studies, DNA constructions are technically simpler, and may consist of endogenous AGP promoter sequence plus reporter gene, with or without AGP-specific sequences. This approach has been used to confirm and characterize the developmental expression of two pollen-specific *Arabidopsis* classical AGPs (AGP6 and AGP11), and one pollen-specific FLA (FLA3), using either GFP, GUS, or the red fluorescent

protein RFP as reporter genes (Coimbra et al., 2008, 2009; Levitin et al., 2008; Li et al., 2010). The temporal expression of AGP6 was determined with great accuracy in *Arabidopsis* plants that were transformed with a ProAGP6::GFP gene construct. GFP fluorescence was absent in all vegetative plant parts, but became clearly visible just after the appearance of the locules in anthers. This stage was identified as corresponding to stage 9 (as described by Smyth et al., 1990). GFP fluorescence was limited to pollen and pollen tubes and could be clearly differentiated from the green yellow autofluorescence characteristic of the exine and of the endothecium lignin thickenings of the anther wall. GFP fluorescence persisted through to the mature pollen grains and was observed in growing pollen tubes (Coimbra et al., 2008; 2009).

4.3 Reverse genetics

The distribution and expression patterns of pollen-specific AGPs have been examined closely in our laboratory. So we tried to identify particular phenotypic traits attributable to either AGP6 or AGP11, or both, in a reverse genetics approach. *AGP6* and *AGP11* are closely related genes, sharing 68% of the amino acid sequence, and therefore seemingly constituting a pair of paralog genes, the function of which may be mutually overlapping. Ds transposon insertion mutant lines for these two genes were available from RIKEN BioResource Center. However, as with so many other plant single gene null mutants, these did not produce recognizable phenotypes (Coimbra et al., 2009). At least in the case of AGP6 and AGP11, a double *agp6 agp11* null mutant did produce identifiable phenotypic traits which could be ascribed to the simultaneous lack of both gene products. In *agp6 agp11* double homozygous mutant lines, but also in plants homozygous for one of the insertions and heterozygous for the other, many of the pollen grains failed to develop normally and collapsed, indicating that the genes are important gametophytically for pollen development (Fig. 6). The collapsed pollen phenotype of *agp6 agp11* was characterized both by scanning electron microscopy and by transmission electron microscopy of pollen grains that clearly showed the degeneration of pollen contents (Coimbra et al., 2009).

Despite the collapsed pollen phenotype typical of the homozygous *agp6 agp11* mutants, a percentage of the pollen grains were able to develop and germinate into functional pollen tubes, as assessed by the presence of seeds in self-pollinated plants. Thus it can be assumed that AGP6 and AGP11 are non-essential for stabilizing pollen grain development, or else that an alternative pathway, potentially involving the ectopic or up-regulation of the expression of other AGP family members, is able partially to compensate for the loss of the two proteins.

In other studies aiming at a more inclusive phenotypic characterization of the *agp6 agp11* double null mutants, it was detected that a number of pollen grains germinated precociously inside the anthers and this phenomenon was dependent upon the relative humidity of the growth chamber (Coimbra et al., 2010). Pollen germination inside anthers was not observed in single *agp6* or *agp11* mutants, and therefore those observations indicated that the double mutation was needed to induce the precocious germination character. Precocious germination of pollen was never found in wild-type plants, even in conditions of high relative humidity, a factor that increased the presence of the phenotypic

trait in the double mutant. AGP6 and AGP11 thus seem to have a role in preventing an early and wasteful germination of pollen inside the anthers. As a rule, untimely germination inside anthers does not occur, so some factor or factors must be preventing it from happening. It is also noteworthy that the minimal conditions for pollen germination and tube growth are reproduced inside the anthers. The fact that pollen tubes can germinate and elongate inside the anther locules poses interesting questions regarding germination control and nutritional requirements necessary to support the high respiration rates generally believed to occur for rapid tube growth. It is indeed interesting and challenging to appreciate that arabinogalactan proteoglycans may be interfering with the timing of pollen germination, maybe by a relatively simple process of modulating access of water for hydration (or for the earlier dehydration process), or by interfering with some kind of signaling pathway.

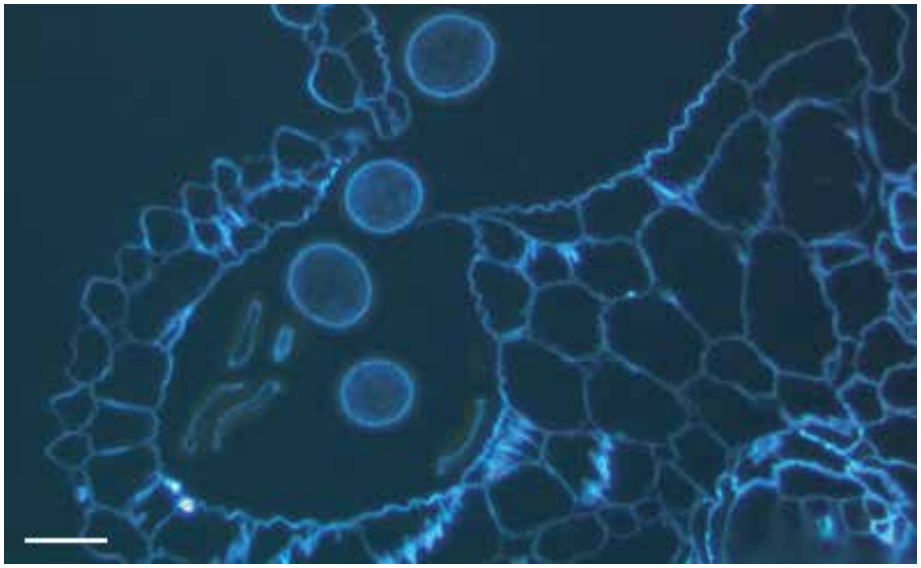

Fig. 6. Light micrograph of an *agp6 agp11* anther showing collapsed pollen grains and some roundish normal pollen grains.

4.4 Microarrays

A series of studies have revealed distinctive transcriptome profiles in microspores, mature pollen and germline cells. The most complete datasets have been generated in the genetic model *Arabidopsis*, where transcriptome analyses have revealed developmental expression profiles from microspores to mature pollen (Honys & Twell, 2004; Pina et al., 2005), the sperm cell transcriptome (Borges et al., 2008) and transcript changes associated with pollen germination and tube growth (Quin et al., 2009; Wang et al., 2008). Sequentially, in our work with the aim of dissecting the biological function of AGPs, we decided to evaluate the whole set of pollen tube expressed genes. For this purpose, we performed microarrays, using the Affymetrix ATH1 genome arrays in the *agp6 agp11* double null mutant pollen tube. We believe that this work is of great general interest for the field of plant science, not only because it highlights interactions between AGPs and other specific gene products but also

because of the surprisingly high number of genes whose expression is altered in the pollen tubes of the mutant line, revealing that these molecules can only be involved in very complex phenomena.

This work identified 1300 genes which have either reduced or elevated expression in the *agp6 agp11* pollen tube as compared to wild-type pollen tube, being some of these genes completely inactivated. These genes can be used as starting points to dissect the gene regulatory networks where AGPs are involved during pollen tube growth.

5. Arabinogalactan proteins in other species pollen development

5.1 Rice

Working with *Oryza sativa indica*, Anand & Tyagi (2010) reported the molecular characterization and the promoter activity of a rice pollen-preferential gene, OSIAGP. The authors isolated this gene and identified it as an arabinogalactan protein gene, during a differential screening of inflorescence-specific cDNA libraries. OSIAGP protein has a secretory domain at its N-terminus and *in silico* analysis revealed it to be a secretory and transmembrane protein. When databases were searched using OSIAGP nucleotide and protein sequences, they showed significant homology with a pollen-preferential gene, AGP23 from *Arabidopsis* (AT3G57690). The eight amino acids of the secretory domain at the N-terminus are 100% conserved among OSIAGP and AGP23. The AGP23 gene is 369 bp long and encodes a 61-amino acid protein with homology with other arabinogalactan proteins from *Arabidopsis*. OSIAGP is 59 amino acids and a pollen-preferential gene falling in the category of late pollen genes and is speculated to play important role in pollen tube growth. Its promoter harboring regulatory elements for pollen expression and light regulation could be of interest to the plant community.

Ma & Zhao (2010) identified 69 AGPs from the rice genome, including 13 classical AGPs, 15 AG peptides, 3 non-classical AGPs, 3 early nodulin-like AGPs (eNod-like AGPs), 8 non-specific lipid transfer protein-like AGPs (nsLTP-like AGPs), and 27 FLAs. The results from expressed sequence tags, microarrays, and massively parallel signature sequencing tags revealed that several rice AGP-encoding genes are predominantly expressed in anthers and display differential expression patterns in response to abscisic acid, gibberellic acid, and abiotic stresses, which is highly in agreement with the microarray results obtained in our group for *Arabidopsis* (manuscript in preparation). The two classical AGP-encoding genes, OsAGP7 and OsAGP10, are highly expressed in pollen, similar to AtAGP6 and AtAGP11. The phylogenetically closest rice gene is OsAGP6, but it has a different expression pattern from that of AtAGP6 and AtAGP11. In this case, it is possible that the genes sharing the same function are not those with higher identity. Therefore, OsAGP7 and OsAGP10 may play a conserved role in pollen development, like AtAGP6 and AtAGP11 which redundantly control pollen development and fertility (Coimbra et al., 2009, 2010; Levitin et al., 2008).

5.2 Brassica

The firstly characterized pollen-specific putative AGP genes, Sta 39-4 and Sta 39-3 were isolated from *Brassica napus* flowers in 1996 (Gerster et al., 1996). These two genes are highly

homologous; they are 95% identical at the nucleic acid level and 98% identical at the amino acid level. Park et al. (2005) isolated and characterized a pollen preferential gene, *BAN102*, from the Chinese cabbage (*B. campestris*). After analyzing its sequence by BLAST search they found that the coding region of *BAN102* gene had great similarity with AGP23 gene from *Arabidopsis*. The similarities of nucleotide and amino acid sequences were 91% (170/186) and 90% (55/61), respectively.

The *BcMF8* (*B. campestris* male fertility 8) gene, possessing the features of classical AGP was later isolated from *B. campestris*. This gene was highly abundant in the fertile flower buds but silenced in the sterile ones of genic male sterile A/B line ('ZUBajh97-01A/B'). Expression patterns analysis suggested *BcMF8* to be a pollen-specific gene, whose transcript started to be expressed at the uninucleate stage and maintained throughout up to the pollination stage. Isolation and multiple alignments of the homologs of BcMF8 gene in the family *Cruciferae* indicated that *BcMF8* was highly conserved in this family sharing high sequence identity with those of the putative pollen-expressed AGPs genes *Sta 39-4* and *Sta 39-3*, and a lower similarity with that of AGP genes *AtAGP11* and *AtAGP6*.

Besides *Sta 39-4, Sta 39-3, AtAGP11, AtAGP6*, and *BAN102*, a pollen-specific AGP gene *PO2* from alfalfa has been characterized by Qiu et al. (1997) using a similar differential screening technology; after performing an alignment of the deduced BcMF8 protein sequence and its homolog from family *Cruciferae* with all these pollen-specific AGPs mentioned above and the other putative AGPs from the *Arabidopsis* database, the authors found that *BcMF8* clustered with those known pollen-specific AGPs, *Sta 39-4, Sta 39-3, AtAGP11* and *AtAGP6*. This result provided further evidence to the hypothesis that *BcMF8* was pollen-specific. Interestingly *BAN102* and *AtAGP23* are closely related to one another. Indeed, AtAGP23 belongs to AG-peptides subclass which differs in sequence composition from classical AGPs. These results may suggest that pollen development requires different members of the AGP family (Huang et al., 2008).

5.3 Nicotiana

Mollet et al. (2002) reported that *Lycopersicon pimpinellifolium, Aquilegia eximia*, and *Nicotiana tabacum* were not labeled with MAb JIM13 at their tube tips nor did the Yariv reagent bind there and arrest pollen tube growth, as opposed to *Lilium longiflorum and Annona cherimola*. The authors stated that the presence or absence of AGPs at the tube tip appeared to be species dependent. However, they do not exclude the possibility that other AGPs which do not possess the epitopes recognized by these antibodies and/or are not bound with β-GlcY may be present at the pollen tube tips of these species.

Qin and coauthors (Qin et al., 2007), also working with *Nicotiana*, showed that abundant AGPs were present in all areas of the pollen tubes after labeling with JIM13, including the tip region. In pollen tubes, immunogold particles were mainly distributed in the cell wall and cytoplasm, especially around the peripheral region of the generative cell wall. β-D-Glucosyl Yariv reagent, which specifically binds to AGPs, caused slow growth of pollen tubes and reduced immunogold labeling of AGPs with JIM13 *in vitro*. These data suggest that AGPs participate in male gametogenesis and pollen tube growth and may be important surface molecules in generative and sperm cells.

5.4 Malus

By searching for anther-specific genes involved in male gametophyte development in apple (*Malus* x *domestica* Borkh. cv. Fuji) by differential display-PCR, three full-length cDNAs were isolated, and the corresponding genomic sequences were determined by genome walking. The identified genes were intron-less with 228- to 264-bp open reading frames and shared 82–90% nucleotide sequence. Sequence analysis identified that they encoded a putative AGP and were designated *MdAGP1*, *MdAGP2*, and *MdAGP3*, respectively. By RT (reverse transcriptase)-PCR the authors showed that the *MdAGP* genes were selectively expressed in stamens. Promoter analysis confirmed that the MdAGP3 promoter was capable of directing anther or pollen specific expression of the GUS reporter in tobacco and apple. Furthermore, expression of ribosome-inactivating protein under the control of the MdAGP3 promoter induced complete sporophytic male sterility as expected (Choi et al., 2010).

6. Mode of action of AGPs; What are the possibilities?

As we have shown, most of the evidence involving AGPs in pollen development is based on immunolocalization of sugar epitopes, in the genetic manipulation of individual AGP backbone peptides or in the binding of AGPs to β-Yariv reagent, a synthetic reagent which binds AGPs in general, perturbing its biological function. Although significant to find modes of action for the AGPs, these observations are currently difficult to interpret. Also important is to compare the biology of AGPs with that of analogous molecules in animals, trying to match up to their modes of action.

6.1 Arabinogalactan proteins as cell wall components

Although precise functions of AGPs remain elusive, they are widely implicated in plant growth and development. When the Yariv reagent is added to seedlings or to cells in culture, a strong inhibition or even a growth arrest is observed, one possibility for this is the involvement of AGPs in cell wall polymer biosynthesis (Seifert & Roberts, 2007).

In plants most cells expand by diffuse growth; whereas root hairs and pollen tubes expand by tip growth. It is now well documented the high expressed number of AGP genes in the root hair transcriptome (Johnson et al., 2003) and on the pollen tube expressed genes profile (Wang et al., 2008). AGPs are deposited into the tip of growing pollen tubes (Dardelle et al., 2010; Pereira et al., 2006) and tip-growing pollen tubes are inhibited by genetic interference with GPI-anchor biosynthesis (Lalanne et al., 2004) and also by β-Yariv reagent treatment, leading to ectopic callose deposition and pectin alteration (Mollet et al., 2002). These results together entail the involvement of AGPs in growth and development.

The presence of AGP carbohydrate epitopes and AGP backbone peptides in secondary wall thickenings suggests that AGPs might be secreted to the cell surface in parallel with cellulose synthase, and they might be released from their GPI anchor and incorporated into cell wall thickening (Seifert & Roberts, 2007).

A well-characterized classical tomato AGP containing a glycosylphosphatidylinositol plasma membrane anchor sequence was used to elucidate functional roles of AGPs.

Transgenic tobacco BrightYellow-2 (BY-2) cells stably expressing GFP-LeAGP-1 were plasmolysed and used to localize LeAGP-1 on the plasma membrane and in Hechtian strands. Cytoskeleton disruptors and β-Yariv reagent were used to examine the role of LeAGP-1 as a candidate linker protein between the plasma membrane and the cytoskeleton. This study used two approaches. First, BY-2 cells, either wild-type or expressing GFP-microtubule (MT)-binding domain, were treated with β-Yariv reagent, and effects on MTs and F-actin were observed. Second, BY-2 cells expressing GFP-LeAGP-1 were treated with amiprophosmethyl and cytochalasin-D to disrupt MTs and F-actin, and effects on LeAGP-1 localization were observed. Collectively, these studies indicated that GPI-anchored AGPs function to link the plasma membrane to the cytoskeleton (Sardar et al., 2006).

6.2 Arabinogalactan proteins as gradients

Plant reproduction involves a series of interactions between the male gametophyte (the pollen grain or pollen tube) and the extracellular matrix molecules secreted by different cell types along the pollen tube growth pathway in the pistil. These interactions are believed to signal and regulate the pollen tube growth process to effect successful delivery of the sperm cells to the ovules where fertilization takes place. AGPs are believed to play a broad range of functions, ranging from providing structural integrity to mediating cell-cell interactions and communication. Upon germination on the stigma, pollen tubes elongate in the stylar transmitting tract, aided by female factors, with speed and directionality not mimicked in *in vitro* pollen tube growth cultures. It was shown that a stylar transmitting tissue arabinogalactan protein from *N. tabacum*, a TTS (Transmitting Tissue Specific) protein, stimulates pollen tube growth *in vivo* and *in vitro* and attracts pollen tubes grown in a semi-*in vivo* culture system. Within the transmitting tissue, TTS proteins display a gradient of increasing glycosylation from the stigmatic end to the ovarian end of the style, coincident with the direction of pollen tube growth (Cheung et al., 1995; Wu et al., 1995).

Gradients of morphogens are a hallmark in animal development, chemoattractants are important to microbial as well as to animal cell motility systems and developmental pathways; they are also believed to function in the directional growth of pollen tubes (Reger et al., 1992). The gradient of increasing TTS protein glycosylation coincident with the direction of pollen tube elongation is a unique protein-based sugar gradient observed in the female reproductive tissue.

6.3 Arabinogalactan proteins as signaling molecules

AGPs have frequently been hypothesized to be sources of soluble signal molecules, in the form of sugar chain fragments, it is now well established that sugars act as signaling molecules (Hanson & Smeekens 2009) and/or as sources of lipid signal molecules, in the form of the lipid chains that are liberated upon fracture of the GPI anchor, and which may diffuse in the plane of the plasma membrane. Despite the scarcity of experimental evidence to support such roles, it is indeed the combination of AGP main features, namely cell surface localization, likely presence in membrane lipid rafts through their GPI anchor, possible controlled release from the plasma membrane upon stimuli, and the complexity of their

sugar content, that makes AGPs, or specific fragments of AGPs, as likely candidates to perform signaling functions in plants, and in plant reproductive processes, in particular. In mammals, species specificity in fertilization occurs through the interactions between sperm cell and egg cell, with cell surface proteins acting as key determinants (Vieira & Miller, 2006). Although flowering plants and mammals have evolved very divergent mechanisms for fertilization and reproductive species recognition, it is highly likely that in both cases GAPs may be used to regulate key steps in fertilization phenomena.

PTs with their unique type of growth restricted to the tip and with an intense secretion and endocytic activity needed for this rapid cell expansion at the apex are always depending on the communications with the pistil molecules. Most probably, the AGPs released to the membrane as part of this exocytic activity are fundamental pieces in the signaling network that directs with precision, pollen tubes to their target, the embryo sac cells.

7. Conclusions and future perspectives

Experiments performed integrating reverse genetics and other experimental approaches, led us to believe that the observed fertility reduction in *agp6 agp11* double null mutant was due to abortion of pollen grains during development (Coimbra et al., 2009). We have further characterized the anthers and pollen of *agp6 agp11* and concluded that both AGPs, AGP6 and AGP11, are necessary for the proper pollen tube growth as well as for preventing untimely pollen grain germination (Coimbra et al., 2010). Further details on the biology of pollen-specific AGPs are expected to emerge from a microarray experiment performed on RNA isolated from pollen tubes of *agp6 agp11* mutant, and which is currently under analysis.

Whether AGPs are predominantly structural, or nutrient-providers, or signaling molecules, is yet to be determined. We are committed to search for AGP-specific ligands recently identified for tobacco pistil AGPs (Lee et al., 2008), reason why we performed microarrays in the double null mutant, *agp6 agp1* pollen tubes, to try to bring some clarification for the biological way of action of this ubiquitous class of plant proteoglycans.

Key future challenges are also the elucidation of the enzymatic machinery that synthesizes the AG carbohydrate structure and the molecular nature and biological role of endogenous AG-specific carbohydrate hydrolases. AGP galactosyltransferase (GalT) activities in tobacco and *Arabidopsis* microsomal membranes were studied with an *in vitro* GalT reaction system. This *in vitro* assay reported to detect GalT activities using AGP peptide and glycopeptide acceptor substrates provides a useful tool for the identification and verification of AGP-specific GalT proteins/genes and an entry point for elucidation of arabinogalactan biosynthesis for AGPs (Liang et al., 2010).

8. Acknowledgment

This work was supported by funding from FCT (Fundação para a Ciência e Tecnologia, Portugal) within the project PTDC/AGR-GPL/67971/2006.

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17

Trichome Specific Expression: Promoters and Their Applications

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1. Introduction

As often reminded to the readers in articles or reviews which deal with plant adaptation to their environment, higher plants are sessile organisms, a life habit which does not allow them to escape danger or to move to avoid adverse conditions. This environmental pressure has led to a myriad of adaptations, which are reflected in the vast diversity of plant habitats, morphologies, life cycles and physiological adaptations among others. The surface of the aerial parts of plants is a major interaction domain between the plant and its environment and as such is the site of many adaptations, be they chemical or anatomical. Among those adaptations, the leaf hairs or trichomes, which cover the surface of a large number of plant species, play a prominent role. Plant trichomes constitute a world of their own, so great is their diversity. In a review published in 1978 and entitled "A glossary of plant hair terminology", Payne compiles a comprehensive list of more than 490 terms used to describe trichome morphology (Payne, 1978). Despite this extensive diversity, two major classes of trichome may be distinguished on the basis of their capacity to produce and secrete or store significant quantities of secondary metabolites, namely glandular or non-glandular. Non-glandular trichomes, or leaf hairs, are poorly metabolically active and provide protection mainly through physical means, for example by restricting access to insects, but also by preventing water losses, or protecting against UV radiation. *Arabidopsis thaliana* has been a model for the study of non-glandular trichome development and many genes involved in non-glandular trichome initiation and development could be identified and characterized (Uhrig and Hulskamp, 2010). The metabolic activity of these non-glandular trichomes is however fairly limited and offers little potential for metabolic engineering. A particular class of hairs is the fibers which are present in various species. Cotton seed trichomes are the most economically important since they are the basis of the cotton fiber, but other species such as cottonwood also have fiber hairs. Glandular trichomes are present in many different plant families and can also be divided in two main classes. The capitate trichomes typically have 1 to 10 glandular cells located at the tip of the trichome stalk, and the secretion is directly exuded from the top cells. The secreted material is in general fairly viscous, and in many cases it makes the leaves sticky. Those trichomes are encountered for example in the Solanaceae (tobacco, tomato, potato, etc.) and in some Lamiaceae species (e.g. *Salvia*). Peltate trichomes have the capacity to synthesize and store volatile compounds (mono- and sesquiterpenes, phenylpropenes) in a subcuticular cavity. Typical representative examples

are those from mint and other Lamiaceae, which are valued for the essential oil produced in their trichomes. In both cases, the massive metabolic fluxes that take place in the secretory cells may lead to the accumulation of metabolites which represent up to 10-15% of the leaf dry weight (Wagner *et al.*, 2004). These cells can thus be considered like true cell factories and therefore constitute attractive targets for metabolic engineering (Schilmiller *et al.*, 2008).

1.1 Why trichome specific promoters?

Whether they are cotton fibers or glandular trichomes producing essential oils or resins, the availability of genes and promoters which are specifically expressed in those structures provides material both for more in-depth studies of trichome specific processes and for high precision engineering of trichome traits. A number of genes which are highly expressed in trichomes may also be expressed in other organs because they are involved in similar processes there. The promoters from these genes are not ideal for the study of trichome specific processes for obvious reasons. Using these promoters will lead to expression outside of the trichomes and may lead to undesirable effects because of the toxicity of the compounds produced. A trichome specific promoter may be used in several ways to further investigate trichome processes. One is to search for transcription factors by one-hybrid screening or other related methods. Unbiased search for upstream regulators may also be achieved in mutant screens in plants expressing promoter:reporter gene fusions. Although not necessarily practical in the species of interest (for example in mint which is a sterile polyploidy species), a convenient host with conserved features but which is more amenable to transformation and screening, may be chosen for this purpose.

Another major motivation to isolate and characterize trichome specific promoters is genetic engineering, in particular for the expression of metabolic pathway genes. When expressed under a strong ubiquitous promoter, like the Cauliflower Mosaic Virus (CaMV) 35S, perturbation of metabolic pathways in the whole plant may have deleterious consequences on plant development and physiology. The trichomes, as a distinct entity with restricted communication to the rest of plant, represent therefore a particularly interesting target for metabolic engineering.

Besides metabolic engineering, the availability of trichome specific regulators may help to modify trichome related traits. For example, modulating the expression of transcription factors specifically controlling trichome differentiation and/or development could lead to an increase in trichome density, an improvement of the productivity of trichome-based secretions (e.g. essential oils) or a boost in trichome-mediated resistance to insect pests or other pathogens.

2. Cotton

2.1 Genomics of cotton fibers

Cotton fibers are specialized single-celled hairs which develop on ovules. The cotton hairs are among the longest plant cells reported and are coated with cellulose fibers which confer its value to the cotton crop. Because cotton hairs are single-celled, it has been proposed that their development is controlled by similar gene networks as those of Arabidopsis leaf trichomes, which are also single-celled but branched. It should be noted however, that Arabidopsis seeds do not have trichomes and thus cannot be considered as an ideal

surrogate model to evaluate the specificity of expression of cotton fiber genes. The development of seed trichomes is a synchronized process with several easily distinguishable phases. These have been well documented in previous reviews and will be briefly summarized here. The initiation of fiber cells takes place early on at the onset of anthesis, which is conveniently used as the reference time point expressed in days post anthesis (DPA) (Lee *et al.*, 2007). Already after 2 DPA, the fibers start elongating, a process which lasts until 20 DPA. This is followed by secondary wall biosynthesis until 45-50 DPA and concluded by the maturation phase. The synchronized process has allowed the preparation of RNA from these different phases. Initially, fiber specific genes were isolated by differential screening of cDNA library. This led to the successful identification of several genes with strong and specific expression in fibers, including E6, genes encoding Lipid Transfer Proteins (LTPs), a Proline Rich Protein and other genes with no obvious sequence similarity (John and Crow, 1992; Ma *et al.*, 1995; Orford and Timmis, 1995; Rinehart *et al.*, 1996; Orford and Timmis, 1997; Orford and Timmis, 1998; Orford *et al.*, 1999). Already, Northern or RT-PCR analysis showed that genes can be expressed during distinct phases of development of the fiber cells or throughout the life of these cells. This is relevant since the promoters from these genes should allow to direct the expression of transgenes during given stages of development of the fibers, which may have important practical consequences depending on the engineering objective. These early studies were followed by genomics approaches, including Expressed Sequence Tag (EST) library sequencing and microarray hybridization. In particular, EST libraries corresponding to various stages of development were produced and these provide invaluable resources for the identification of fiber specific genes (Li *et al.*, 2002a; Arpat *et al.*, 2004; Udall *et al.*, 2006; Yang *et al.*, 2006). As genes from these EST collections start being characterized, more information has become available on the pattern of expression and the importance of some transcription factors in fiber development (Lee *et al.*, 2007). For some of the genes, the promoters have been cloned and characterized by transgenesis or transient assays. Because cotton transformation is a lengthy process, alternative hosts have been used to characterize cotton promoter:GUS fusions. In most cases, these are either *Arabidopsis thaliana* or tobacco (*Nicotiana tabacum*). These hosts are far from ideal when it comes to characterize seed fiber specific expression because they are both devoid of seed trichomes. Arabidopsis is perhaps a little better because its trichomes are single-celled, like those of cotton, whereas those of tobacco are typically multicellular. There is, in addition, evidence that single celled trichomes from Arabidopsis, which, like cotton, belongs to the Rosids, and multicellular trichomes of the Solanaceae or other Asterids (Antirrhinum) are under the control of distinct regulatory network (Serna and Martin, 2006). A list of available cotton fiber promoters is provided in Table 1. This list is probably not exhaustive, but contains already 28 promoters, underscoring the high interest in characterizing such promoters. The expression range, expressed in DPA was compiled, and illustrates the diversity of promoters available, from the differentiation stage to the late secondary wall synthesis phase. Thus, targeting engineering to specific phases of fiber development is theoretically possible. It is difficult to compare the strength of these promoters between them, as they were often assessed in independent studies using different methods (Northern, semi-quantitative and quantitative RT-PCR,). Nonetheless, it can be assumed that genes with a function in cell wall biosynthesis, e.g. cellulose synthase, are probably among the most highly expressed.

Gene	Protein description	Expression measured by RT-PCR or Northern	Expression window in cotton fibers (in days)	Expression in other tissues	References
GhE6	hypothetical	Y	15-24	N	(John and Crow, 1992)
GhLTPx_GH3	Lipid transfer protein	Y	5-20	N	(Ma *et al.*, 1995)
FbL2a	Hypothetical	Y	25-45	N	(Rinehart *et al.*, 1996)
pGhEX1	Expansin	Y	6-20	N	(Orford and Timmis, 1998; Harmer *et al.*, 2002)
GhLTP6	Lipid transfer protein	N	10-20	N	(Ma *et al.*, 1995; Hsu *et al.*, 1999)
GhLTP3	Lipid transfer protein	N	5-20	N	(Liu *et al.*, 2000)
GhTUB1	beta-tubulin	Y	0-14	early seedling development (cotyledons, root tips)	(Li *et al.*, 2002b)
GhCTL1-2	Chitinase-like	Y	8-31	xylem, pollen, cells with secondary walls (weak)	(Zhang *et al.*, 2004)
GaRDL1	RD22_like	Y	3-12	N	(Wang *et al.*, 2004)
GhACT1	Actin	Y	4-21	Cotyledons	(Li *et al.*, 2005)
GhDET2	Steroid reductase	Y	3-14	Roots	(Luo *et al.*, 2007)
GhGlcAT1	glucuronosyltransferase	N	NA	NA	(Wu *et al.*, 2007)
Fsltp4	Lipid transfer protein	Y	6-14	N	(Delaney *et al.*, 2007)
GhTUA9	alpha-Tubulin	Y	5-10	N	(Li *et al.*, 2007)
GaHOX1/2	Transcription factor	Y	3-12	N	(Guan *et al.*, 2008)
GaMYB2	Transcription factor	Y	0-9	trichomes in other organs	(Wang *et al.*, 2004; Shangguan *et al.*, 2008)
GhMYB109	Transcription factor	Y	4-8	N	(Suo *et al.*, 2003; Pu *et al.*, 2008)
GhSCFP	Protease	N	2-25	N	(Hou *et al.*, 2008)
GhH6L	Arabinogalactan	Y	3-20	N	(Wu Y, 2009)
GhMYB25	Transcription factor	Y	0-5	trichomes of other tissues, pollen, anthers, root epidermis, root initials	(Machado *et al.*, 2009)

Gene	Protein description	Expression measured by RT-PCR or Northern	Expression window in cotton fibers (in days)	Expression in other tissues	References
GhSUS3	Sucrose synthase	Y	0-5	NA	(Ruan *et al.*, 2009)
GhXTH1	Xyloglucan endotransglycosylase/hydrolase	Y	10-25	N	(Michailidis *et al.*, 2009)
GbML1	Transcription factor	Y	-3-8	Petal	(Zhang *et al.*, 2010)
GhRING1	Ubiquitin Ligase	Y	0-20	NA	(Ho *et al.*, 2010)
GhXTH1	Xyloglucan endotransglycosylase/hydrolase	Y	10-15	Petal	(Lee *et al.*, 2010)
ADPGp_SSU2	ADP-glucose pyrophosphorylase	Y	10	meristem, immature stem, roots	(Taliercio, 2011)
GhCesA4	Cellulose synthase	Y	16-24	root vascular tissue	(Wu *et al.*, 2009; Kim *et al.*, 2011)

Table 1. Promoters expressed in cotton fibers. DPA: days post-anthesis. Y: yes; N: no; NA: not available

2.2 Examples of engineering of cotton trichomes

The first attempts at genetic engineering of cotton fibers were performed in the late 1990s, soon after the first specific promoters were identified. The objective was to introduce poly-hydroxybutyrate (PHB) into cotton fibers, via the expression of two genes *phaB* and *phaC* from the bacterium *Alcaligenes eutrophus*, which naturally produces PHB in inclusion bodies. *phaB* encodes the acetoacetyl-CoA reductase and *phaC* the PHB synthase. Expression of both genes in *Arabidopsis thaliana* was previously shown to support *de novo* biosynthesis of PHB in plants for the first time (Poirier *et al.*, 1992). In cotton, this was achieved by expressing *phaB* under the control of the promoters from the fiber specific genes FbL2a or E6, and *phaC* with the FbL2a or 35S promoters. Since the substrate for the PHB synthase does not occur naturally in plants, the expression of *phaC* under 35S should not have deleterious effects on whole plants. The transgenic plants were reported briefly in a first paper (Rinehart *et al.*, 1996) and analyzed in more detail in a second article (John and Keller, 1996). Production of PHB in the lumen of cotton fiber cells could be shown as evidenced by staining, electron microscopy, HPLC and GC-MS. PHB accumulated in the form electron-translucent granules. Quantification of crotonic acid released after hydrolysis indicated levels of up to 3440 µg/g dry fiber in the best lines. The majority of the PHB produced (68.3 %) had a MW above 0.6×10^6 Da, which is similar to PHB produced in bacteria. PHB synthesis peaked at 10 DPA and did not increase nor decrease afterwards, indicating the absence of major PHB degrading activity in cotton fibers. The thermal properties of the transgenic fibers were also assessed and indicated that they had higher heat retention capacity (John and Keller, 1996). However, although promising, those modified properties were apparently not significant enough to warrant commercialization. This was due to the relatively low level of PHB produced (0.34% of fiber weight), which would need to increase several fold to be considered for commercialization.

In a more recent attempt at metabolic engineering, melanin biosynthesis was introduced in cotton fibers (Xu *et al.*, 2007). Dyeing cotton fibers has a heavy imprint on the environment and solutions to reduce its polluting impact are desirable. Naturally colored cotton fibers exist but the choice of colors is limited and the colored cotton varieties have low producing capacity. An alternative is to use biotechnology to engineer colors into cotton fibers. As a proof of concept, Xu and co-workers (2007) expressed two genes, *TyrA* and *ORF438*, from *Streptomyces antibioticus*, which are required and sufficient to synthesize melanin. Both genes were codon optimized for expression in cotton, fused to a vacuolar targeting peptide and cloned under the control of a fiber specific promoter from the *Ltp3* gene (Liu *et al.*, 2000). The same construct was used to transform tobacco and cotton. Both in tobacco and in cotton transgenic plants the change in color in the leaf trichomes (tobacco) or in the seed fibers (cotton) was distinctly visible although no dosage of melanin was reported (Xu *et al.*, 2007). In addition to its color, melanin also absorbs UV light and could therefore provide UV-protection properties to cotton fabrics.

3. Tobacco

Tobacco (*Nicotiana tabacum*) is an allotetraploid species which is grown worldwide for its leaf which is processed and used for various products, from which the most widely sold and consumed are cigarettes. It is well established that regular tobacco smoking is a health-damaging habit with associated increased risks of cancer and cardio-vascular diseases. Health-promoting uses of tobacco could provide alternative revenue sources for tobacco farmers, for example by producing pharmaceutical ingredients in tobacco through genetic engineering. Plant Made Pharmaceuticals (PMPs) have mostly concerned therapeutic proteins, such as antibodies or hormones like insulin. Plants are also known to provide many natural small molecules to the pharmacopeia or as drug leads. These belong to the secondary, or specialized, as they are now sometimes called, classes of metabolites. The huge diversity of these compounds provides a phenomenal reservoir of chemical structures whose biosynthesis pathways are now beginning to be elucidated thanks to the contribution of genomics approaches in plant biochemistry studies. One issue which is frequently raised about plant natural products is the availability of the raw material and the cost associated to extraction and purification of the compound. Pharmaceutical companies will shy away from substances whose supply cannot be safely guaranteed, which is likely to be the case if the chemical is produced in one rare plant of the Amazon forest for example. But the plant does not need to come from tropical forest to be endangered. The story of Taxol is a good example in this respect. Taxol is a diterpenoid extracted from yew tree with potent anticancer activity. Initially, Taxol was extracted from the barks of pacific yew trees (*Taxus brevifolia*), where it was present in less than 0.01% of the dry matter, with many related taxoids to separate it from, making it an extremely expensive chemical to produce. Chemical synthesis was too complex to be exploited commercially. Since the extraction was destructive, natural populations of Taxus were threatened through commercial exploitation of the trees. Fortunately, a semi-synthetic method starting from a precursor abundant in the twigs, 10-deacetyl-baccatin III, was developed. This allowed a durable and renewable procedure since twigs can be harvested without felling trees.

The presumed progenitors of *N. tabacum* are *N. sylvestris* and *N. tomentosiformis*. All three species have glandular capitate trichomes on their leaf and stem surfaces, with distinct exudate profiles. Cultivated tobacco (*Nicotiana tabacum*) and its wild relatives, *Nicotiana sylvestris* and *N. tomentosiformis* produce diterpenes in large amounts in their glandular capitate trichomes. *N. tomentosiformis* secretes large quantities of labdanoid diterpenes. In *N. tabacum*, these may have two types, either macrocyclic cembranoid or bicyclic labdanoids. The cembranoids are also produced by *N. sylvestris* trichomes and include the cembratrien-diols (α- and β-CBT-diols) and their precursors the cembratrien-ols (α- and β-CBT-ols). Labdanoids include Z-abienol and labdene-diol. Depending on the variety, these diterpenoids may be present in varying amounts and combinations. The terpenoid biosynthesis capacity of tobacco glandular trichomes is massive. In the appropriate conditions, the amount of CBT-diols produced by a *N. sylvestris* leaf may represent up to 10% of the leaf dry weight. Early studies performed on tobacco glandular trichomes concluded that the biosynthesis of the diterpenes takes place in the trichome heads themselves (Keene and Wagner, 1985; Kandra and Wagner, 1988; Guo *et al.*, 1994). This, together with the high productivity of tobacco trichomes makes them an ideal target for terpenoid metabolic engineering.

Terpenes are hydrocarbon molecules whose structure is based on repeated units of isoprene. They are derived from two C5 precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP units can be sequentially added to DMAPP by isoprenyl transferases, thus leading to the major short chain isoprenyl diphosphates, geranyl diphosphate (GPP- C10), farnesyl diphosphate (FPP – C15) and geranylgeranyl diphosphate (GGPP – C20). These isoprenyl diphosphates are the substrates of terpene synthases, which in many cases make cyclic products. These are the origin of the skeletal diversity of terpenes. In tobacco, the pathway to the cembratrien-diols was elucidated and shown to involve two steps. The first is encoded by a multigene family of diterpene synthases, the cembratrien-ol synthases (CBTS), which altogether account for the mix of the two stereoisomers of CBT-ol (α and β) (Wang and Wagner, 2003; Ennajdaoui *et al.*, 2010). The second step is carried out by a cytochrome P450 mono-oxygenase which hydroxylates the CBT-ols at a specific position (Wang *et al.*, 2001; Wang and Wagner, 2003). Since the biosynthesis of trichome diterpenoids specifically takes place in the glandular cells, one way to get trichome specific promoters is to clone the corresponding genes.

3.1 Tobacco trichome specific promoters

The first tobacco trichome specific promoter identified was that of the *CYP71D16* gene, which encodes the CBT-ol hydroxylase (Wang *et al.*, 2002). The gene was itself identified through subtractive cDNA library construction which was followed by the cloning of the promoter. 1.8 kb of the promoter was sufficient to confer a highly specific expression of the GUS reporter gene to the trichomes. Remarkably, only the glandular cells were stained, highlighting the distinct differentiation status between the glandular cells of the head and the non-glandular cells of trichome stalk.

The CBTS genes provided another set of trichome specific promoters. It was found that the CBT-ol synthase activity is encoded by a family of 3 closely related genes, which arose via recent duplication event. These genes share over 90 % identity at the nucleotide level, including in the promoter regions. One of those promoters (pCBTS2a) was further studied

with sequential and internal deletions. This allowed the identification of a positive regulatory region and a negative regulatory region. When the inhibitory region is deleted, expression can be detected in the whole leaf epidermis as well as in patches in roots. On the contrary, when the activating region is deleted, no expression at all can be detected (Ennajdaoui *et al.*, 2010). This indicates that the cell specific expression is the result of the unique combination of a broad activating region with an inhibitory region which restricts expression to the desired cells.

Genes involved in the other tobacco labdanoid pathway have been recently identified and one promoter was also identified and characterized as trichome specific (Tissier, unpublished results).

The availability of several distinct promoters with identical specificity and different strengths should broaden the possibilities for metabolic engineering.

3.2 Strategies and example for tobacco trichome engineering using specific promoters

Since tobacco produces diterpenoids, one logical possibility for metabolic engineering is to use tobacco trichomes for the production of heterologous diterpenoid. The substrate, namely GGPP, should be available in non-limiting quantities and in addition, the glandular cells have a machinery which allows them to excrete hydrophobic compounds like diterpenes. To facilitate the detection of heterologous terpenoids, it may be useful to eliminate or reduce the endogenous diterpenoids. This can be achieved by inactivating the *CBTS* genes. Because they are members of a multigene family which are most likely located at the same chromosomal locus, the most efficient way to achieve this is to use gene silencing technologies. This was done with an antisense construct under the control of a 35S promoter (Wang and Wagner, 2003). However, more efficient silencing was obtained with intron-hairpin constructs targeting the exon 2 of the *CBTS* genes, under the control of the *CBTS2a* gene itself. In this case, the best transgenic lines had almost no CBT-diols detectable (Ennajdaoui *et al.*, 2010). There is also the possibility to exploit natural variation in *N. tabacum*. During a survey of the metabolic profiles of tobacco leaf exudates we have noticed that some cultivars produce labdanoids and no cembranoids, while others produce cembranoids and no labdanoids. Thus, by crossing these cultivars it is theoretically possible to breed new varieties which produce no diterpenoids at all, but which still have the capacity to produce new ones. Once a diterpene-free background has been established by either of these approaches, heterologous diterpene synthases may be cloned behind trichome specific promoters for targeted expression to the glandular cells. This was successfully done for taxadiene synthase (Rontein *et al.*, 2008) with yields of up to 10 µg/g fresh weight.

3.3 Glandular trichome expression as a gene function discovery tool

Several steps of Taxol biosynthesis have been investigated, including the early oxidations of taxadiene which lead to the synthesis of the important semi-synthesis precursor, 10-DABIII (Croteau *et al.*, 2006). The first of these oxidations was shown to be at the C5 position of the taxadiene core, which is necessary to form the so-called oxetane ring (Hefner *et al.*, 1996). Subsequently, a gene encoding taxadiene 5-α-hydroxylase (*T-5-OH*) was identified and

characterized (Jennewein *et al.*, 2004). In order to reconstitute these early steps of the taxol biosynthesis pathway, both genes were expressed in tobacco under the control of trichome specific promoters. Taxadiene synthase (TS) was cloned downstream of the CBTS2a promoter as described above while the T-5-OH was cloned 3′ of the CYP71D16 promoter. Surprisingly, no taxadien-5-α-ol could be identified in the transgenic plants expressing both genes. Instead, a new product, which was later found to be 5(12)-oxa-3(11)-cyclotaxane, derived from a complex rearrangement of the taxadiene core upon oxidation, could be identified (Rontein *et al.*, 2008). The activity of the enzyme was then further proved from a protein expressed in yeast. This shows that the tobacco trichome platform was useful to produce sufficient quantities of the product to be structurally characterized. The initial functional assignment was likely misguided by the presence of small amounts of T-5-OH as a by-product in the enzyme assays. Since T-5-OH was the compound that was looked for, the major product may have been ignored. The novel assignment derived from the initial tobacco expression, was later confirmed by expression in *E. coli*, although in this case significant amounts of T-5-OH could be detected (Ajikumar *et al.*, 2010).

In another example, the function of the genes required for the biosynthesis of *Z*-abienol in tobacco could be confirmed by expression in *N. sylvestris* trichomes. *Z*-abienol is a labdane diterpenoid whose biosynthesis was predicted to require two successive enzymatic steps (Guo *et al.*, 1994), first a copalyl-diphosphate synthase like then a kaurene synthase like enzyme (Peters, 2010). Two candidate genes were thus identified and expressed in *N. sylvestris*, which does not produce *Z*-abienol. The exudate of these transgenic plants contained significant amounts of *Z*-abienol of up to 100 µg/g FW (Sallaud and Tissier, unpublished results).

The supply of isoprenyl diphosphates IPP and DMAPP in tobacco trichomes should allow also engineering of other terpenoid classes, like mono- or sesquiterpenes. In those cases, the appropriate isoprenyl transferases (i.e. geranyl diphosphate or farnesyl diphosphate synthases) should be expressed in addition to the terpene synthases. This was done for a sesquiterpene synthase from tomato, the santalene and bergamotene synthase, which uses an unusual isoprenyl diphosphate precursor, *Z,Z*-FPP (Sallaud *et al.*, 2009). Both enzymes are naturally targeted to the plastids, which is where IPP and DMAPP from the methyl-erythritol pathway (MEP) are synthesized. In this case, the sesquiterpenes could not be identified in the leaf exudate, rather in the headspace collected from transgenic plants (Sallaud *et al.*, 2009). This indicates that tobacco trichomes are suitable for the biosynthesis of sesquiterpenes, but not for their storage. For these, and other volatile compounds, such as monoterpenes or phenylpropenes, glandular trichomes with a storage compartment for volatile compounds, such as the peltate trichomes of mint, should be used (see below).

3.4 Other examples of tobacco trichome engineering

As in cotton, the genes for the biosynthesis of the pigment melanin were expressed in tobacco under the control of the cotton trichome promoter LTP3 (Xu *et al.*, 2007). This promoter was previously shown to be active in tobacco trichomes (Liu *et al.*, 2000). Based on the color of trichomes, the presence of melanin could be detected, however no quantification was performed. This, however, shows that glandular trichomes which are normally producing terpenoids may also be used as an engineering platform for other classes of compounds.

4. Mint and other lamiaceae

4.1 Mint

Peppermint (*Mentha x piperita*) is an aromatic plant which is grown worldwide for its essential oil whose distinctive character is imparted by its most well known compound, (-)-menthol. The essential oil of mint, and of many other aromatic plants form the Lamiaceae, is stored in glandular trichomes of the peltate type (Gershenzon *et al.*, 1987; Gershenzon *et al.*, 1989). Peltate trichomes are composed of 8 glandular cells topped by a subcuticular space where the secretion products are stored. When the cuticule is ruptured, by pressing the leaf between the fingers, or by an insect, the volatile compounds are released and may reach their target. It was shown that the peltate trichomes are not just a site of storage, but also that the terpenoids are produced in the peltate glandular cells (Gershenzon *et al.*, 1989). A technique for the purification of intact peltate glands was developed to allow the production of a trichome specific EST library (Gershenzon *et al.*, 1992). This EST library provided sequence information for the characterisation of the (-)-menthol biosynthetic pathway, which was completely elucidated over the years by the research group from Prof. Croteau (Alonso *et al.*, 1992; Gershenzon *et al.*, 1992; Rajaonarivony *et al.*, 1992; Lupien *et al.*, 1999; Turner *et al.*, 1999; Gershenzon *et al.*, 2000; McConkey *et al.*, 2000; Bertea *et al.*, 2001; Wust *et al.*, 2001; Croteau *et al.*, 2005). Thus, peltate trichomes are extremely well adapted for the production and storage of volatile compounds, in particular mono- and sesquiterpenoids. Metabolic engineering of mint trichomes should therefore yield particularly interesting results for these volatile compounds. Mint transformation by *Agrobacterium tumefaciens* was independently reported by several groups (Diemer *et al.*, 1998; Weller *et al.*, 1998). However, although the use of trichome specific promoters was proposed as early as 1999 as a pre-requisite for metabolic engineering in mint (Lange and Croteau, 1999), to our knowledge no characterization of trichome promoters from mint has been published to date. The promoter of the Arabidopsis GL1 transcription factor was shown to be functional in tobacco and peppermint (Gutierrez-Alcala *et al.*, 2005). However, whether the strength of this promoter will be sufficient for metabolic engineering remains to be seen. Nonetheless, the whole menthol pathway from spearmint provides a set of genes with trichome specific expression and the identification of their promoters should not raise major difficulties.

4.2 Basil

Like mint, Basil (*Ocimum basilicum*) is grown for its aromatic properties which are due to volatile compounds produced in similar peltate trichomes. Following the successful approach developed in mint, trichome specific EST libraries from different cultivars of basil, corresponding to distinct chemotypes, were produced. These were used to elucidate the pathways to volatile phenylpropenes and monoterpenes (Gang *et al.*, 2001; Gang *et al.*, 2002a; Gang *et al.*, 2002b; Iijima *et al.*, 2004a; Iijima *et al.*, 2004b). As in mint, the enzymes of the pathway are likely to be highly specific to the peltate glandular cells, and therefore the promoters of the corresponding genes should drive specific expression to these cells. Like mint, basil could prove an interesting host for the metabolic engineering of volatile compounds, with the additional option of the capacity to engineer phenylpropanoid metabolism in accessions which produce phenylpropenes. However, to date no promoters of basil trichome genes have been characterized. One could also assume that promoters from mint should operate in basil, and reciprocally, because of the similarity of their trichomes and the fact that mint and basil both belong to the Lamiaceae.

4.3 Sage

Sage (*Salvia sp.*) is a large genus with a number of species which are grown commercially for the extraction of fragrant or aromatic oils. One of the most important is *Salvia sclarea* (clary sage), a biennial plant which produces both an essential oil rich in linalyl acetate and linalool, and a concrete with high amounts of the labdanoid diterpene sclareol. Sclareol is currently used in the fragrance industry as a synthesis precursor for Ambrox®, a highly valued compound with amber-like fragrance und excellent fixative properties (Decorzant *et al.*, 1987; Martres *et al.*, 1993; Koga *et al.*, 1998; Moulines *et al.*, 2001; Barrero *et al.*, 2004; Moulines *et al.*, 2004). *Salvia sclarea* possesses two types of glandular trichomes, capitate and peltate. The capitate trichomes are likely to produce sclareol, which is secreted onto the surface of the inflorescences while the volatile compounds like linalyl acetate are more likely to be produced in peltate trichomes (Lattoo *et al.*, 2006; Schmiderer *et al.*, 2008). The productivity of sclareol by *Salvia sclarea* is very high, making it an attractive target for metabolic engineering of terpenoids. Recently, massive sequencing of calyx RNA, where peltate and capitate glands are highly abundant, was carried out (Legrand *et al.*, 2010). A number of genes encoding proteins with clear similarities to terpene synthases and enzymes isoprenoid metabolism could be identified, thus providing genes with potentially highly specific pattern of expression, notably restricted to trichome glandular cells. Although transformation and regeneration of transgenic *Salvia sclarea* plants has not been achieved to date, hairy root cultures were established (Kuzma *et al.*, 2006; Kuzma *et al.*, 2008), and transformation of a related species (*Salvia miltiorrhiza*) by *Agrobacterium tumefaciens* could be successfully demonstrated (Yan and Wang, 2007; Lee *et al.*, 2008). These results suggest that trichome specific metabolic engineering of clary sage is technically feasible.

4.4 Lavender

Lavender (*Lavandula angustifolia, L.* x *intermedia* and other species) is a perennial plant grown in the Mediterranean area for its highly fragrant and characteristic essential oil, which is a complex mixture of mono- and sesquiterpenoids. As for other Lamiaceae species discussed above, the essential oil is produced in peltate glandular trichomes located mostly on the inflorescences (Guitton *et al.*, 2010). Here also, genomics approaches have been initiated to better understand the molecular basis of essential oil production. In one study, EST libraries from flowers and leaves were sequenced by the Sanger method to yield a total of 14,000 sequences (Lane *et al.*, 2010), thus providing the foundation to identify trichome specific genes. A recent study also showed that the oil profile changes over the course of flower development correlated with changes in expression of certain terpene synthases, providing important information regarding harvest time (Guitton *et al.*, 2010). In addition, a trichome specific promoter from the linalool synthase of *L. angustifolia* (LaLIS) was recently isolated and characterized (Biswas *et al.*, 2009). Lavender transformation is also well established, having been reported by two independent groups (Mishiba *et al.*, 2000; Nebauer *et al.*, 2000). Attempts were also made at metabolic engineering of essential content by overexpressing 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) and a limonene synthase with a constitutive 35S promoter (Munoz-Bertomeu *et al.*, 2007; Munoz-Bertomeu *et al.*, 2008). Overexpression of HMGR lead to an increase of both monoterpenes and sesquiterpenes, indicating that the cytosolic mevalonate pathway may contribute to both types of terpenes, although monoterpenes are synthesized in the plastids (Munoz-Bertomeu *et al.*, 2007). Overexpression of the spearmint limonene synthase on the other hand led to a strong

increase in limonene while not affecting the other constituents of the oil, indicating that the supply of isoprenyl diphosphate precursors is likely not to be limiting in glandular trichomes (Munoz-Bertomeu *et al*., 2008). These results bode well for the metabolic engineering of lavender trichomes, and no doubt that the availability of specific promoters should allow more precise manipulation of essential production in these species.

5. Tomato

Like tobacco, tomato (*Solanum lycopersicum*) belongs to the family Solanaceae, which is rich in species with trichomes. Wild species of tomatoes, such as *Solanum pennellii*, *S. habrochaites*, and *S. peruvianum* among others, have different trichome types including non-glandular and glandular types. Altogether up to 7 different types could be described, of which 3 main glandular types could be described (Luckwill, 1943)(See Figure 1).

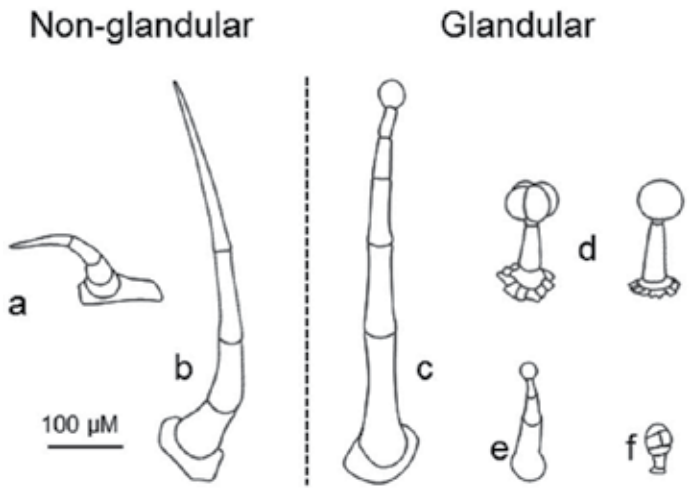

Fig. 1. Trichome types from various tomato species. a. type II non-glandular trichomes from *S. lycopersicum*. b. Type III long hairs (non-glandular). c. Type I long glandular trichomes with single secretory cell at the tip. d. Type VI glandular trichomes. On the left, trichomes from the cultivated tomato, *S. lycopersicum*. On the right, trichomes from the wild species *S. habrochaites*. The type VI trichomes from *S. lycopersicum* have four secretory cells on one plane, which can be easily distinguished from each other. The type VI trichomes from *S. habrochaites* also have four secretory cells, but they are wrapped in a common cuticular envelope, making it look like a single cell from the outside. In addition there is an intercellular space in the middle of these type VI trichomes, where the metabolites are stored. e. type IV trichomes. In some species, like *S. habrochaites*, these trichomes have a single glandular head, while in others like *S. pennellii* they look more like tobacco glandular trichomes with several glandular cells. F. type VII short glandular trichomes.

The type VII are short glandular trichomes with a single stalk cell and a berry-shaped glandular head composed of 7-10 cells. Tobacco also has similar trichomes, and it was shown in tobacco that these trichomes secrete short proline rich proteins, called phylloplanins which have antifungal activities (Shepherd *et al*., 2005). These trichomes do not appear to secrete small metabolites, and thus seem to be specialized for peptide

synthesis. Type I and type IV are capitate trichomes with few or a single glandular cell at the tip. The Type I are extremely long trichomes which can be easily seen with a naked eye, while the type IV trichomes are shorter. Type I trichomes are rare in cultivated tomatoes, while fairly abundant in some wild species, like *S. habrochaites*. Type IV trichomes are absent from cultivated tomatoes, and very abundant in *S. habrochaites*. These trichomes seem to be involved in the synthesis and secretion of secondary metabolites, mostly terpenoids (McDowell *et al.*, 2011). Type VI trichomes are present in both *S. lycopersicum* and *S. habrochaites* but they present distinct morphologies in each species. In *S. lycopersicum*, the four secretory cells are distinctly visible, forming a four-leaf clover shape when viewed from above with a total width of ≈ 60 µM. In *S. habrochaites*, the four secretory cells are encased in an envelope made of cuticule and cell wall materials, so that they appear as a single unit from the outside. The diameter of this ball-shape structure is also about 60 µM, and in contrast to the type VI trichomes from the cultivated tomato, it contains a cavity, most likely of intracellular space resulting from cell wall degradation, between the 4 cells. This storage cavity is reminiscent of the subcuticular space of the peltate trichomes of mint and is likely to contain the secretion products of the glandular cells.

Tomato trichomes have attracted major interest because of their roles in biotic interactions, in particular with arthropods. There are many reports of the roles of trichome secretions in the resistance to insect or arthropod pests (Kennedy, 2003). Most of the resistances to insects are found in wild species, like *S. pennellii* and *S. habrochaites*. For example the white fly *Bemisia tabacci*, which can transfer viruses, can be overcome thanks to glandular trichome secretions (Heinz and Zalom, 1995; Rubinstein and Czosnek, 1997; Snyder *et al.*, 1998; Vendramim *et al.*, 2009). *Tuta absoluta* is another important pest which is recently causing increasing damages to tomato crops. Again sources of resistance have been identified in wild accessions of *S. habrochaites* (Gilardon *et al.*, 2001; Maluf *et al.*, 2010).

The origin of the resistance lies both in the nature and in the quantity of the chemicals secreted. These wild species can be crossed to *S. lycopersicum*, and they can be used to introgress agriculturally relevant traits (disease and abiotic stress resistance, flavor, yield, etc.) into the cultivated tomato genome. However, introgression of complex traits which involve not only biosynthetic pathways, but also regulatory factors controlling may prove difficult and could lead to the introduction of undesirable genes from the wild species which adversely affect yield traits for example, and which may be difficult to eliminate because of the lower level of recombination between wild and cultivated tomato genomes. An alternative is then to introduce the required genes by genetic engineering. To avoid the synthesis of these compounds in the whole plant, which may cause undesirable side effects, trichome specific promoters are required.

In early studies, it was found that polyphenol oxidases are strongly expressed in tomato type VI glandular trichome (Kowalski *et al.*, 1992; Yu *et al.*, 1992; Thipyapong *et al.*, 1997). However, the promoters of this complex multigene family are not specific to trichomes and are also expressed in many other tissues upon stress (Yu *et al.*, 1992; Thipyapong *et al.*, 1997).

5.1 Omics of tomato glandular trichomes

Subsequently, as interest in elucidating the biosynthesis pathways of tomato glandular trichomes increased, trichome specific EST libraries from tomato were produced, in

particular from the wild species with abundant trichome secretions. The first libraries were released in 2001 from *S. habrochaites* (accession LA1777))(van Der Hoeven *et al.*, 2000) and *S. pennellii* (accession LA716). They were produced by Sanger sequencing and contained around 2000 sequences each (see Table 2).

Species	accession	Trichome type	Sequencing type	# of ESTs	Reference
S.habrochaites	LA1777	mixed	Sanger	2,656	(van Der Hoeven *et al.*, 2000; Fei *et al.*, 2004)
S. habrochaites	PI126449	mixed	Sanger	5,494	(Fridman *et al.*, 2005)
S. lycopersicum	NA	mixed	Sanger	7,254	(Besser *et al.*, 2009)
S. pennellii	LA716	mixed	Sanger	2,917	(Fei *et al.*, 2004)
S. lycopersicum	LA3475	mixed stems	NGS	278,000	(McDowell *et al.*, 2011)
S. lycopersicum	LA3475	type VII	Sanger	791	(McDowell *et al.*, 2011)
S. lycopersicum	LA3475	type VI	NGS	225,000	(McDowell *et al.*, 2011)
S. lycopersicum	LA3475	type I	Sanger	831	(McDowell *et al.*, 2011)
S. habrochaites	LA1777	mixed leaves	NGS	108,000	(McDowell *et al.*, 2011)
S. habrochaites	LA1777	type I	Sanger	978	(McDowell *et al.*, 2011)
S. habrochaites	LA1777	type IV	Sanger	1,425	(McDowell *et al.*, 2011)
S. habrochaites	LA1777	type VI	NGS	224,000	(McDowell *et al.*, 2011)
S. habrochaites	PI126449	Type VI	Sanger	15,000	(McDowell *et al.*, 2011)
S. pimpinellifolium	LA1589	type VI	NGS	227,000	(McDowell *et al.*, 2011)
S. pennellii	LA0716	type IV	Sanger	1,277	(McDowell *et al.*, 2011)
S. pennellii	LA0716	type VI	Sanger	1,137	(McDowell *et al.*, 2011)
S. pennellii	LA0716	mixed leaves	NGS	275,000	(McDowell *et al.*, 2011)
S- arcanum	LA1708	mixed stems	NGS	415,000	(McDowell *et al.*, 2011)
Total				**1,791,760**	

Table 2. A summary of currently available EST libraries from tomato trichomes. The accession numbers are those according to the Tomato Genetics Resource Center nomenclature (preceded with LA), or from the USDA germplasm collection (preced with PI).

Other similar sequence libraries were produced (Fridman *et al.*, 2005; Slocombe *et al.*, 2008), followed by the recent release of trichome specific libraires from several Solanum species and from distinct trichome types (McDowell *et al.*, 2011). Some of these were sequenced by next generation sequencing technologies, thus affording much larger numbers of EST sequences (up to 278 000 in some cases) (McDowell *et al.*, 2011). These sequence databases have been extremely useful in identifying and characterizing genes for the trichome specific biosynthesis pathways, in particular for terpenes (van Der Hoeven *et al.*, 2000; Sallaud *et al.*, 2009; Schilmiller *et al.*, 2009) and methylketones (Fridman *et al.*, 2005; Ben-Israel *et al.*, 2009; Yu *et al.*, 2010). A summary of the available EST sequences from various tomato species and trichome types is provided in Table 2. With a total of 1 791 760 ESTs, tomato trichomes are probably the trichomes with the best sequence resources currently available.

The availability of the tomato (*S. lycopersicum*) genome sequence makes it possible now to rapidly have access to promoter sequences, although the most interesting promoters will undoubtedly come from the wild species. However, the high sequence similarity between *S. lycopersicum* and *S. pennellii* or *S. habrochaites* should allow the facile identification of the promoters in those wild species.

There is at this stage and to the best of our knowledge, no reported example of metabolic engineering in tomato trichomes. Since tomato is a food crop grown for its fruit, much more has focused on fruit metabolism. There was even a report of taxadiene synthase (TS) expression in tomato fruit under the control of a fruit specific promoter (Kovacs *et al.*, 2007). From a purely metabolic point of view, this makes sense since tomato fruits are rich in carotenoids which derive from the same substrate as diterpenes, namely GGPP. However, the overexpression of TS caused sterility and growth defects which are undesirable side effects. In addition, the presence of potentially toxic secondary compounds in edible vegetables or fruits is a potential source of incidents by contamination of the food supply chain which must be avoided.

6. Artemisia

Artemisia annua, or sweet wormwood, a biennial plant from the Asteraceae family, has attracted attention as the source of an alternative to quinoline drugs for the treatment of malaria. The emergence of foci of resistance to quinine and related drugs in strains of *Plasmodium falciparum* requires the use of durable alternative treatments. Sweet wormwood was long known in Chinese traditional medicine to treat fevers. It was rediscovered in the 1970s for the treatment of malaria. The active ingredient is artemisinin, a sesquiterpene lactone, but semi-synthetic derivatives (Artemeter, Artesunate) have been developed as drugs by the pharmaceutical industry. Artemisia, like many other species from the Asteraceae, produces sesquiterpene lactones in glandular capitate trichomes localized on the leaves, stems and flowers. As with other trichome specific biosynthetic pathways, the elucidation of the first steps of the artemisinin biosynthesis pathways was made possible after sequencing trichome specific cDNA libraries, with the exception of the very first committed step, the sesquiterpene amorphadiene synthase (AaAS). AaAS was initially identified from a leaf cDNA library by similarity to known sesquiterpene synthases from plants (Mercke *et al.*, 2000; Wallaart *et al.*, 2001) and its specific pattern of expression in trichomes was later confirmed (Bertea *et al.*, 2005; Olofsson *et al.*, 2011). A succession of oxidation steps requiring a P450 mono-oxygenase (CYP71AV1) and an aldehyde dehydrogenase leads to artemisinic acid, while the synthesis of de-hydro-artemisinic acid requires the intervention of reductase (DBR2) (Zhang *et al.*, 2008; Liu *et al.*, 2009; Teoh *et al.*, 2009; Wang *et al.*, 2009; Zhang *et al.*, 2009; Weathers *et al.*, 2011). Although much progress has been achieved in the elucidation of the artemisinin pathway, relatively little was done with regards to promoter identification. Although most genes of the pathway are likely to be trichome specific (Liu *et al.*, 2009; Olsson *et al.*, 2009; Wang *et al.*, 2009), only one study reports on the cloning of the *AaAS* promoter and the identification of a WRKY transcription factor (AaWRKY1) which binds to the promoter of *AaAS* (Ma *et al.*, 2009). So far, to the best of our knowledge, no attempt at engineering of *A. annua* trichome metabolism has been reported. Given the importance of this compound as a pharmaceutical ingredient, attempts

at metabolic engineering of the artemisinic acid pathway in other plants and in microorganisms will be here briefly reviewed. Artemisinic acid can be used as a precursor for the semi-synthesis of artemisinin and related compounds. In parallel to these pathway elucidation efforts, different approaches were proposed and undertaken to improve the supply of the ingredient. Increasing demand, as well as requirements of reliable quality and supply to maintain stable prices, have spurred the search for either improvement of the available crop plant or transferring the production in heterologous hosts by metabolic engineering.

The first strategy is plant-based with the objective of improving artemisinin production by breeding using existing natural variation or induced mutagenesis. To reach this goal, a high density genetic map based on markers derived from transcriptome deep sequencing was created and used to map QTLs for artemisinin production (Graham *et al.*, 2010). This work showed that next generation sequencing technologies allow the rapid production of dense genetic maps in species where there is little or no prior genetic knowledge.

Another set of approaches is based on the expression of artemisinin biosynthesis genes in heterologous hosts. Reconstitution of the pathway to artemisinic acid was tested in tobacco but using ubiquitous promoters such as CaMV 35S (Zhang *et al.*, 2011). Previous sesquiterpene engineering studies in tobacco had revealed that targeting a FPP synthase together with a sesquiterpene synthase to plastids gave the best results (Wu *et al.*, 2006). With the same strategy and using different combinations of genes, it could be shown that amorphadiene, artemisinic and de-hydroartemisinic alcohol could be produced in the range of µg/g FW, but no artemisinic aldehyde or acid could be detected (Zhang *et al.*, 2011). Further analysis indicated that an endogenous reductase in tobacco prevents accumulation of artemisinic aldehyde and acids (Zhang *et al.*, 2011), thus questioning the relevance of tobacco for such metabolic engineering. The use of trichome specific promoters may solve this issue, or perhaps an even better solution would be to use other Asteraceae hosts which are able to accumulate sesquiterpene lactones in large quantities, such as chicory for example. Another explored strategy was the transient expression in *N. benthamiana*. This system was shown to be quite successful for the transient expression of proteins at very high levels (Marillonnet *et al.*, 2005), however the requirements for successful metabolic engineering are likely to be different. Nonetheless, transient expression of AaAS with CYP71AV1, together with HMGR to increase isoprenyl precursor supply, resulted in the production of artemisinic acid-12-β-diglucoside at levels up to 39.5 mg/kg FW (equivalent 16.6 mg/kg artemisinic acid). This indicated that artemisinic acid is indeed produced to significant levels, and highlights the importance of the host and the tissues targeted for expression. While altogether these results are promising and suggest that metabolic engineering of advanced terpenoid metabolites in plants is feasible, much progress is required to reach levels which will make commercial exploitation a reality. Many combinations of constructs with different promoters, sub-cellular targeting (plastids, cytosol, mitochondria), and hosts will have to be tested to identify the best solutions. However, in plants, even with transient expression systems, this is a highly time consuming tasks.

In comparison, micro-organisms allow a much higher throughput to test a multiplicity of constructs in a short time frame. Highly successful engineering endeavors have been achieved in *E. coli* and yeast by Keasling and co-workers. Through introduction of

mevalonate pathway genes in *E. coli*, production levels of amorphadiene of up to 0.5 g/L could be reached (Martin *et al.*, 2003; Newman *et al.*, 2006). Even artemisinic acid could be produced in *E. coli* after extensive modification of the P450 CYP71AV1 (Chang *et al.*, 2007). However the best results were obtained in yeast, where production levels of up to 100 mg/L could be reached (Ro *et al.*, 2006).

7. Conclusion

Trichomes have been used as a model to study cell differentiation and organ development in *Arabidopsis thaliana*, where the power of molecular genetics and genomics has made possible numerous advances in this area. However, Arabidopsis trichomes offer little opportunities for the development of novel products or applications, essentially because Arabidopsis trichomes are devoid of metabolic or structural properties of interest. However, trichomes play important roles in several crop species, where they are at the origins of important agricultural derived products, like cotton fibers and essential oils and fragrance ingredients of the Lamiaceae. In addition, trichome-borne resistances to insects and microorganisms in plants like tomatoes have attracted interest to restrict the use of pesticides. In those species (cotton, tomatoes, Lamiaceae), extensive EST resources were created and have proved valuable tools to identify and characterize trichome specific genes involved in development or metabolic pathways. Nonetheless, examples of trichome engineering using trichome specific promoters are still scarce and are limited to a handful of cases in cotton and tobacco. It seems that one limitation is to reach levels of productions for the metabolite of interest which are in the same range as those of endogenous metabolites. To reach those levels, it is necessary to understand more about how gene expression in those specialized cells is regulated, so as to be able to design and construct appropriate expression vectors enabling to reach these targets.

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18

Comparative Metabolomics of Transgenic Tobacco Plants (*Nicotiana tabacum* var. *Xanthi*) Reveals Differential Effects of Engineered Complete and Incomplete Flavonoid Pathways on the Metabolome

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1. Introduction

Anthocyanins and proanthocyanidins (PAs) are two groups of end products of the plant flavonoid pathway (Fig. 1). Biochemical and genetic evidences have demonstrated that they share the same upstream pathway beginning with phenylalanine through a series of enzymatic reaction to anthocyanidins. Anthocyanidins are either modified by glycosylation, methylation, or other reactions to form diverse anthocyanins (Springob et al. 2003) or catalyzed into flavan-3-ols by an anthocyanidin reductase (ANR) (Xie et al. 2003; Xie et al. 2006). In addition, leucoanthocyanidin reductase (LAR) has been enzymatically demonstrated to catalyze leucoanthocyanidins into catechin (Tanner et al. 2003). To date, whether or not this branch catalyzed by LAR exists in plants still remains open to genetic studies.

Production of Anthocyanin Pigment 1 (*PAP1)* encodes a R2R3-MYB transcription factor and is a master regulator of anthocyanin biosynthesis in *Arabidopsis thaliana* (Fig. 1) (Borevitz et al. 2000; Tohge et al. 2005; Xie et al. 2006). The constitutive expression of *PAP1* in *Arabidopsis* and tobacco resulted in massive accumulation of targeted anthocyanins and numerous other phenylpropanoid compounds (Borevitz et al. 2000; Tohge et al. 2005). Our recent experiments revealed that the regulatory function of *PAP1* on anthocyanin biosynthesis is closely associated with cellular specificity in tissues. In transgenic tobacco leaves of *PAP1* and *pap1-D Arabidopsis* leaves, anthocyanins accumulated in epidermal cells and parenchymal cells of vascular bundles in veins (Shi and Xie 2010; Xie et al. 2006). It was interesting that the overexpression of *PAP1* led to massive accumulation of anthocyanins in transgenic tobacco leaf trichomes (Xie et al. 2006) but not in leaf trichomes of *pap1-D Arabidopsis thaliana* (Shi and Xie 2010). *In vitro* separation of red cells from other cells has demonstrated that the regulatory function of *PAP1* expression can be inherited by cell culture developed from mother plants (Zhou et al. 2008). Both no anthocyanin-producing white cells and anthocyanin-producing red cells were obtained from transgenic *PAP1*

tobacco leaves. Transcript analysis showed that the level of the *PAP1* overexpression in these two types of cells were similar, indicating that the regulatory function of the *PAP1* expression was dependent upon cell types. (Zhou et al. 2008). The regulatory function of *PAP1* is also highly controlled by environmental factors. Although the *pap1-D Arabidopsis* plants over express *PAP1* leading to high anthocyanin pigmentation in most of tissues (Borevitz et al. 2000), when growth conditions were changed, anthocyanin levels and composition were dramatically altered in leaves of *pap1-D* plants (Rowan et al. 2009; Shi and Xie 2010; Tohge et al. 2005). In the same nutrition condition, high light conditions increased anthocyanin levels in leaves of *pap1-D* plants; under the same light condition, high nitrogen nutrition conditions increased anthocyanin levels in leaves of *pap1-D* plants; although in these conditions, the expression levels of *PAP1* were similar (Shi and Xie 2010). These inconsistent relationships between the expression levels of *PAP1* and anthocyanin levels were likely associated with other transcription factors involved in anthocyanin biosynthesis. PAP1 (MYB75), TT8 (transparent testa 8) (bHLH) / GL3 (glabra 3) (bHLH) and TTG1 (transparent testa glabra 1) (WD40) have been demonstrated to form a regulatory complex of MYB-bHLH-WD40 (MBW) controlling anthocyanin biosynthesis in *Arabidopsis* (Gonzalez et al. 2009; Ramsay and Glover 2005). We recently determined that the PAP1-TT8/GL3-TTG1 complex independently regulated anthocyanin biosynthesis in *pap1-D* cells (Shi and Xie 2011). In addition, there are other MBW regulatory complexes controlling anthocyanin biosynthesis in *Arabidopsis* (Gonzalez et al. 2009). In different growth conditions, the regulatory function of *PAP1* is likely essentially dependent upon these regulatory complexes.

ANR is a NADPH/NADH-dependent flavonoid reductase converting anthocyanidins to flavan-3-ols (e.g. epicatechin) and PAs (Fig. 1) (Xie et al. 2003). ANR is encoded by a *BANYULS* gene that was first cloned from young seeds of *Arabidopsis* (Devic et al. 1999). Its homologs were cloned from different species including a model legume plant *Medicago truncatula* (Xie et al. 2003). The constitutively ectopic expression of *ANR* in tobacco showed the loss of anthocyanins in flowers, in which ANR competitively catalyzed anthocyanidins into flavan-3-ols (e.g. epicatechin) and PAs (Xie et al. 2003). The transgenic vegetative tissues including leaf and stem tissues also expressed *ANR*, but failed to form epicatechin and PAs due to the absence of anthocyanidins (Xie et al. 2003; Xie et al. 2006). To establish a complete pathway of PAs in ANR transgenic leaves and stems, *PAP1* were ectopically expressed in them. The co-expression of *ANR* and *PAP1* produced flavan-3-ols and PAs in leaves and stems of transgenic tobacco plants (Xie et al. 2006). This result demonstrated that the expression of *ANR* alone resulted in production of ANR protein in transgenic leaves and stems and these tissues contained an incomplete PA pathway. This result also demonstrated that the overexpression of *PAP1* provided substrates for ANR; thus formed an effective platform for metabolic engineering of flavan-3-ols and PAs.

Whether the ectopic expression of *PAP1*, *ANR*, and *PAP1::ANR*, which form different pathways in transgenic tobacco vegetative tissues, can impact other metabolisms beyond the flavonoid pathway remains unknown. In this study, metabolic profiles of wild-type (WT) and transgenic tobacco plants expressing *PAP1, ANR, and PAP1::ANR* were examined by GC-MS analysis, showing that these three transgenic events differentially altered accumulation patterns of both targeted and non-targeted metabolites beyond anthocyanins and PAs in both transgenic leaves and stems

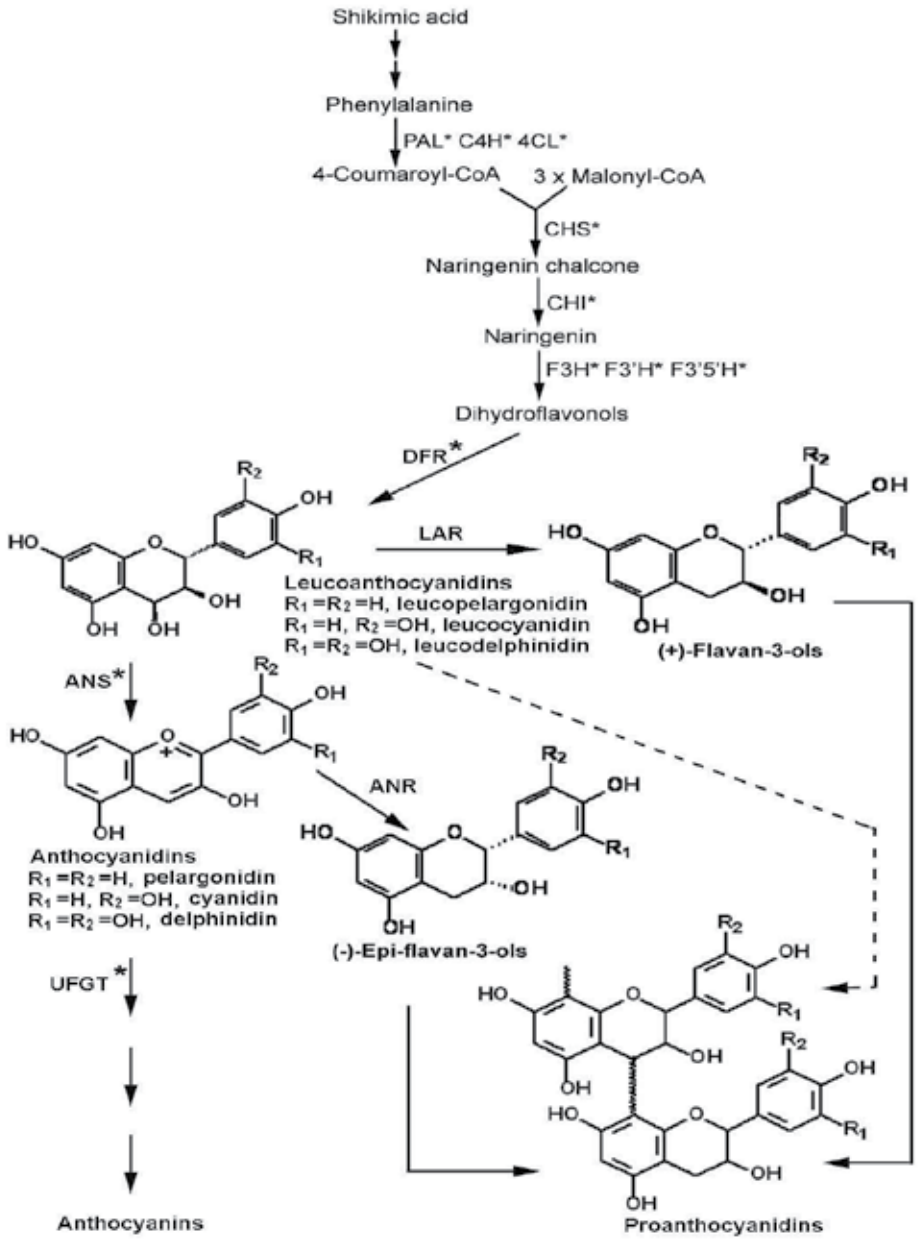

Fig. 1. Biosynthetic pathways of anthocyanins and proanthocyanidins starting with shikimic acid. Enzymes include: PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate: CoA-ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; UFGT: uridine diphosphate glucose-flavonoid 3-O-glucosyltransferase. Two arrows between shikimic acid and phenylalanine means there are multiple steps. Asterisks (*) indicate steps known to be up-regulated by *PAP1* expression.

2. Materials and methods

2.1 Plant growth

Plant materials used for this experiment included *PAP1*, *ANR* and *PAP1:: ANR* (F1 progeny) transgenic plants as well as wild-type (WT) plants. WT plants was the control line #3, which has been used as control plants to characterize *PAP1* gene function in metabolic engineering of PAs (Xie et al. 2006). *PAP1* transgenic plants containing an engineered pathway of anthocyanins were a homozygotic one from the line #292 (Xie et al. 2006). The *ANR* transgenic plants were derived from the line # B-21, which has only one copy of the *MtANR* (*Medicago truncatula* ANR) transgene and produces PAs in flowers, (Xie et al. 2003), but contains an incomplete pathway of PAs in leaves and stems. The *PAP1::ANR* transgenic plants were derived from the line # P-B-13 (PAP1 x ANR) that was obtained by crossing the line # B-21 with the line #292 of *PAP1* transgenic plants. This line produced a relatively high level of PAs in leaves and stems resulting from an engineered PA pathway (Xie et al. 2006).

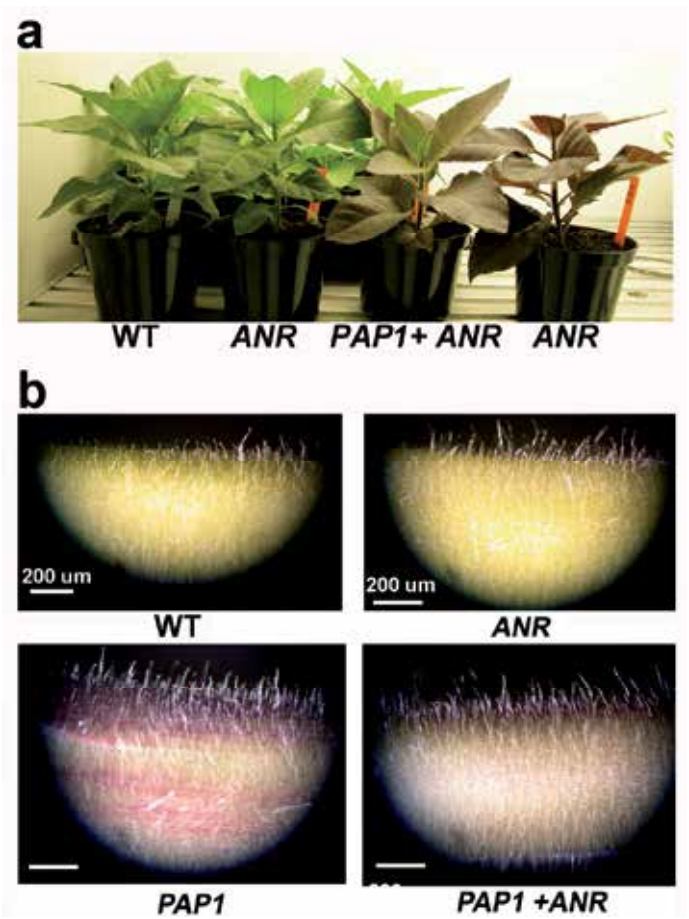

Fig. 2. Phenotypes of plants and stem trichomes. a phenotypes of wild-type (WT) and transgenic tobacco plants; b trichomes from stems; WT: wild type; *ANR*, *PAP1*, and *PAP1* + *ANR*: *ANR*, *PAP1*, and *PAP1::ANR* transgenic plants.

Ten individual plants from each of four genetic background lines were vegetatively propagated by cutting. Finally, 37 plants (10 for wild-type and *PAP1* and *PAP1::ANR* transgenic plants, respectively, and 7 for *ANR* transgenic plants) were grown in a growth chamber for metabolic profiling. To minimize effects of physical factors (e.g. temperature, lighting, and humidity) on *in vivo* biochemical variability, plants were grown in pots with a size of three inches in radius and eight inches in height in a growth chamber at a constant temperature of 25°C, 70% humidity, 16:8 hr light: dark cycle, and under fluorescent epi-illumination. The possible effects of shading on plant growth were reduced by rotating the pots. The growth of all plants was morphologically similar (Fig. 2 a).

Sixty-two day old plants, which were approximately 30 cm in height with 10-13 leaves, were used for metabolome analysis. Plant tissues were harvested directly into liquid nitrogen. Plant tissues collected included old leaves, young leaves, and stems. Old leaves were defined as those that were fully expanded, 15-20 cm in length, from each individual plant, while young leaves were defined as those with 5-10 cm in length, not yet fully expanded. Old leaves, young leaves or stems from a single plant was pooled to create one sample, thus 111 samples in total were obtained from 37 plants. The frozen samples were stored at -80°C, freeze-dried at -80°C, and then ground into a fine powder, which was stored at -80°C until use.

2.2 Tissue extraction and GC-MS analyses

A polar and a lipophilic extraction for each sample were profiled in this experiment. Metabolites were extracted from lyophilized plant material as described previously (Broeckling et al. 2005). Briefly, freeze-dried homogenized tissue (6.0 +/- 0.05 mg) was weighed into a vial (4.0 ml) for extraction of metabolites by adding $CHCl_3$ (1.5 ml) that contained an internal standard (heptadecanoic acid methyl ester). Vials were thoroughly vortexed and incubated at 50°C for 45 min, followed by addition of HPLC grade H_2O (1.5 ml) containing a second internal standard (ribitol). The biphasic system was thoroughly vortexed and then incubated for an additional 45 min at 50°C. The sample was then centrifuged at 3000 x *g* for 30 min at 4°C. One ml of each phase was collected and the solvent was then evaporated either using speed vacuum (aqueous phase) or dried under liquid nitrogen stream ($CHCl_3$ phase). The polar phase extraction was methoximated in 120 µl of 15 µg/ µl methoxyamine HCl in pyridine for 120 min at 50°C and then trimethylsilyalted by adding 120 µl of MSTFA + 1% TMCS (Pierce Biotechnology, Rockford, IL, USA) and incubating for 60 min at 50°C. The non-polar phase extraction was derivitized in 100 µl of 50% MSTFA + 1% TMCS in pyridine at 50°C for 60 min. One µl of each was injected onto an Agilent 6890 GC coupled to a 5973 MS. The polar sample was split at 15:1 and the non-polar sample split 1:1. The oven program was 80°C (2 min) and ramped at 5°C/min to 315°C (12 min). Separation was performed on a 60m DB-5MS (J&W Scientific – 0.25 mm ID and 0.25 µM film thickness) at a flow rate of 1.0 ml/min. Metabolites were identified by comparison to a library of electron impact mass spectra and GC retention time as described previously (Broeckling et al. 2005). Identification was performed by using AMDIS deconvolution and identification software (NIST). The same GC separation parameters were used for obtaining positive and negative-ion chemical ionization spectra for tentative identification of the

cembratienols using methane as the ionization gas on an Agilent 5973N MS. Quantification was performed as described previously (Broeckling et al. 2005).

2.3 Data processing, statistical analysis and heatmap

Peak detection and deconvolution were performed with AMDIS (Halket et al. 1999) for 2-3 samples of each treatment. Resultant peak lists were imported and compiled in MET-IDEA (Broeckling et al. 2006) and then used to extract quantitative peak area values for polar and non-polar metabolites. Redundant peaks were removed from the dataset, peak area values were scaled to mean zero and standard deviation 1.0. The resulting data matrix was statistically analyzed with discriminant function analysis, ANOVA, and principal component analysis in JMP (SAS institute, Cary, North Carolina). One-Way ANOVA was performed to extract significant different levels of metabolites (p-values < 0.05), then followed by Tukey's HSD pos-hoc analysis to compare all pair-wise mean difference (p-value < 0.05). To visualize metabolite accumulation patterns and the effect of transgenes on metabolite levels in tissues, heatmaps were established from excel using vision functions in macro.

2.4 Histological analysis of anthocyanin accumulation

Both young leaves and stems were dissected to 10-20 μm in thickness by hands and cellular localization of anthocyanin accumulation was examined with light microscope as described previously (Xie et al. 2006).

3. Results

3.1 Plant growth and cellular localization patterns of anthocyanin accumulation

The growth of all plants was morphologically similar in the growth chamber except for color difference (Fig. 2 a). Red pigmentation patterns were examined with a microscope. Trichomes, epidermis, and hypodermis of *PAP1* transgenic stems highly accumulated anthocyanins (Figs. 2 b and 3). Parenchyma cells around vascular bundle and particularly near phloem were clearly red or pink resulting from high accumulation of anthocyanins (Fig. 3 b, c and e). In addition, parenchymal cells around xylem were clearly red or pink due to the high accumulation of anthocyanins (Fig. 3 b, c and e). Cells in the pith and cortex were not observed to produce anthocyanins (Fig. 3 b, c and e). In contrast, all cells in WT stems did not produce anthocyanins in the same growth conditions (Fig. 2 a-b and Fig. 3 a, d and f). In addition, *ANR* transgenic plants did not produce anthocyanins either (Fig. 2 a and b). As reported previously for greenhouse-grown plants (Xie et al. 2006), *PAP1::ANR* transgenic plants showed obviously reduced levels of anthocyanins in plants (Fig. 2 a and b).

We previously reported the features of anthocyanin accumulation in *PAP1* transgenic leaves of greenhouse-grown plants. Anthocyanins were mainly localized in trichomes, epidermal cells, hypodermal cells, and parenchyma cells in veins (Xie et al. 2006). In this experiment, the accumulation patterns of anthocyanin in leaves were the same as ones in leaves of greenhouse-grown plants (Xie et al. 2006).

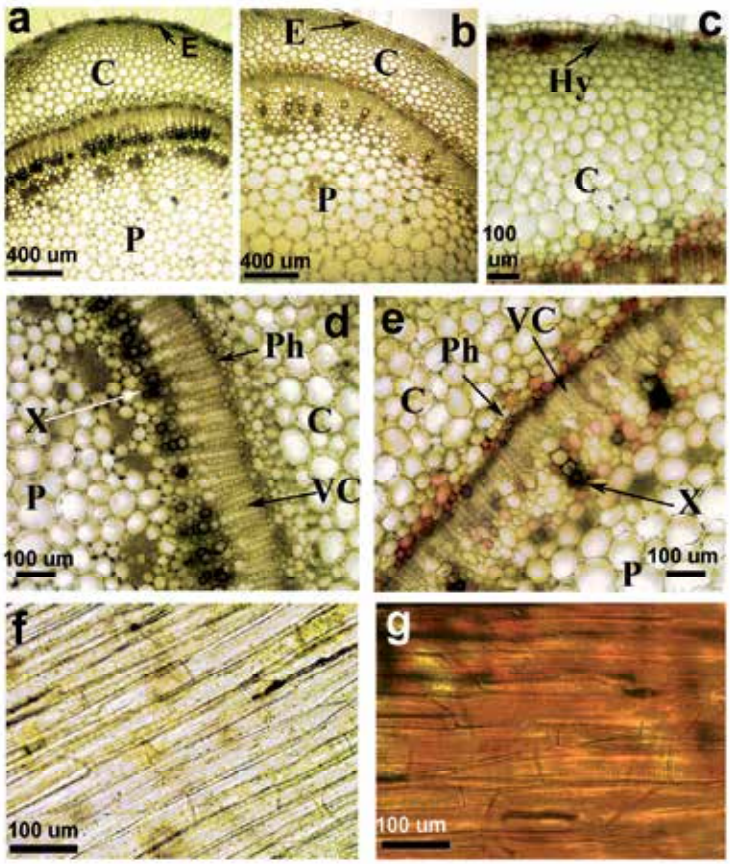

Fig. 3. Microscopic images show cellular specificity of anthocyanin accumulation in stems of *PAP1* transgenic plants. a-b cross sections of young stems of wild-type (a) and *PAP1* transgenic (b) plants; c a magnified image showing anthocyanin accumulation in hypodermal cells of *PAP1* transgenic stems; d a magnified image showing vascular bundle of WT stems; e a magnified image showing anthocyanin accumulation patterns in or around vascular bundles of *PAP1* transgenic stems; f epidermal cells of WT stems; g epidermal cells of *PAP1* transgenic stems. Abbreviations: C, cortex; E, epidermis; Hy: hypodermis; P: pith; Ph: phloem; VC: vascular cambium; X: xylem.

3.2 Metabolites identified and their profile properties in wild-type tobacco

Young leaves, old leaves, and stem tissues of WT tobacco plants were used to analyze metabolites and examine their accumulation patterns. In total, eighty-seven metabolites including both water (polar molecular compounds) and chloroform soluble compounds (low or non-polar molecular compounds) were characterized based on their mass spectra profile identity and chromatographic retention times in comparison to authentic standards. ANOVA significance tests indicated that seventy-one metabolites were featured with tissue-related accumulation patterns, which was shown in a heatmap (Fig. 4). Fourteen metabolites did not show significant differences among the three tissues examined. In addition, two metabolites, ribose and trihydroxybutyrate showed an interesting pattern in the three

tissues. ANOVA analysis showed that the levels of the two compounds were not obviously different between young leaves and stems as well as between old leaves and stems but significantly higher in young leaves than in old ones.

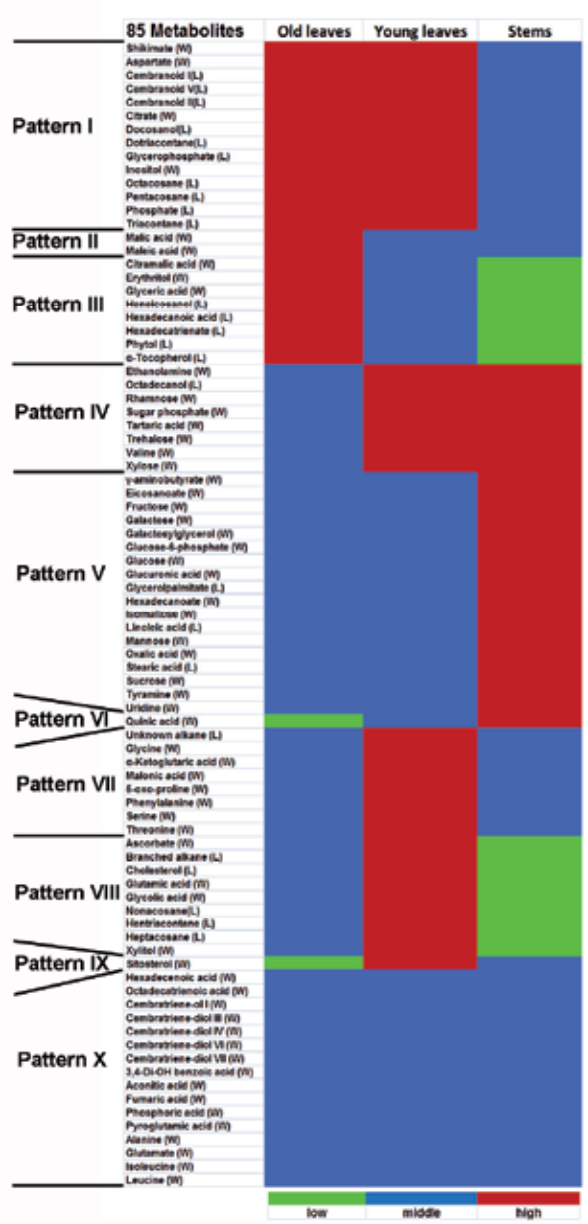

Fig. 4. A heatmap shows differential accumulation patterns of 85 metabolites in stems, young leaves, and old leaves. In each pattern, red color means the highest levels of metabolites in the tissue ($P < 0.05$), and blue color means significantly ($P<0.05$) higher levels of metabolites in the tissue than in the other tissue indicated by green colors. (L): lipophilic metabolites extracted in chloroform phase; (W): water soluble metabolites in extracted in water phase.

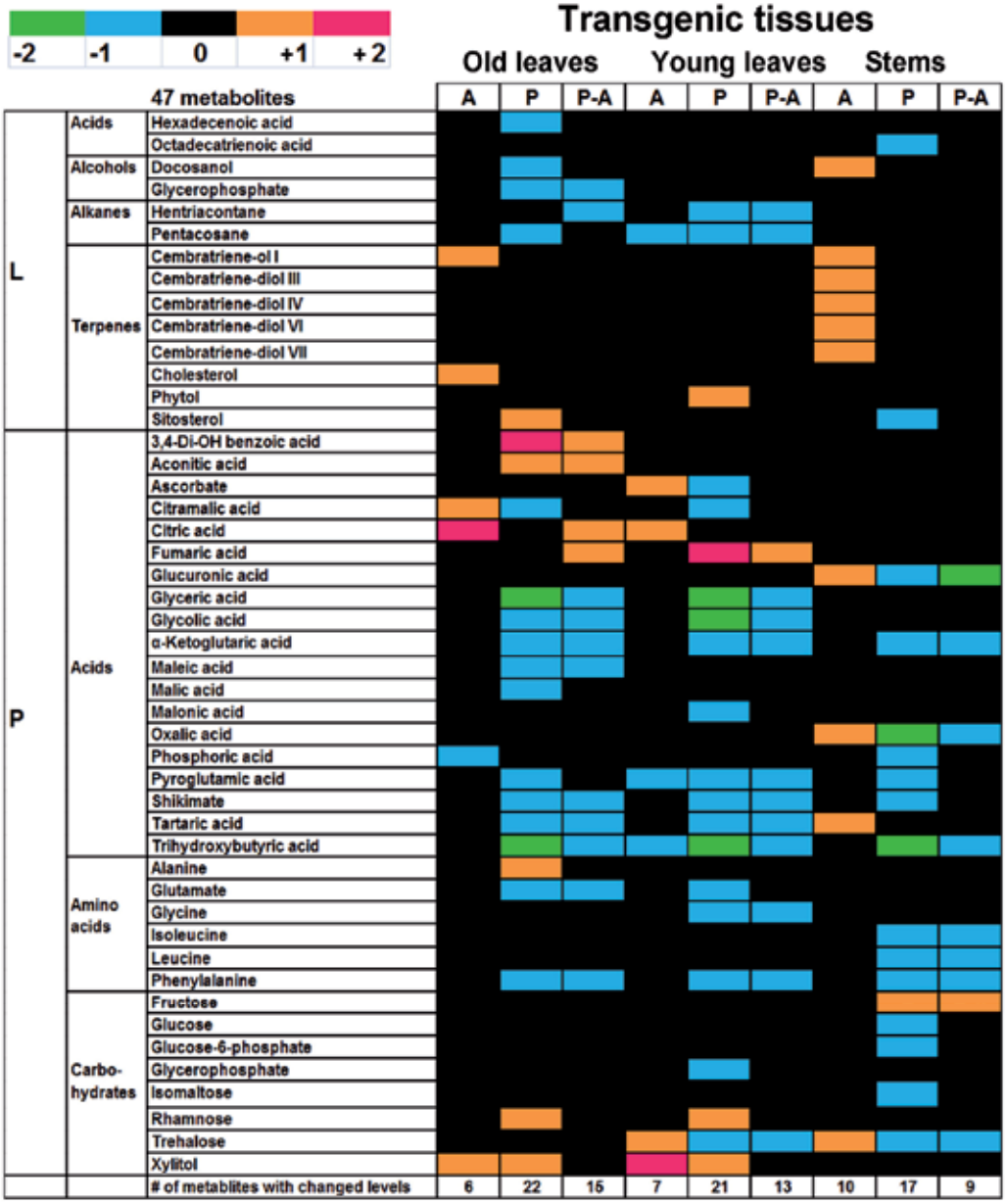

Fig. 5 A heatmap shows significant increase or decrease of levels for metabolite in *PAP1*, *ANR*, and *PAP1::ANR* transgenic stems and young and old leaves. Black color (0) indicates no differences in levels of metabolites between transgenic tissues and wild-type tissues. Red (+2) color indicates highly significant (P<0.05) increase. Bright brown color (+1) indicates significant (P<0.05) increase. Blue color (-1) indicates significant (P< 0.01) decrease. Green color (-2) indicates highly significant (P<0.05) decrease. A: *ANR*; P: *PAP1*; P-A: *PAP1:: ANR*; L: lipophilic metabolites extracted in chloroform phase; W: water soluble metabolites in extracted in water phase.

3.3 Metabolites with significantly altered levels in *PAP1* transgenic plants

PAP1 is a master transcriptional regulator of the anthocyanin pathway (Fig. 1) (Xie et al. 2006). *PAP1* transgenic plants highly produced anthocyanins in the growth chamber (Fig. 2 a). GC-MS analysis of the eighty-seven metabolites showed that the levels of thirty-nine were altered, which included nine metabolites with increased levels, twenty-nine metabolites with reduced levels, and one metabolite, sitosterol, the level of which was increased in old leaf but decreased in stem (Fig. 5).

Phenylalanine, malonic acid and shikimic acid, which are three early pathway precursors of the anthocyanin biosynthesis (Fig. 1), were dramatically reduced (Fig. 5). The decreases of phenylalanine and shikimic acid were apparent in all three analyzed tissues, while malonic acid was significantly reduced only in young leaves (Fig. 5).

Thirty-six metabolites with altered levels were not immediately metabolically related to plant phenylpropanoids (Fig. 5). These metabolites included two clusters consisting of seven non-polar and twenty-nine polar compounds either in old leaves, young leaves, or stems. The seven non-polar metabolites, which were composed of acids, alkanes, alcohols, and terpenes, included 4 in old leaf, 3 in young leaf, and 2 in stem. For example, the level of non-polar compound docosanol was increased only in old leaf, while the abundance of non-polar compound sitosterol was reduced in stem but increased in old leaf. The twenty-nine polar metabolites, which were composed of acids, amino acids, and carbohydrates, included 18 in old leaf, 18 in young leaf, and 15 in stem. Examples of decreased accumulation included α-ketoglutaric acid, citramalic acid, glyceric acid, glycine, maleic acid, and trihydroxybutyric acid in a tissue specific fashion.

3.4 Metabolites with altered levels in *ANR* transgenic plants

Our previous work discovered that ANR (anthocyanidin reductase) was a pathway enzyme of the PA biosynthesis (Xie et al. 2003) . Analyses of variance followed by Tukey's HSD post-hoc comparisons were conducted to assess the alteration extent of metabolites in *ANR* transgenic plants. The levels of nineteen identified metabolites including eleven polar and eight non-polar metabolites were altered in tissue-dependent accumulation patterns (Fig. 5). The eleven polar metabolites included 4 in old leaf, 6 in young leaf, and 4 in stem, the levels of eight of which were increased in either leaves or stems, while, the levels of three of which were decreased in *ANR* transgenic leaves. The eight non-polar metabolites included seven with increased levels but one, pentacosane (a lipophilic odd-chained alkane) with a decreased level (Fig. 5). Among the seven metabolites with increased levels, cembratriene-ol I, cembratriene-diol III, cembratriene-diol IV, cembratriene-diol VI, and cembratriene-diol VII are defensive compounds against aphids (Wang et al. 2001).

3.5 Metabolites with altered levels in *PAP1: ANR* transgenic plants

PAP1::ANR transgenic plants were the F1 hybrid progeny of *ANR* and *PAP1* transgenic plants. These plants formed both anthocyanins and PAs (Xie et al. 2006). The levels of twenty-four metabolites including three non-polar and twenty-one polar metabolites were altered (Fig. 5). Among them, twenty-three were included in those metabolites with altered levels in *PAP1* transgenic plants as described above (Figs. 5 and 6). Phenylalanine and shikimic acid are two early precursors of the anthocyanin and PA pathways (Fig. 1). As observed in *PAP1* transgenic plants described above, the accumulation level of

phenylalanine was significantly reduced in all analyzed tissues. The accumulation level of shikimic acid was also dramatically reduced in leaf tissues. Twenty-two metabolites with altered levels were not directly related to plant flavonoid biosynthesis, twenty-one of which were included in those impacted by*PAP1*alone transgenic plants (Fig. 5). Therefore, these results supported the metabolite profile alteration in *PAP1* alone transgenic plants.

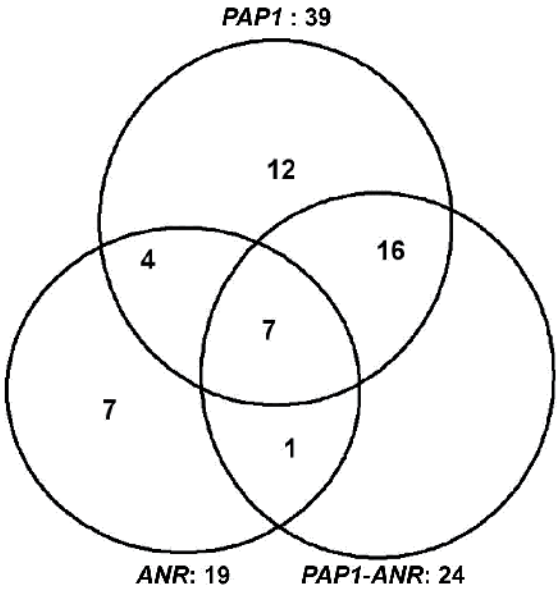

Fig. 6. A Venn diagram shows separate and overlaid properties of metabolites with altered levels in the *PAP1*, *ANR*, and *PAP1::ANR (PAP1-ANR)* transgenic plants. In total, the levels of forty-seven out of eighty-seven metabolites analyzed were impacted in the three transgenic events.

3.6 Principal component analysis

Principal component analysis (PCA) was performed with the eighty seven identified metabolites to examine the ordination relationships for all three tissues between WT plants and transgenic plants. The first principal component accounted for nearly 25%, 23% and 20% of total variation in old leaves, young leaves and stems respectively. The second principal component was nearly 12% of total variation in old leaves, young leaves, and stems. From PCA results for old and young leaves, it was obvious that *PAP1* transgenic plants and WT type plants were clearly separated each other in the first principal component (Fig. 7 a and b). *ANR* transgenic plants were scattered in the same cluster with WT plants. *PAP1::ANR* transgenic plants were between *PAP1* transgenic and *ANR* transgenic or WT plants (Fig. 7 a and b). It was interesting that in stems the ordination relationship patterns of four groups of plants were different from those observed in old and young leaves (Fig. 7 c). *PAP1* and *ANR* transgenic stems were of the most separation in the first principal component. WT and *PAP1* transgenic stems were also clearly different each other in the first principal component. As expected, *PAP1* and *PAP1::ANR* transgenic stems were relatively close (Fig. 7 c). These PCA results showed that the impacts of *PAP1* and *ANR* on metabolite profiles in transgenic plants were different.

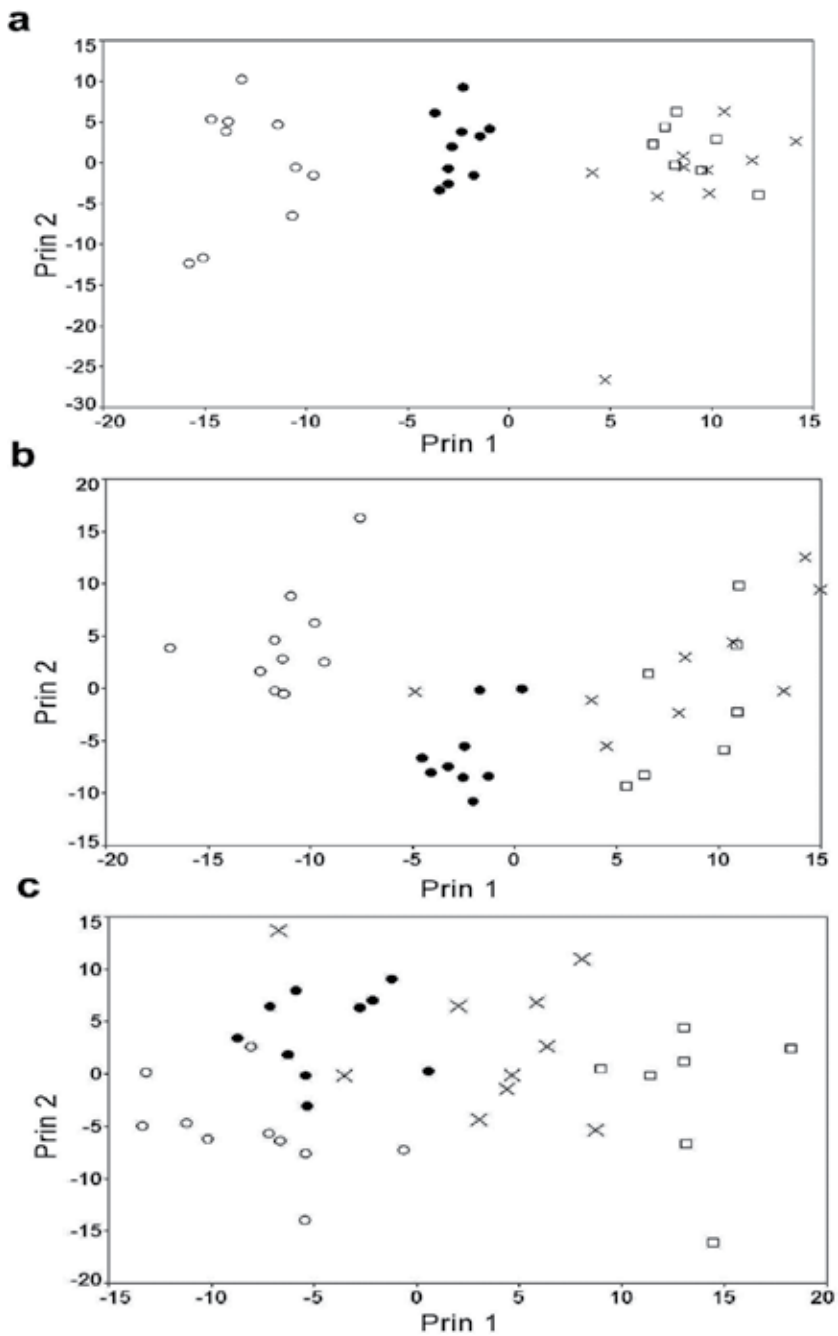

Fig. 7. Principal component analysis of wild-type plants ("x"), *ANR* (empty squares), *PAP1* (empty circles), or *PAP1::ANR* (solid circles) for (a) old leaf, (b) young leaf, and (c) stem tissue. Principal components 1 and 2 account for (a) 24.5 and 12.1 % of the total variation in old leaf, (b) 23.1 and 12.1% of the total variation in young leaf, and (c) 20.2 and 11.8% of the variation in stem tissue samples.

4. Discussion

4.1 Impacts of *PAP1* transgene on metabolic profiles

Our recent report showed that genome-wide transcriptional programs in red *pap1-D Arabidopsis* cells cultured *in vitro* were dramatically different from cultured wild-type cells (Shi and Xie 2011). On the one hand, massive numbers of genes involved in multiple metabolic networks were highly expressed in red cells. On the other hand, the expression levels of large number of genes involved in multiple metabolic networks were also reduced in red cells. We hypothesized that the dramatic alterations of genome-wide gene expression must have led to massive alterations of metabolic profiles beyond anthocyanin biosynthesis in red cells and specifically programmed red cells established by the overexpression of *PAP1* must have led to transcriptional and metabolic reprogramming (Shi and Xie 2011).

In this study, GC-MS analysis revealed that levels of thirty-nine metabolites excluding flavonoids were altered in *PAP1* transgenic plants (Figs 5 and 6). These metabolites are composed of seven non-polar molecules and thirty-two molecules including sugars, amino acids, and organic acids (Fig. 5). Based on their roles in anthocyanin biosynthesis, these metabolites can be characterized as targeted and non-targeted metabolites. Targeted metabolites are precursors in the anthocyanin pathway (Fig. 1). Phenylalanine, shikimic acid, and malonic acid are three early precursors of the anthocyanin biosynthesis. Therefore, the high production of anthocyanins must result from an enhanced metabolic flux from shikimic acid to anthocyanins (Fig. 1) and thus lead to the high consumption of shikimic acid, phenylalanine, and malonic acid. As expected, the levels of the three metabolites were reduced in *PAP1* transgenic plants. This result metabolically supported the previous reports about the effect of *PAP1* on gene expression profiles at the transcriptional level involved in anthocyanin biosynthesis. The transcriptional levels of the *PAL1* and several other anthocyanin pathway genes were increased 1.2-6.7 folds by the overexpression of the *PAP1* gene (Borevitz et al. 2000; Lillo et al. 2008; Tohge et al. 2005). In addition, this result supported our recent report that most of the pathway genes involved in the anthocyanin pathway in red cells over expressing *PAP1* were dramatically increased leading to alterations of multiple metabolic networks (Shi and Xie 2011). Therefore, the alterations of metabolic profiles of the three molecules resulted from the ectopic expression of *PAP1* transgene.

Most of metabolites with altered levels in *PAP1* transgenic tissues were not directly related to the biosynthesis of anthocyanins. They are non-targeted metabolites. These metabolites included acids, amino acids, and carbohydrates (Fig. 5). Two studies showed that the transcriptional levels of several genes involved in sugar transport, glutathione metabolism, calcium binding, and carbohydrate metabolism were up-regulated by PAP1 in *Arabidopsis* (Borevitz et al. 2000; Tohge et al. 2005). However, no changes in the levels of amino acids, sugars, or anions profiled by capillary electrophoresis or liquid chromatography were observed (Tohge et al. 2005). This result was not in agreement with our current observation. We observed dramatic reduction of levels of several amino acids and carbohydrates in response to *PAP1* expression in transgenic tobacco plants (Fig. 5). The discrepancy likely resulted from different experimental designs. We separated young leaves from old leaves in our experiments, while Tohge and co-workers (2005) did not in their study. More importantly, we recently isolated red cells from *pap1-D Arabidopsis*. Those red cells showed

dramatically different transcriptional profiles from wild-type cells (Shi and Xie 2011) and these global gene expression alterations were not observed in Tohge's experiments either. Particularly, in red cells, expression levels of a large number of genes involved in glycolysis, amino metabolisms, photosynthesis and other metabolic pathways were significantly decreased (Shi and Xie 2011). In addition, the discrepancy might result from the species-specific responses to the over-expressed PAP1 protein. The results of ectopic expression of the *A. thaliana PAP1* gene in tobacco plants might differ from the consequences of its overexpression in its native plant species. The evidence was that we recently observed dramatically differential response of anthocyanin biosynthesis to chemical factors in tissue cultures of *PAP1* transgenic *Arabidopsis* and tobacco (Shi and Xie 2011; Zhou et al. 2008). Therefore, the alterations of these non-targeted metabolite profiles most likely resulted from the ectopic expression of *PAP1* transgene.

4.2 Impacts of ANR transgene on metabolic profiles

It was very interesting that our data showed the altered levels of nineteen metabolites in leaf and stem tissues of the *ANR* transgenic plants. Obviously, the accumulation of five cembranoids was enhanced in the stem of the *ANR* alone transgenic plants (Fig. 5). Cembranoids are diterpenoids derived from the isoprenoid pathway and involved in defense against aphids (Wang et al. 2001). In *Nicotiana spp.,* relatively high levels of cembranoids were found to localize to secretory glandular trichomes (Wang et al. 2004; Wang et al. 2001). In addition, oxalic acid accumulated at higher levels in *ANR* plants in a pattern similar to that of the cembranoids. Oxalate, which accumulates in glandular trichomes and idioblast cells of *Nicotiana* species (Choi et al. 2001; Sarret et al. 2006; VolkA and Franceschi 2000), is the calcium salt of oxalic acid and has important defensive activities against chewing insects (Korth et al. 2006). We microscopically examined trichomes, but did not observe changes of their morphology and density on the surfaces of stems and leaves between transgenic and WT tobacco plants (Fig. 2 b), suggesting that their level increase most likely resulted from the *ANR* transgene expression.

The mechanism behind the metabolic alteration in *ANR* transgenic plants is unclear. Little has been reported regarding the mechanism through which ectopic expression of a single biosynthetic reductase can dramatically alter cellular metabolome. Here, we would provide certain perspective discussion to potentially interpret this result observed from our experiments. A few studies revealed the effects of marker genes on *in vivo* cellular biochemical processes. Constitutive expression of marker genes such as *NPTII* and *GUS* genes resulted in changes of certain transcriptome profiles in transgenic *A. thaliana*, although there was no impact on the development and growth of plants (Ouakfaoui and Miki 2005). The constitutive presence of Bt protein was observed to unexpectedly increase the production of lignin in insect-resistant transgenic corn plants (Saxena and Stotzky 2001). However, the mechanism of such observed effects caused by the presence of introduced foreign proteins has not been investigated in detail thus remains unknown. It is interesting that a report demonstrated that an overexpression of a rice dihydroflavonol reductase (DFR) led to enhanced levels of NAD (H) and NADP (H) in rice cell suspension and seedlings, which increased the tolerance of transgenic rice to bacterial pathogens and reactive oxygen species (ROS)-induced cell death (Hayashi et al. 2005). DFR is a NADPH/NADH dependent

reductase essential for both anthocyanin and PA biosynthesis (Fig. 1) (Xie et al. 2004a). Although Hayashi et al (2005) did not report whether or not their transgenic rice seedlings and cells had *in vivo* substrates for DFR enzyme, nor did they measure changes in unrelated metabolic pathways, the constitutive presence of DFR protein increased the levels of the two coenzymes by enhancing the enzymatic activities of NAD synthetase, NAD kinase and ATP-NMN adenylyltransferase (Hayashi et al. 2005). ANR is a DFR-like reductase using NADH/NADPH as coenzyme to convert anthocyanidins to two isomers of flavan-3-ols, e.g. (-)-epicatechin and (-)-catechin (Devic et al. 1999; Xie et al. 2003). *In vitro* experiments showed that ANR bound NADPH and NADH with Km values of 0.5 mM and 1.0 mM respectively (Xie et al. 2004b). In addition, our previous studies demonstrated the constitutive expression of the enzyme and its function to using *in vivo* NADPH/NADH as co-factor (Xie et al. 2006). The enzyme was recently localized to the cytoplasm as predicted (Pang et al. 2007). The concentration of cytosolic NADPH/NADH in tobacco plants is unknown, but a report showed that the cellular NADPH concentrations during photosynthesis were estimated to range from 0.1 to 0.5 mM in the blue-green algae *Anacystis nidulans* (Grossman and McGowan 1975). We hypothesize that the presence of ANR in transgenic tobacco plants may alter metabolic homeostasis of NAD or NADP metabolism as the observed DFR expression in rice (Hayashi et al. 2005). Thus, metabolism may be impacted by the changes of cellular homeostasis of NADPH/NADH. For example, sugar alcohol biosynthesis in celery is thought to be regulated by cytosolic NADPH levels (Gao and Loescher 2000). Consequently, the alterations of tissue-dependent patterns of numerous compounds observed in our experiment, e.g. trichomes-secreted cembranoids, likely result from the changes of coenzyme homeostasis or other factors.

4.3 Impacts of the coupled expression of *PAP1* and *ANR* transgenes on metabolic profiles

The twenty-four metabolite profiles altered in the *PAP1::ANR* transgenic F1 progeny plants were also impacted in *ANR* or *PAP1* transgenic plants (Figs. 5 and 6). It was interesting that obviously overlaid properties of metabolic profiles were observed between *PAP1* or *ANR* and *PAP1::ANR* plants (Fig. 5, Fig. 6). Without considering the effects of tissue specificity, it was observed that the accumulation levels of eleven identical metabolites, i.e. pentacosane , ascorbic acid, citramalic acid, glucuronic acid, oxalic acid, pyroglutamic acid, tartaric acid, trehalose, trihydroxybutyric acid, and xylitol, which were altered in *PAP1::ANR* transgenic plants, were increased or decreased in *PAP1* or/and *ANR* transgenic plants (Figs. 5 and 6). Twenty three of the twenty-four metabolites, which were showed their accumulation pattern alterations in *PAP1::ANR* transgenic plants, were also with altered levels in *PAP1* transgenic plants (Figs. 5 and 6).

It was interesting that a counteracting consequence on metabolite accumulation patterns was observed in *PAP1::ANR* transgenic plants due to the co-expression of *PAP1* and *ANR*. On the one hand, metabolite accumulation patterns altered in *ANR* transgenic plants were counteracted by the expression of *PAP1* in *PAP1::ANR* transgenic plants. Eleven metabolites with altered levels in *ANR* transgenic plants were not found to have the alteration in *PAP1::ANR* transgenic plants. For example, the levels of cembratriene-diol III, IV, VI and VII were increased in *ANR* transgenic stems, but in *PAP1::ANR* transgenic stems, reduced to

similar levels as the ones in WT plants (Fig. 5). In addition, the levels of cembratriene-ol I, cholesterol, citramalic acid, and xylitol were increased in old leaves of the *ANR* transgenic plants but were reduced in the *PAP1::ANR* transgenic plants (Fig. 5). Other examples include citric acid, oxalic acid, ascorbic acid, and citramalic acid (Fig. 5). On the other hand, metabolite accumulation alterations caused by the expression of *PAP1* were counteracted by the expression of *ANR*. Levels of thirty-nine metabolites were altered in *PAP1* transgenic plants, among which the alteration of the levels of fifteen metabolites was diminished in the *PAP1::ANR* transgenic plants (Fig.5). For example, the levels of glucose-6-phosphate and glucose were reduced in *PAP1* transgenic stems, but in *PAP1* × *ANR* transgenic stems, returned to the normal levels as the ones in WT plants (Fig. 5). No evidence has been reported describing counteracting impacts of the co-expression of a regulatory gene and a structure gene from the flavonoid pathway on cellular metabolomes. We suggest that this phenomenon results from the reduction of transgene dosage in *PAP1::ANR* F1 progeny plants. Given that *PAP1* transgenic plants were homozygotic plants, the transgene dosage was higher in the mother plants than *PAP1::ANR* F1 progeny plants. In addition, the mechanism of counteracting phenomenon is likely more complicated than the additive of the two transgenes. This may be also due to the metabolon formation (metabolic channeling) consisting of multiple proteins required for every single secondary metabolite and for avoiding metabolic interference (Jorgensen et al. 2005; Winkel-Shirley 1999).

5. Conclusion

The *PAP1* gene encodes a R2R3-MYB transcription factor and its ectopic expression led to the formation of a complete anthocyanin pathway in all tissues of tobacco (*Nicotiana tobacum*) plants. Anthocyanidin reductase (ANR) catalyzes anthocyanidins to flavan-3-ols and its ectopic expression of *ANR* formed an incomplete pathway of flavan-3-ols and proanthocyanidins (PAs) in leaves and stems of tobacco plants. The co-expression of *PAP1* and *ANR* constructed a complete biosynthetic pathway of PAs in tobacco leaves, stems and flowers. Gas chromatography-mass spectrometry (GC-MS) analysis identified eighty-seven metabolites in wild-type tobacco leaves and stems, and revealed tissue-specific and development-dependent patterns of the abundance of seventy-one metabolites in plants. In *PAP1* transgenic plants, the accumulation patterns of thirty-nine metabolites (out of eighty-seven) including both primary and secondary metabolites were altered in either leaves or stems. In *PAP1::ANR* transgenic plants, twenty-four metabolites showed accumulation pattern alterations. Shikimic acid, phenylalanine, and malonic acid, which are three early precursors of the flavonoid pathway, were altered in their abundance in these two transgenic events. In ANR transgenic plants, nineteen metabolites showed abundance alterations. The array of metabolites altered in ANR transgenic plants was distinct from that in *PAP1* transgenic plants. The array of metabolites altered in *PAP1::ANR* transgenic plants were intermediate to the two transgenic events. Principal component analysis showed that there was close ordination relevance in metabolic profile alterations between *PAP1* alone and *PAP1::ANR* transgenic plants but distant ordination relevance between *PAP1* and *ANR* transgenic plants or wild-type plants. These results indicated that the ectopic expression of *PAP1*, *ANR*, or *PAP1::ANR* leading to different branch pathways of plant flavonoids differentially impacted accumulation patterns of metabolites.

6. Acknowledgments

The completion of this work benefited from the support of the Noble Foundation and the support of an USDA grant (USDA 2006-35318-17431). We appreciate constructive advice provided by Lloyd Sumner and Richard Dixon. We appreciate Dr. Bettina E. Broeckling for her critical reading and suggestions.

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19

Effect of Antisense Squalene Synthase Gene Expression on the Increase of Artemisinin Content in *Artemisia anuua*

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1. Introduction

Artemisinin, a sesquiterpene lactone endoperoxide, is a valuable and powerful antimalarial drug obtained from the aerial parts of a Chinese herb, *Artemisia annua* (Liu et al., 1979). Artemisinin and its derivatives show few or no side effects with the existing antimalarial drugs, it has consequently been regarded as the next generation of antimalarial drugs (Looaresuwan, 1994). Currently, commercial production of artemisinin mainly based on its extraction and purification from plant material, however, the endogenous production of artemisinin is very low (0.01%-0.8% dry weight) (Wallaart et al., 1999). In view of the limited availability of artemisinin and the increased demand, the synthetic preparation of artemisinin becomes an attractive proposition. However due to its complex structure, the complete chemical synthesis is very difficult (Schmid & Hofheinz, 1983). *Artemisia annua* as the only valid source, many research groups have directed their investigations toward the enhancement of artemisinin production in *A. annua* cell cultures or whole plants by biotechnological approaches. However these approaches were still proved to be not successful (Ghingra et al., 2000). Recently, several genes in artemisinin biosynthesis have been cloned, and important advances in artemisinin biosynthesis have been achieved, which makes it possible to regulate artemisinin biosynthesis in a direct way, for example, by metabolic engineering (Abdin et al., 2003).

Artemisinin is synthesized by the isoprenoid pathway. In the cytosol, isoprenoids are synthesized via the classical acetate/mevalonate pathway (Fig. 1). In this pathway, farnesyl diphosphate (FDP) occupies a central position and serves as a common substrate for the first committed reactions of sterols and sesquiterpenes, such as artemisinin. Therefore, this point represents a potentially important controlling point for balancing sterol synthesis and sesquiterpenes synthesis. From metabolic engineering point of view (Fig. 1), there are two

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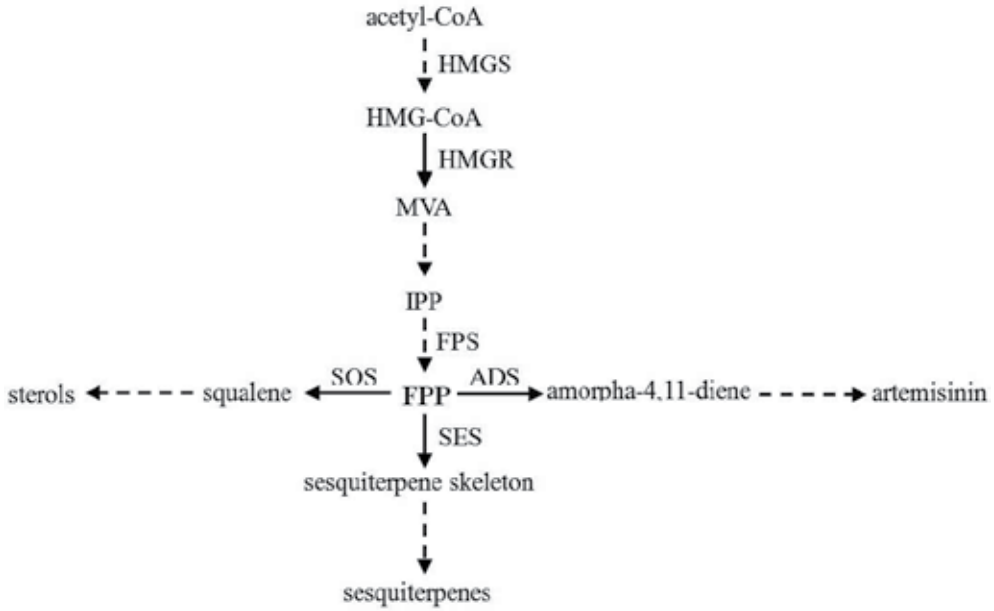
ways to increase the flux to artemisinin biosynthesis, on the one hand, we can overexpress the key genes involved in the biosynthesis of artemisinin; on the other hand, we can inhibit the genes involved in other pathways competing for its precursors.



ADS: amorpha-4,11-diene synthase; FPP: farnesyl diphosphate; FPS: farnesyl diphosphate synthase; HMG-CoA: 3-hydroxy-3-methylglutaryl CoA; HMGR: HMG-CoA reductase; HMGS: HMG-CoA synthase; IPP: isopentenyl diphosphate; MVA: mevalonate; SES: sesquiterpene synthase; SQS: squalene synthase.

Fig. 1. Diagram of the mevalonate pathway leading to the biosynthesis of sesquiterpenes and sterols.

Squalene synthase (SQS) catalyzes the condensation of two molecules of farnesyl diphosphate (FDP) to form the linear 30 carbon compound squalene, the first committed precursor for sterol biosynthesis (Goldstein & Brown, 1990). SQS is generally described as a crucial branch point enzyme for synthesizing sterol intriguing as a potential regulatory point that controls carbon flux into either sterol or into non-sterol isoprenoids (such as sesquiterpenes). So if the SQS gene expression is inhibited by genetic manipulation, the carbon flux into sterol may be diverted to sesquiterpenes, and the biosynthesis of sesquiterpenes may be increased. With the purpose to increase artemisinin production, we have cloned squalene synthase cDNA (SQS) (Liu et al., 2003). In this chapter, we report the construction of the antisense SQS plant expression vector, and its effects on inhibition of SQS gene expression on squalene and artemisinin biosynthesis.

2. Materials and methods

2.1 Plant materials

A high artemisinin producing *Artemisia annua* L. strain 001 was collected from Sichuan Province of China. The seeds were surface sterilized and cultured on the Murashige &

Skoog (1962) basal medium with 0.7% agar and 3% sucrose in growth chamber at 26 °C and 16 h photoperiods. Leaves of 2-week-old seedlings were used for *Agrobacterium*-mediated transformation. After transformation, the transgenic plants and control plants were grown in green house with natural light and watered manually.

2.2 Construction of antisense plant gene expression vector

The original plasmid pSQF2 containing squalene synthase cDNA (SQS) gene, cloned from *A. annua* by our laboratory (Liu et al., 2003), was used as a template for the amplification of SQS fragment, then the amplified fragment was used for the construction of antisense plant expression vector. The PCR primers were designed according to the sequence at the 5′- and 3′-terminal region of SQS gene: 5′-GAC GGA TCC AAC AAA CAG TAC AAT TGG TG-3′ (*Bam*H I restriction site underlined) and 5′-GCA GAG CTC GGA TTT GGA TCT TGA AGA AG-3′ (*Sac* I restriction site underlined). The amplified SQS fragment was 1.5 kb in length, after digested with *Bam*H I and *Sac* I, the resulting large fragment was collected. The binary vector pBI121, containing both the *NPT II* gene controlled by the NOS promoter and the *GUS* gene controlled by the cauli-flower mosaic virus 35S promoter, was digested with *Bam*H I and *Sac* I, and the resulting large fragment was collected. The above two collected fragments were fused with T4 DNA ligase (Takara) (Fig. 2). The recombinant plasmid pBISQS was transformed into the competent *E. coli* DH5α prepared using $CaCl_2$ method and pBISQS was extracted using alkaline lysis method then sequenced by Genecore Company.

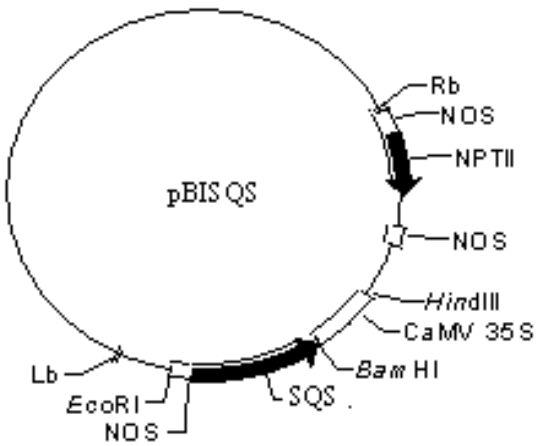


Fig. 2. Structure and diagram of recombinant plasmid pBISQS.

2.3 *Agrobacterium* preparation and genetic transformation

The binary vector pBISQS was introduced into *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method and used for genetic transformation. Transformants were selected on LB medium supplemented with 50 mg/L kanamycin (Kan) and 50 mg/L rifampicin. The corresponding wild type strain was cultured in LB medium containing 50 mg/L rifampicin.

Plant transformation was basically performed according to the method of Han et al (2005). The leaves of 2-week-old aseptic seedlings were immersed in 50 mL *A. tumefaciens*, which was at the log phase of growth and was diluted 10-fold with MS medium. After 20 min, the

infected leaves were taken out, blotted with sterile paper and co-cultured on solid MS medium at 26 °C for 2 or 3 d. After co-cultivation, the leaves were transferred to shoot-inducing medium (MS medium supplemented 1.0 mg/L 6-BA and 0.05 mg/L NAA) containing 20 mg/L Kan to induce shoot and 500 mg/L cefotaxine (Cefo) to kill residual *Agrobacterium*. The medium without Kan was used as a control. Here the key difference with Han's method is that a sheet of sterile filter paper was placed on the shoot-inducing medium, since we found by such a simple action, the shoot induction frequency can be noticeably increased. One week later, the leaves were transferred to fresh MS selection medium with 400 mg/L Cefo. After 4-6 weeks selection Kan resistant shoots were obtained and transferred to 100 mL Erlenmeyer flask with 40 mL regeneration medium (MS basal medium supplemented with 0.05 mg/L NAA) to induce roots. All the rooted seedlings were kept in the greenhouse at 25 °C under the fluorescent lamps (with a light intensity of 3000 lx, 16h), with a relative humidity of 40%.

2.4 PCR detection

The integration of SQS gene into *A. annua* genome was confirmed by the polymerase chain reaction (PCR). The CTAB method was used to purify the genomic DNA from transgenic *A. annua* leaves. The forward primer was 5´-CCA CGT CTT CAA AGC AAG TGG ATT -3´, designed according to the sequence of CaMV 35S promoter, and the reverse primer was 5´-GCA GAG CTC GGA TTT GGA TCT TGA AGA A G -3´, designed according to the sequence at the 3´-terminal region of SQS gene. 30-cycle reactions, each consisted of heated denaturation (94 °C for 40 s), annealing (55 °C for 30 s) and extension (72 °C for 2 min), were carried out and the reaction mixtures were subjected to agarose gel electrophoresis.

2.5 RNA isolation and RT-PCR detection

For reverse transcription-polymerase chain reactions (RT-PCR), 1 g of total RNA isolated from the leaves of the transformed and non-transformed plants was used. The RNA extraction protocol was done as described in Sambrook et al (1989). The first strand cDNA was synthesized by using a first-strand cDNA synthesis kit (TaKaRa), according to the manufacturer's instructions. The resultant first-strand cDNA was used as a template, and the PCR primers, P1: 5´-GGA ACC ATG GGT AGT TTG AAA GCA GTA TTG-3, and P2: 5´-GCC TGG ATC CCT TGA CTC TCT CTT AAC TAT-3´, were designed according to the SQS gene sequence of *A. annua*. The PCR was performed in the same way as described in the Section of PCR detection. At the same time, to normalize the amount of mRNA in each PCR reaction, a PCR product of actin in *A. annua* was amplified, and the primers were: 5´-AAC TGG GAT GAC ATG GAG AAG ATA T-3´, and 5´-TCA CAC TTC ATG ATG GAG TTG TAG G-3´.

2.6 Squalene analysis

Squalene analysis was carried out according to the method described by Wentzinger et al (2002). For all the plants analyzed, young leaves was collected. Extraction and purification of the samples were in accordance with Wentzinger et al (2002), and the samples were analyzed by GC, the GC injection port was operated at 120 °C. The oven temperature programmed from 120 °C to 180 °C at 15 °C min^{-1} and from 180 °C to 260 °C at rate of 25 °C min^{-1}. The final temperature was maintained for 25 min. The results were compared to standards.

2.7 Determination of artemisinin

The detection of artemisinin was performed according to the method of Zhao & Zeng (1986). Fresh leaves of the transformed and non-transformed plants were collected and dried to constant weight in an oven at 50 °C. Then the dried leaves were ground to fine powder. Exactly 0.05 g powder was added to an extraction bottle containing 40 mL petroleum ether (30-60 °C) and treated in a supersonic bath for 2 min. The extraction mixture was filtered and the petroleum ether was evaporated. The residue was dissolved in 1 mL methanol and centrifuged at 12000 r/min to precipitate the undissolved components. The supernatant was used for detection of artemisinin by HPLC.

200 μL of the above prepared methanol solution was placed in a 10 mL tube and 800 μL methanol and 4 mL 0.2% sodium hydroxide were added, mixed and maintained in a 50 °C water bath for 30 min, then the reaction mixture was cooled to room temperature. 0.5 mL of the reaction mixture was placed in a 1.5 mL Eppendorf tube, and 100 μL methanol and 400μL 0.05 M acetic acid were added, mixed and the sample purified by filtering on a NC filter (40 μm). The artemisinin standard (Sigma, MO) solutions with concentrations of 3, 6, 12, 24 and 48 μg/mL were prepared in the same way as the sample.

C18 reverse column was 4.6×250 mm, 5 μm. The mobile phase was 0.01 M phosphate buffer (pH 7.0) : methanol (55:45), with flow rate 1 mL/min. The wavelength of the UV detector was 288.6 nm and the injection volume was 20 μL. Artemisinin standard appeared at 4 min 30 s under the above mentioned conditions.

3. Results

3.1 Regeneration of transgenic *A. annua*

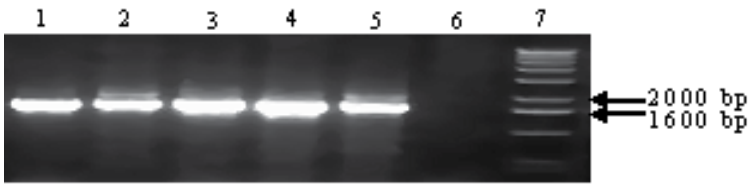
Leaf discs, which were infected with *A. tumefaciens* strain EHA105 harboring the binary vector pBISQS, were co-cultured for 36-48 h at 26 °C in dark, then transferred to shoot-inducing medium. Shoots usually start to appear within 2-3 weeks on this medium. In order to obtain higher frequency of shoot induction, a sheet of filter paper was placed on the shoot-inducing medium during the transformant selection step, which was proved to be a very effective means for shoot induction (Song et al., 2006). Regenerated shoots are rooted on MS medium with 0.05 mg/L NAA, followed by transferring to greenhouse (16 h light at 25 °C).

3.2 Molecular analysis of transgenic plants

To investigate the presence of antisense SQS gene in the putatively transformed plants, genomic DNA of 4 Kan-resistant plants regenerated from the leaves inoculated with EHA105 was isolated, and PCR analysis was performed. The antisense SQS gene was detected as 1660-bp fragments in all 4 analyzed plants (Fig. 3). The amplified fragments were of the same size as the predicted one. The fragment in the nontransformed plant was not amplified.

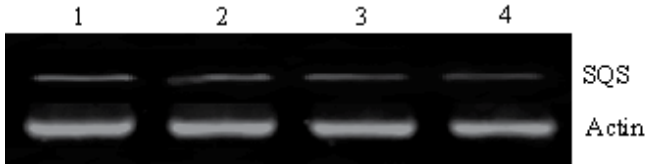
RT-PCR was performed using specifically designed primers according to the squalene synthase cDNA sequence in *Artemisia annua*. These primers allow specific amplification of *A. annua* squalene synthase cDNA. The results showed that the suppressed expression of the

endogenous *A. annua* squalene synthase gene in lines SQS3 and SQS5, but the transcriptional level in line SQS2 had no noticeable difference to that of the control (Fig. 4).



1. positive control; 2-5. transformed lines; 6. negative control; 7. DNA ladder.

Fig. 3. PCR amplification of the transformed plants and the control.

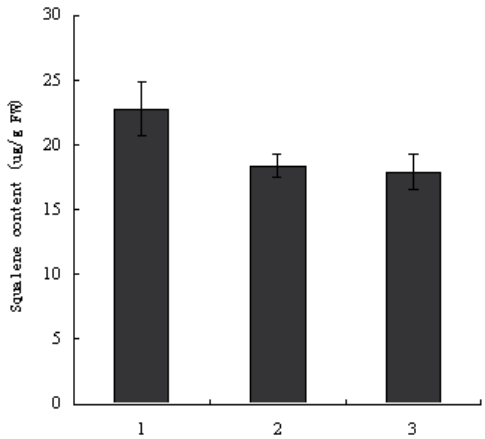


1. 001 line (non-transgenic control); 2. SQS2; 3. SQS3; 4. SQS5.

Fig. 4. RT-PCR analysis of the transformed plants and the control.

3.3 Detection of squalene content

In order to determine the effects of inhibiting squalene synthase gene expression on sterol biosynthesis, the leaves of transgenic lines SQS3, SQS5, and these of the control 001 were selected to detect squalene content. The results of GC-MS showed that in SQS3 and SQS5 transgenic lines, squalene content is decreased by 19.4% and 21.6% respectively in comparison with the control (Fig. 5).



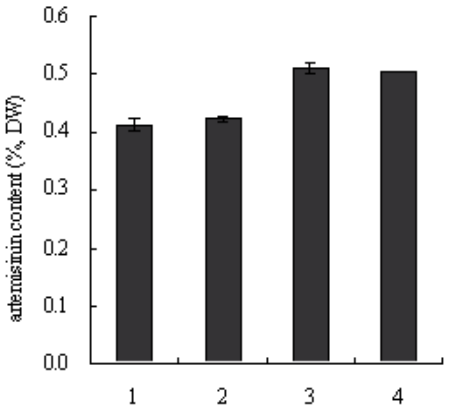
1. 001 line (control); 2. SQS3; 3. SQS5.

Fig. 5. Analysis of squalene content of the transgenic plants and the control.

3.4 Determination of artemisinin

Artemisinin was detected by HPLC. The results indicated that artemisinin content of SQS3 and SQS5 transgenic lines was increased by 23.2% and 21.5%, respectively compared with that of the control (Fig. 6) and in SQS2 transgenic line, the artemisinin content manifested no obvious variation compared with the control.

The above results demonstrated a clear negative correlation between squalene content and artemisinin content, which implies that the inhibiting of squalene synthase gene expression caused part of the flux for squalene biosynthesis diverting to artemisinin biosynthesis.



1. 001 line (control); 2. SQS2; 3. SQS3; 4. SQS5

Fig. 6. Analysis of artemisinin content of the transgenic plants and the control.

4. Discussion

Since squalene synthase is commonly depicted as the incipient and crucial branch point enzyme of the isoprenoid pathway to sterol biosynthesis, it has attracted considerable interest as a potential regulatory point that controls carbon flux into sterols. Several researchers reported the induction of sesquiterpene phytoalexins biosynthesis had been correlated with suppression of sterol biosynthesis in elicitor-treated tobacco cell cultures (Chappell et al., 1989; McGarvey & Croteau, 1995; Yin et al., 1997). The induction of one enzyme and suppression of the other are thought to be one mechanism that regulates the production of squalene and sesquiterpene (Devarenne et al., 1998). The biosynthesis of artemisinin belongs to the isoprenoid pathway, in this pathway, squalene synthase and amorpha-4,11-diene synthase are positioned at putative branch points in isoprenoid metabolism, these two enzymes catalyze the common farnesyl diphosphate to form squalene and amorph-4,11-diene, respectively. Furthermore, amorpha-4,11-diene synthase is considered as a key enzyme in artemisinin biosynthesis (Bouwmeester et al., 1999), so squalene synthase can be considered as a competitive enzyme of artemisinin biosynthesis. Therefore, the inhibiting of SQS gene expression may increase the biosynthesis of artemisinin.

In order to increase artemisinin content, we introduced antisense squalene synthase gene into *A. annua* via *Agrobacterium*-mediated transformation and transgenic plants were obtained. It has been shown that the transgenic plants had an increase of artemisinin content in lines SQS3 and SQS5 and a reduction in squalene content. This may be due to the suppression of squalene synthase gene expression caused part of the carbon flux to squalene biosynthesis diverting to artemisinin biosynthesis. At the same time, in correspondence with the decline in squalene content, the endogenous squalene synthase transcript level of the SQS3 and SQS5 transgenic lines were reduced compared with the control. These results strongly supported that the overexpression of squalene synthase in antisense orientation had a relevant effect on endogenous squalene metabolism in transgenic tobacco plants (Zhang et al., 2005). Though the SQS gene expression of the transgenic plants was inhibited, both the growth and the phenotype of the transgenic plants showed no obvious difference to those of the control, and this was in consistent with the results of transgenic tobacco (Zhang et al., 2005).

Our results as well as other related reports all indicated that it is possible to increase artemisinin content of *A. annua* by inhibiting the expression of genes competing precursors with artemisinin in transgenic plants (Yang et al., 2008; Zhang et al., 2009; Chen at el., 2011). But it has caused only a limited increase in artemisinin content, perhaps this is because of the biosynthesis of artemisinin is controlled by multi-genes and the contribution of manipulating one gene to artemisinin biosynthesis is limited. Recently, the study of artemisinin has made great progress, and more and more genes of artemisinin biosynthesis, such as cytochrome P450 monooxygenase (CYP71AV1), double bond reductase 2 (DBR2) and aldehyde dehydrogenase 1 (ALDH1) (Teoh et al., 2006; Covello et al., 2007; Zhang et al., 2008; Teoh et al., 2009), have been cloned, this makes it possible to regulate multi-genes of artemisinin biosynthesis in the future.

5. Conclusion

The antisense squalene synthase (SQS) gene was transferred into *A. annua* via *Agrobacterium*-mediated transformation, and the artemisinin content of one of the transgenic lines showed an increase of 23.2% in comparison to the wild-type control. The results demonstrated that inhibiting pathway competing for precursor of artemisinin by anti-sense technology is an effective means of increasing the artemisinin content of *A. annua* plants.

6. Acknowledgements

This work was supported by the National Natural Science Foundation of China (NSFC) (No. 61173098) and the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-EW-J-29).

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Part 4

Biosafety

20

Transgenic Plants – Advantages Regarding Their Cultivation, Potentially Risks and Legislation Regarding GMO's

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1. Introduction

Transgenic plants are the results of modern biotechnology. Biotechnology represents "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use" as it was defined by the United Nations Convention on Biological Diversity. So, modern biotechnology represents the technology by which the genetic material of an organism is modified. This technique can be applied to micro-organisms, plants, animals and even to humans. This modern technology by which the genes are transferred from one organism to another is called genes technology, genetic modification, genetic engineering or bioengineering and the transferred genes are called transgenes. The receptor organism, which is transformed by the insertion of the new genes into its genome, is named transgenic organism or genetic modified organism. The donor and receptor organisms may belong to very different species which can not cross by natural means. This fact makes the differentiation of the modern biotechnology by the conventional amelioration techniques. So, the transgenic organisms can not be obtained free in nature, in natural breeding, they can be obtained just with the help of modern biotechnologies.

Genetic engineering appeared as an own scientific discipline at the beginnings of '70 and till today it has many definitions, but the most comprehensive looks to be the following: genetic engineering represents an ensemble of methods and technologies made "in vitro" with genes, chromosomes and some times with entire cells, on the purpose of "building" of some new genetic structures with premeditated hereditary properties (Popa L. and col., 1982, as cited Vlaic A., 1998).

Genetic engineering has created two new concepts such as: genetic modified organism and the term of transgenesis.

The concept of genetic modified organism represents a legislative term which groups all the organisms resulted by genetic engineering techniques, obtained by different methods of genetic recombinations.

The transgenic organism term implies the incorporation of some exterior genes, considered as useful, obtained by the recombinant DNA technology in the genome of some zygotes, resulting transgenic organisms with modified genetical properties. This gene transfer is

called transgenesis because it presumes crossing the barriers (transgression) meaning the gene transfer between different species, especially belonging to different genus (a human gene transferred in the bacteria genome, a bacterial gene transferred into the genome of a plant or animal).

Practically the term of genetic modified organism designate every organism of which genetic patrimony was modified using the specific methods of genetic engineering.

2. Obtaining of the genetic modified plants (transgenic plants)

The genetic manipulation of plants has been going on since the dawn of agriculture, but until recently this has required the slow and tedious process of cross-breeding varieties. Genetic engineering promises to speed the process and broaden the scope of what can be done.

Progress is being made on several fronts to introduce new traits into plants using recombinant DNA technology. Recombinant DNA is DNA that has been created artificially. DNA from two or more sources is incorporated into a single recombinant molecule.

In order to obtain transgenic plants it is necessary to perform the following steps:

a. transfer of the gene of interest in the host cell;
b. selection of the host cells which have integrated the transgenes in their genome;
c. regeneration of some whole plants, starting from plantulas obtained by „in vitro" cultivation of the host cells;
d. cultivation of the transgenic plants in protected environments (green houses, etc.).
e. experimental cultivation of the new plants in the fields.

Each step of the operation requires some special aspects, such as:

a. transfer of the foreign gene of interest in the genome of the vegetal cell can be realized both indirectly or directly.

The indirect technique presume the using of a biologic vector, represented by some bacteria, plasmids or viruses, capable to introduce, naturally, a part of their DNA in the host cells, which provides new properties for the receptor plant. The direct method uses some techniques as the cellular microinjection of the recombinant DNA or the electro-perforation of the cellular membrane by electric shocks which produce micro-pores allowing the transgenic DNA to penetrate the new cell.

b. Selection of the host cells of the gene of interest, no matter the transfer method used. The success rate is always very low and for this reason it is necessary to associate the gene of interest with a marker gene, favorising the selection of the cells in which the transgene was integrated. The most used marker genes by the transgenic seeds producers are the ones which are codifying the resistance against an antibiotic or herbicide.
c. The regeneration and the cultivation of the transgenic plants in protected areas are made after they have been obtained *in vitro*. The plants obtained by cultivation in vitro, must be transferred into green houses or rooms with controlled climate. This process must be repeated for several generations, to control the expression of the new character, its hereditary transmission and the absence of the unwanted effects.
d. The experimental cultivation in field has the main aim to test the behavior of the new transgenic plant in natural conditions. Another aim is to cross the transgenic plant with

the conventional elite type (with the best performances at the time), to obtain varieties of GMO with an increase productive efficiency. Theoretically speaking, at least, these experimental cultures should also evaluate the impact of the GMO regarding the environment and human health.

All the experimental cultures of transgenic plants are conditioned by an authorization issued by a group of official experts. The name of the transgenic plant, the nature of the experiment, the location and the dimensions of the cultivated experimental areas must be communicated to the local authorities and to the public. In average, about one decade passes between the first laboratory manipulations and the commercial cultivation of the plants, all these experiments being done with high costs.

Finally, we still mention that all the transgenic plants are protected by industrial type registered marks. All the farmers who buy GM seeds are obliged, by contract, not to keep a part of their crop for seeds and the penalties for nonobservance of the law are very high.

3. New properties of the transgenic plants

The obtaining and, after this, the production of the transgenic plants, with commercial aim, determined a lot of enthusiasm within the researchers. The initial enthusiasm was then followed by the opposition of the skeptics, who tried, during the time, to argue against the cultivation of transgenic plants.

The supporters of transgenic plants cultivation base their arguments on the new properties of the transgenic plants. Among these, the most important are: resistance against the herbicides (more than half of the tries), followed by the resistance against the illnesses (mostly the viral ones) and against the insects.

We will shortly describe now the properties of transgenic plants already cultivated on a large scale.

a. Resistance against the herbicides

It is manifested by the capacity of the plant to live and to develop after it was sprayed with a strong insecticide substance. This property is due to the transfer of the *Bar* gene which determines different enzymatic actions materialized by the transformation of the herbicide into a non toxic element. In the case of transgenic plants, the resistance against the herbicides is uni-specific, so the plant is resistant only against the herbicide for which it was created and for this reason the producer of the transgenic plant delivers also the characteristic herbicide for each transgenic plant.

b. Resistance against the insect pests

The plants having this property are permanently synthetize in their tissue an insecticide protein that determine the death of the phytophage insects. The gene codifying the resistance against the insects originates in a soil bacteria named *Bacillus thuringiensis. Bacillus thuringiensis* is a bacterium that is pathogenic for a number of insect pests. The lethal effect is determined by a proteic toxine which is produced. Through recombinant DNA methods, the toxin gene can be introduced directly into the genome of the plant where it is expressed and provides protection against insect pests of the plant.

c. Resistance against illnesses

Genes that provide resistance against plant viruses have been successfully introduced into such crop plants as tobacco, tomatoes, and potatoes. By transferring the gene codifying the protein of the viral capside there were obtained plants resistant against the illnesses produced by some viruses because it blocks the propagation of the viruses in the transgenic plant.

d. Resistance against freezing

Even not on a large scale, but there were obtained GMO resistant against freezing. This type of resistance was obtained by two methods. First, the conventional cultures (especially strawberries) were treated with transgenic "antifreezing" bacteria. The second method consisted in insertion of some genes obtained from fish living in cold water, as *Hippoglossus hippoglossus*, a fish living in the North Sea, transferred into strawberries.

e. Improved nutritional quality

Milled rice is the staple food for a large fraction of the world's human population. Milling rice removes the husk and any beta-carotene it contained. Beta-carotene is a precursor to vitamin A, so it is not surprising that vitamin A deficiency is widespread, especially in the countries of Southeast Asia. The synthesis of beta-carotene requires a number of enzyme-catalyzed steps. In January 2000, a group of European researchers reported that they had succeeded in incorporating three transgenes into rice that enabled the plants to manufacture beta-carotene in their endosperm.

f. Delayed maturation

This property was first conferred to tomatoes. In this case, there were also used two main methods:

- insertion of a gene that blocks the galacturonase, an enzyme producing the fruits softening;
- blockage of the maturating hormone synthesis; the maturation is than started by treatment with ethylene before transferring on the market.

g. Salt tolerance

A large part of the land is so laden with salt that it cannot be used to grow most important crops. However, researchers at the University of California Davis campus have created transgenic tomatoes that grow well in saline soils. The transgene was a highly-expressed sodium/proton antiport pump that sequestered excess sodium in the vacuole of leaf cells.

Due to the new properties of the transgenic plants, their supporters have valid commercial arguments to support their production, cultivation and trading.

4. Advantages of transgenic plants presented by their producers and supporters

The supporters of transgenic plants producing and trading say that these have a lot of advantages both for producers, for farmers, for industry, for consumers and for the environment and the human future.

a. For the producers of the new varieties

A high efficiency in plants amelioration is obtained. The techniques of gene transfer are more precise because they allow the insulation and the propagation of the interest gene, while the classical hybridization techniques use the entire parental genomes and for this reason are needed back-crossings to emphasize the manifestation of a parental gene or to eliminate some secondary unwanted effects determined by the action of the gene in the genome. Furthermore, the number of the new characters susceptible to be conferred by gene transfer is much higher because the entire genetic information could be used despite its origin (viral, bacterial, vegetal or even human).

b. For farmers

First of all, the process of pests destroying is simplified due to the elimination of herbicides in the pre-emergent period and in the vegetation period. For the GMO only one total herbicide is necessary.

On the other hand, the production output is increasing as well as the profits of the transgenic cultures, even the obtaining cost of the GMO is rather high.

c. For industry

Due to the new properties of the transgenic plants, their processing could be also improved, as is the case of the modified starch, of low lignin content wood (in this case the paper manufacturing is less pollutant), of bio-plastics, of some human protein production (easier and in higher quantities, for therapeutic aim).

d. For consumers

Nowadays the fruits and the vegetables with delayed maturation are easier stored, with minimum losses. The maturation moment can be controlled according to the demands of the market.

In the future it is considered that transgenic plants can determine an improved human health due to the higher content of vitamins, minerals, essential aminoacids, by using the vaccine plants, the rice enriched in pro-vitamin A, etc.

e. For the environment and human future

First of all, transgenic plants imply lower pollution due to lower quantities of pesticides. Then, higher agricultural productions are obtained and people hope to eliminate the starving in the world (by extension of the areas cultivated with GMO resistant against salted soils, acid soils, lower temperatures, etc.)

5. Risks related to the cultivation of the transgenic plants

Despite of their advantages, it seems that the obtaining and mostly the cultivation of transgenic plants also imply some risks. The most important risks are the followings:

a. Risks related to the nowadays techniques of vegetal gene transfer

- Secondary unwanted effects. The first obtained transgenic tomato with delayed maturation was floury, with metallic taste and difficult to be transported due to its very fragile skin. Due to these reasons, the American consumers rejected it.

- "weaknesses" in the transgene expression. For example, in USA, in 1996, the fields with transgenic cotton were destroyed in proportion of about 60% by some insects against which the plants were considered to be resistant. Similar, also in cotton, after the second treatment with herbicide it was noticed a deformation of the capsule. This could have happened because the producers have had no enough time to check the stability of the transgenic character on an enough great number of experimental fields.

b. Ambiental risks

- Limitation of the risk evaluation by experimental cultures
- Risks related to the health, materialized by some allergies and resistance to some antibiotics.
- Risks related to the biodiversity of the ecosystems by
  - dissemination of the transgenic pollen to the similar spontaneous plants;
  - crossings between transgenic and conventional varieties of species;
  - apparition of some pest plants resistant against total herbicides
- Risks related to plants resistant to the insects attack:
  - apparition of some pests resistant against the insecticide-protein of the Bt maize;
  - intoxication of other insects by the transgenic plants
  - toxicity for the enemies of the pest insects.
- Risks regarding the circuit of the insecticide toxins in soils and in the trophic chains;
- Risks determined by the cultivation techniques of the plants resistant against herbicide, insects, viruses.
- Risk of destruction of the spontaneous flora and of the plants in the neighborhood of the cultivated fields, by the total herbicides.

6. Cultivation of the genetic modified plants worldwide

The first commercial transgenic plants cultures started in the middle of 1990 years, in USA, where they had the fastest evolution.

Immediately after followed Argentina and Canada, where the increasing of the surfaces cultivated with transgenic plants stabilized beginning with 1999. Both countries were followed by Brazil and China.

After only 7 years from their official start, the commercial cultures of transgenic plants reached a total surface of 60 million ha at which we could add the illegal GM soy cultures in Brazil of about 1 million ha.

During the fourteen years of commercialization 1996 to 2009, the global area of biotech crops increased almost 80-fold (78.8), from 1.7 million hectares in 1996 to 134 million hectares in 2009. This rate of adoption is the highest rate of crop technology adoption for any crop technology and reflects the continuing and growing acceptance of biotech crops by farmers in both large as well as small farms and resource-poor farmers in industrial and developing countries. In the same period, the number of countries growing biotech crops quadrupled , increasing from 6 in 1996 to 12 countries in 1999, 17 in 2004, 21 countries in 2005, and 25 in 2009 (Clive J., 2010).

Taking into account that approximately 21% of the 134 million hectares had two or three traits (planted primarily in the USA, but also increasingly in ten other countries, Argentina, Canada, the Philippines, South Africa, Australia, Mexico, Chile, Colombia, Honduras, and Costa Rica), the true global area of biotech crops in 2009 expressed as "trait hectares" was 180 million compared with 166 million "trait hectares" in 2008. Thus, the real growth rate measured in "trait hectares" between 2009 (180 million) and 2008 (166 million) was 8% or 14 million hectares compared with the apparent growth rate of 7% or 9 million hectares when measured conservatively in hectares between 2008 (125 million hectares) and 2009 (134 million hectares). (Clive J., 2010).

The list of the higher transgenic plants which are produced includes: (http://www.molecular-plant-biotechnology.info/transgenic-plants/list-of-higher-plants-where-transgenic-plants-have-been-produced.htm)

Herbaceous dicotyledons, such as: Nicotiana tabacum (tobacco), N. plumbaginifolia (wild tobacco), Petunia hybrida (petunia), Lycopersicon esculentum (tomato), Solanum tuberosum (potato), Solanum melongena (eggplant), Arabidopsis thaliana, Lactuca sativa (lettuce), Apium graveolens (celery), Helianthus annuus (sunflower), Linum usitatissimum (flax), Brassica napus (oilseed rape; canola), Brassica oleracea (cauliflower), Brassica oleracea var (cabbage), Brassica rapa (syn. B. campestris), Gossypium hirsutum (cotton), Beta vulgaris (sugarbeet), Glycine max (soybean), Pisum sativum (pea), Medicago sativa (alfalfa), M. varia, Lotus corniculatum (lotus), Vigna aconitifolia, Cucumis sativus (cucumber), Cucumis mew (muskmelon), Cichorium intybus (chicory), Daucus carota (carrot), Armoracia sp. (horse radish), Glycorrhiza glabra (licorice), Digitalis' purpurea (foxglove), Ipomoea batatas (sweet potato), Ipomoea purpurea (morning glory), Fragaria sp. (strawberry), Actinidia sp. (Kiwi), Carica papaya (papaya), Vitis vinifera (grape), Vaccinium macrocarpon (cranberry), Dianthus caryophyllus (carnation), Chrysallthemum sp. (chrysanthemum), Rosa sp. (rose);

Woody dicotyledons: Populus sp. (poplar), Malus sylvestris (apple), Pyrus communis (pear), Azadirachta indica (neem), Juglans regia (walnut);

Monocotyledons: Asparagus sp. (asparagus), Daclylis glomerata (orchard grass), Secale cereale (rye), Oryza sativa (rice), Triticum aestivum (wheat), Zea mays (corn), Avena sativa (oats), Festuca arundinacea (tall fescue);

Gymmosperms (a conifer) Picea glauca (white spruce).

Analysing the list it can be noticed that nowadays genetic engineering helped the producing of a wide scale of different types of plants but just a few of them are nowadays cultivated at large scale.

The worldwide market of transgenic plants consists almost exclusively, in four species: soy, cotton, maize and rapes. The other transgenic plants (potato, papaya, tobacco, pumpkin) are cultivated only on small surfaces, non-relevant for the total cultivated ones.

The list of the EU registered GM products which are used as food or food additives comprise the following plants:

(http://ec.europa.eu/food/dyna/gm_register/index_en.cfm)

- Soybean (MON40-3-2), MON-Ø4Ø32-6, Monsanto, genetically modified soybean that contains: cp4 epsps gene inserted to confer tolerance to the herbicide glyphosate.
- Soybean (A2704-12), ACS-GMØØ5-3, Bayer, genetically modified soybean that contains: pat gene inserted to confer tolerance to the glufosinate-ammonium herbicide.
- Soybean (MON89788), MON-89788-1, Monsanto, genetically modified soybean that contains: cp4 epsps gene inserted to confer tolerance to the herbicide glyphosate.
- Cotton (MON1445), MON-Ø1445-2, Monsanto, genetically modified cotton that contains: cp4 epsps gene inserted to confer tolerance to the herbicide glyphosate.
- Cotton (MON15985) MON-15985-7, Monsanto, genetically modified cotton that contains: cry1Ac and cry2Ab2 genes inserted to confer insect-resistance highly selective in controlling Lepidopteran insects.
- Cotton (MON15985 x MON1445), MON-15985-7 x MON-Ø1445-2, Monsanto, Genetically modified cotton that contains: cry1Ac and cry2Ab2 genes inserted to confer insect-resistance highly selective in controlling Lepidopteran insects and cp4 epsps gene inserted to confer tolerance to the herbicide glyphosate.
- Cotton (MON531), MON-ØØ531-6, Monsanto, genetically modified cotton that contains: cry1A(c) gene inserted to confer insect-resistance.
- Cotton (MON531 x MON1445), MON-ØØ531-6 x MON-Ø1445-2, Monsanto, Genetically modified cotton that contains: cry1A(c) gene inserted to confer insect-resistance and cp4 epsps gene inserted to confer tolerance to the herbicide glyphosate.
- Cotton (LLCotton25), ACS-GHØØ1-3, Bayer, genetically modified cotton that contains: pat gene inserted to confer tolerance to the glufosinate-ammonium herbicide.
- Cotton (GHB614), BCS-GHØØ2-5, Bayer, genetically modified cotton that expresses: 2mepsps gene inserted to confer tolerance to the glyphosate herbicides.
- Maize (Bt11), SYN-BT Ø11-1, Syngenta, genetically modified maize that contains: the cryIA (b) gene inserted to confer insect-resistance and the pat gene inserted to confer tolerance to the herbicide glufosinate-ammonium.
- Maize (DAS59122), DAS-59122-7, Pioneer and Dow AgroSciences, genetically modified maize that contains: the cry34Ab1 and cry35Ab1 genes inserted to confer protection against certain coleopteran pests such as corn rootworm larvae (Diabrotica spp.) and pat gene inserted to confer tolerance to the glufosinate-ammonium herbicide.
- Maize (DAS1507), DAS-Ø15Ø7-1, Pioneer and Dow AgroSciences, genetically modified maize that contains: cry1F gene inserted to confer resistance to the European corn borer and certain other lepidopteran pests and pat gene inserted to confer tolerance to the herbicide glufosinate-ammonium.
- Maize (DAS1507xNK603), DAS-Ø15Ø7-1xMON-ØØ6Ø3-6, Pioneer and Dow AgroSciences, genetically modified maize that contains: cry1F gene inserted to confer protection against certain lepidopteran pests such as the European corn borer (Ostrinia nubilalis) and species belonging to the genus Sesamia, pat gene inserted to confer tolerance to the glufosinate-ammonium herbicide and cp 4epsps gene inserted to confer tolerance to the glyphosate herbicide.
- Maize (GA21), MON-ØØØ21-9, Syngenta, genetically modified maize that contains: mepsps gene inserted to confer tolerance to herbicide glyphosate.
- Maize (MON810) , MON-ØØ81Ø-6, Monsanto, genetically modified maize that contains: cryIA (b) gene inserted to confer resistance to lepidopteran pests.
- Maize (MON863), MON-ØØ863-5, Monsanto, genetically modified maize that contains: a trait gene cry3Bb1 inserted to confer insect- resistance and nptII gene inserted as a selection marker.

- Maize (NK603), MON-ØØ6Ø3-6, Monsanto, genetically modified maize that contains: cp4 epsps gene inserted to confer tolerance to the herbicide glyphosate.
- Maize (NK603 x MON810), MON-ØØ6Ø3-6 x MON-ØØ81Ø-6, Monsanto, genetically modified maize that contains: cp4 epsps gene inserted to confer tolerance to glyphosate herbicides and the cry1Ab gene inserted to confer protection against certain lepidopteran insect pests (Ostrinia nubilalis, Sesamia spp.).
- Maize (T25), ACS-ZMØØ3-2, Bayer, genetically modified maize that contains: pat gene inserted to confer tolerance to the herbicide glufosinate-ammonium.
- Maize (MON88017), MON-88Ø17-3, Monsanto, genetically modified maize that contains: modified cry3Bb1 gene inserted to confer protection to certain coleopteran pests and cp4 epsps gene inserted to confer tolerance to glyphosate herbicides.
- Maize (MON89034), MON-89Ø34-3, Monsanto, genetically modified maize that contains: cry1A.105 and cry2Ab2 genes inserted to confer protection to certain lepidopteran pests.
- Maize (59122xNK603), DAS-59122-7xMON-ØØ6Ø3-6, Pioneer, genetically modified maize that contains: cry34Ab1 and cry35Ab1 genes inserted to confer protection against certain coleopteran pests; pat genes inserted to confer tolerance to the glufosinate-ammonium herbicides and cp4 epsps genes inserted to confer tolerance to glyphosate herbicides.
- Maize (MIR604), SYN-IR6Ø4-5, Syngenta, genetically modified maize that contains: modified cry3A gene inserted to confer protection against certain coleopteran pests and pmi gene inserted as selection marker.
- Maize (MON863xMON810xNK603) , MON-ØØ863-5xMON-ØØ81Ø-6xMON-ØØ6Ø3-6, Monsanto, genetically modified maize that contains: cry3Bb1 gene inserted to confer protection against certain coleopteran pests; cry1Ab gene inserted to confer protection against certain lepidopteran insect pests; cp4 epsps gene inserted to confer tolerance to glyphosate herbicides and nptII gene inserted as a selection marker.
- Maize (MON863 x MON810) , MON-ØØ863-5 x MON-ØØ81Ø-6, Monsanto, genetically modified maize that contains: cry3Bb1 gene inserted to confer protection against certain coleopteran pests; cry1Ab gene inserted to confer protection against certain lepidopteran insect pests and nptII gene inserted as a selection marker.
- Maize (Bt11xGA21), SYN-BTØ11-1xMON-ØØØ21-9, Syngenta, genetically modified maize that expresses: the cry1Ab gene which confers protection against certain lepidopteran pests ; the pat gene which confers tolerance to the glufosinate-ammonium herbicides and the mepsps gene which confers tolerance to glyphosate herbicides.
- Maize (MON863 x NK603), MON-ØØ863-5 x MON-ØØ6Ø3-6, Monsanto, genetically modified maize that contains: cry3Bb1 gene inserted to confer protection against certain coleopteran pests; cp4 epsps gene inserted to confer tolerance to glyphosate herbicides and nptII gene inserted as a selection marker.
- Maize (MON88017xMON810), MON-88Ø17-3xMON-ØØ81Ø-6, Monsanto, genetically modified maize that expresses: the cry1Ab gene which confers protection against certain lepidopteran pests ; the cry3Bb1 gene which provides protection to certain coleopteran pests and the cp4 epsps gene which confers tolerance to glyphosate herbicides.
- Maize (MON89034 xNK603), MON-89Ø34-3x MON-ØØ6Ø3-6, , Monsanto, genetically modified maize that expresses: the cry1A.105 and cry2Ab2 genes which provide protection to certain lepidopteran pests and the cp4 epsps gene which confers tolerance to glyphosate herbicides.

- Maize (59122x1507xNK603), DAS-59122-7xDAS-Ø15Ø7xMON-ØØ6Ø3-6, Pioneer, genetically modified maize that expresses: the cry1F gene which confers protection against certain lepidopteran pests; the cry34Ab1 and cry35Ab1 genes which provide protection to certain coleopteran pests; the pat gene which confers tolerance to the glufosinate-ammonium herbicides and the cp4 epsps gene which confers tolerance to glyphosate herbicides.
- Maize (1507x59122), DAS-Ø15Ø7x DAS-59122-7, Pioneer, genetically modified maize that expresses: the cry1F gene which confers protection against certain lepidopteran pests; the cry34Ab1 and cry35Ab1 genes which provide protection to certain coleopteran pests and the pat gene which confers tolerance to the glufosinate-ammonium herbicides.
- Maize (MON89034 xMON88017), MON-89Ø34-3x MON-88Ø17-3, Monsanto, genetically modified maize that expresses: cry1A.105 and cry2Ab2 genes which provide protection to certain lepidopteran pests; cry3Bb1 gene which provides protection to certain coleopteran pests and cp4 epsps gene which confers tolerance to glyphosate herbicides.
- Oilseed rape (GT73), MON-ØØØ73-7, Monsanto, genetically modified oilseed rape that contains: cp4 epsps and goxv247 genes inserted to confer tolerance to the herbicide glyphosate.
- Oilseed rape (T45), ACS-BNØØ8-2, Bayer, genetically modified oilseed rape that contains: pat gene inserted to confer tolerance to the herbicide glufosinate-ammonium.
- Swede-rape (MS8, RF3, MS8xRF3), ACS-BNØØ5-8ACS-BNØØ3-6ACS-BNØØ5-8 x ACS-BN003-6, Bayer, genetically modifieds oilseed rape that contains:a bar (pat) gene inserted to confer tolerance to herbicides based on glufosinate ammonium; barnase gene inserted to leads to lack of viable pollen and male sterility and barstar gene inserted to leads to lack of viable pollen and male sterility.
- Starch potato (EH92-527-1), BPS-25271-9, BASF, genetically modified starch potato that contains: an inhibited gbss gene responsible for amylase biosynthesis. As a result, the starch product has little or no amylase and consists of amylopectin and nptII gene inserted as a selection marker.
- Sugar beet (H7-1), KM-ØØØ71-4, KWS SAAT and Monsanto, genetically modified sugar beet that expresses: a CP4 EPSPS protein confers tolerance to glyphosate containing herbicides.

Beside the GMO plants there also exist genetic modified microorganisms, such as: (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm)

Bacterial biomass, (pCABL- Bacterial biomass), Ajinomoto Eurolysine SAS, Bacterial protein, by-product from the production by fermentation of L-Lysine HCl obtained from (Brevibacterium lactofermentum) the recovered killed microorganisms. The source is the Brevibacterium lactofermentum strain SO317/pCABL, used for feed produced from GMO bacteria: " bacterial biomass".

Yeast biomass, (pMT742 or pAK729-Yeast biomass), NOVO Nordisk A/S, NOVO Yeast Cream is a product produced from genetically modified yeast strains (Saccharomyces cerevisiae) cultivated on substrates of vegetable origin. The source is the Saccharomyces cerevisiae strain MT663/pMT742 or pAK729, used for feed materials produced from GMO yeast: "yeast biomass".

The global impact of the genetic modified plants presents the following aspects *(www.biotech-gmo.com):* the additional brut margin realized by the farmers by cultivation of the genetic modified plants is of 22 billion USD, and the reduction of the pesticides quantities applied on the soil for the modified plants is about 172 million kg, resulting an impact coefficient on the environment of 14%.

Herbicide tolerance continues to be the most common transgenic trait. Herbicide tolerance is available for all of the major GM crops, including soybean, maize, rapeseed, and cotton. In 2005, the first herbicide tolerant sugar beets were approved in the US, Australia, Canada, and the Philippines. Herbicide tolerant rice and wheat already have been developed, but currently are not in use. In 2006, there was wide cultivation of herbicide tolerant alfalfa for the first time in the USA (80,000 hectares). In most of the cases the tolerance is to the following herbicides glyphosate (*Roundup*) or glufosinate-ammonium (*Liberty*). Such crops make up 70 percent of the 102.0 million hectares of GM crops worldwide (2006).

(http://www.gmo-compass.org/eng/agri_biotechnology/gmo_planting/145.gmo_cultivation_trait_statistics.html)

Global area of genetically engineered crops, 1996 to 2006: By trait (million hectares)					
Trait	HT	IR (Bt)	IR/HT	VR/Others	**Total**
1996	0.6	1.1	--	<0.1	**1.7**
1997	6.9	0.4	<0.1	<0.1	**11.0**
1998	19.8	7.7	0.3	<0.1	**27.8**
1999	28.1	8.9	2.9	<0.1	**39.9**
2000	32.7	8.3	3.2	<0.1	**44.2**
2001	40.6	7.8	4.2	<0.1	**52.6**
2002	44.2	10.1	4.4	<0.1	**58.7**
2003	49.7	12.2	5.8	<0.1	**67.7**
2004	58.6	15.6	6.8	<0.1	**81.0**
2005	63.7	16.2	10.	<0.1	**90.0**
2006	69.9	19.0	13.1	<0.1	**102.0**
Source: ISAAA, Clive James, 2006.					

HT	Herbicide tolerance
IR	Insect resistance (mostly Bt)
VR	Resistance to virus diseases

Table 1. Global area of genetically engineered crops

Insect resistance is the second most common genetically modified trait. Herbicide tolerance and insect resistance (Bt) often are introduced simultaneously to a crop in one transformation event. This is called trait stacking. The third most commonly grown transgenic crop was stacked insect resistant/herbicide tolerant maize. Combined herbicide

and insect resistance was the fastest growing GM trait from 2004 to 2005, grown on over 6.5 million hectares in the US and Canada and comprising seven percent of the global biotech area. The recent expansion of Bt crops is mainly due to the increasing Bt maize and Bt cotton production in China, India, and Australia (Table 1 and 2).

Trait-crop combinations, 1996 to 2005 (million hectares)						
Trait	IR maize	HT maize	IR/HT maize	IR/HT cotton	HT cotton	IR cotton
1996	0.3	0.0	--	0.0	< 0.1	0.8
1997	3.0	0.2	--	< 0.1	0.4	1.1
1998	7.0	2.0	--	--	--	1.0
1999	7.5	1.5	2.1	0.8	1.6	1.3
2000	6.8	2.1	1.4	1.7	2.1	1.5
2001	5.9	2.4	2.5	1.9	1.8	2.1
2002	7.7	2.5	2.2	2.2	2.2	2.4
2003	9.1	3.2	3.2	2.6	1.5	3.1
2004	11.2	4.3	3.8	3.0	1.5	4.5
2005	11.3	3.4	6.5	3.6	1.3	4.9
2006	--	--	--	--	--	3.8
Source: ISAAA, Clive James, 2006.						

HT	Herbicide tolerance
IR	Insect resistance (mostly Bt)
VR	Resistance to virus diseases

Table 2. Trait –crop combinations in GMOs

7. Legislation regarding the transgenic plants

The purpose of a legislation system regarding the utilisation and cultivation of transgenic organisms obtained by modern biotechnology is the protection of the environment and the human health. The legislation has a preventive role and not a corrective one.

The legislation regulating the obtaining, testing, utilisation and commercialisation of the organisms obtained by modern biotechnology was elaborated in 1990 with three main objectives:

- to protect the human and animal health;
- to protect the environment;
- to assure the circulation of the GMO in EU;

The most important legislative act regarding GMO's in EU is the Directive 2001/18/EC and the Regulation (EC) No. 178/2002.

7.1 Directive 2001/18/EC

The directive presents the legislative framework regarding the deliberate release of the genetically modified organisms (GMOs) into the environment and the placing of GMOs on the market in accordance with the precautionary principle.

(http://europa.eu/legislation_summaries/agriculture/food/l28130_en.htm).

The main aim of this Directive is to make the procedure for release and placing on the market of genetically modified organisms (GMOs) and to introduce compulsory monitoring after GMOs have been placed on the market.

It also provides a common methodology to assess case-by-case the risks for the environment associated with the release of GMOs (the principles applying to environmental risk assessment are set out in Annex II to the Directive).

Public consultation and GMO labelling are made compulsory under the new Directive. Rules on the operation of these registers are laid down in Decision 2004/204/EC

The Directive invited the Commission to present a proposal for implementing the Cartagena Protocol on biosafety, which led to the adoption of Regulation (EC) No 1946/2003 on transboundary movements of genetically modified organisms.

The Directive 2001/18/EC had entry into force in 17.4.2001, the deadline for transposition in the member states of the EU was 17.10.2002 and the Official Journal in which it was published was OJ L 106 of 17.4.2001. The amending acts of Directive 2001/18/EC are represented by two regulations, Regulation (EC) No 1829/2003 and Regulation (EC) No 1830/2003, both entering into force starting with 07.11.2003, and being published into OJ L 268 of 18.10.2003.

(http://europa.eu/legislation_summaries/agriculture/food/l28130_en.html).

7.2 Regulation (EC) No. 178/2002

Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority (EFSA) and laying down procedures in matters of food safety.

This Regulation provides a framework for food and feed Law within the EC. It applies to all stages of production, processing and distribution of food and feed, but does not apply to primary production for private domestic use or to the domestic preparation, handling or storage of food for private domestic consumption.

(http://www.food.gov.uk/scotland/regsscotland/regulations/scotlandfoodlawguide/sflg 200501/).

The legislation regarding the authorization for introduction of the GMO's in the environment for the experimental purpose is presented in the Directive 2001/18/EC (part B).

Important information, regulated in the part C, are about the general aspects of GMO's labelling, free circulation and information to the public.

(http://www.biosafety.be/GB/Dir.Eur.GB/Del.Rel./2001_18/2001_18_TC.html)

7.3 Other legislation regarding GMO's

Other legal instruments regarding GMO's are the followings:

- Directive 90/220/EEC on the deliberate release into the environment of genetically modified organisms entry into force on the 17-th of October 2002;
- Directive 98/81/EC amending Directive 90/219/EEC regarding the use of GMO's (http://europa.eu.int/eur-lex/pri/en/oj/dat/1998/1_330/1_33019981205en00130031.pdf)
- Decision 2002/623/EC establishing guidance notes supplementing Annex II to Directive 2001/18/EC. (http://europa.eu.int/eur-lex/pri/en/oj/dat/2002/1_200/1_20020020730en00220033.pdf)
- Decision 2002/813/EC115 establishes the format to be used by competent authorities when they provide summaries of notifications to the Commission under Article 11 of Directive 2001/18/EC. (http://europa.eu.int/eur-lex/pri/en/oj/dat/2002/1_280/1_28020021018en00620083.pdf)
- Decision 2003/701/EC116 establishes the format to be used by notifiers in the reporting of the results of the deliberate release to the competent authorities, as required by Article 10 of Directive 2001/18/EC (http://europa.eu.int/eur-lex/pri/en/oj/dat/2003/1_254/1_25420031008en00210028.pdf)
- Decision 2002/812/EC establishing pursuant to Directive 2001/18/EC the summary information format relating to the placing on the market of genetically modified organisms as or in products. (http://europa.eu.int/eur-lex/pri/en/oj/dat/2002/1_280/1_28020021018en00370061.pdf)
- Decision 2004/204/EC laying down detailed arrangements for the operation of the registers for recording information on genetic modifications in GMO, provided in Directive 2001/18/EC. (http://europa.eu.int/eur-lex/pri/en/oj/dat/2002/1_280/1_28020021018en00270036.pdf)
- Decision 2002/811/EC establishing guidance notes supplementing Annex II to Directive 2001/18/EC. (http://europa.eu.int/eur-lex/pri/en/oj/dat/2002/1_200/1_20020020730en00220033.pdf)
- Regulation (EC) No. 178/2002 regarding the general principles and requirements of food law. (http://europa.eu.int/eur-lex/pri/en/oj/dat/2002/1_031/1_03120020201en00010024.pdf)
- Regulation (EC) No. 1829/2003 on genetically modified food and feed; (http://europa.eu.int/eur-lex/pri/en/oj/dat/2003/1_268/1_26820031018en00010023.pdf)
- Regulation (EC) No. 1830/2003 concerning the traceability and labelling of genetically modified organisms and traceability of food and feed products produced from genetically modified organisms. (http://europa.eu.int/eur-lex/pri/en/oj/dat/2003/1_268/1_26820031018en00240028.pdf)

- Regulation (EC) No. 1946/2003 on transboundary movements of genetically modified organisms.
(http://europa.eu.int/eur-lex/pri/en/oj/dat/2003/1_287/1_28720031105en00010010.pdf)

8. Genetical modified flowers on the market

The research regarding the color of the flower is an ongoing process. It was noticed that in some plants the blue pigment is lacking, so the blue colored flowers cannot be obtained by classical cross-breed techniques. Genetic engineering has allowed scientists to produce carnation blue flowers even that the blue pigment is missing.

Moondust' carnation, first grown commercially in 1997, is a mini-carnation with purple-mauve flowers that gets its blue color from petunia genes grafted into the DNA of the carnation. Twelve scientists at an Australian company called Florigene labored for a decade to isolate the gene responsible for blue color in petunia and then transfer it into the carnation.

To date, they have released five carnations with the "Moon" prefix, all with varying shades of mauve (Moonvista), blue (Moonshade), violet (Moonlite) or purple (Monaqua) (Fig.1).

Roses, carnations, lilies and orchids all lack a class of blue pigments called delphinidins, named after the violet-blue we see in delphinium. The gene for delphinidin production is what the Florigene scientists removed from petunia and transferred to the carnation. (http://www.arhomeandgarden.org/plantoftheweek/articles/blue_carnation.htm)

Moonvista Moonshade Moonlite Moonaqua

Fig. 1. Florigene blue carnations

The blue carnations are cultivated in Ecuador, Columbia and Australia and commercialised in USA, Canada, Japan, EU and Australia.

Blue roses were always very desirable flowers because they symbol the mystery, the untouchable, being impossible to be produced free in nature. They were avaible on many markets, from many years, but they were not real blue roses, because they are traditionally created by dyeing white roses with blue dye.

It was in 2004 when the first blue rose was obtained by genetic engineering (Fig. 2). It was the result of the researches made by Japanese (Suntory Limited Research Centre) and Australian (Florigene) researchers and the first blue roses were officially presented to the specialists and to the market at the World Rose Convention in Osaka 2006.

http://www.flowermeaning.info/Blue.php

Fig. 2. Blue rose presented at World Rose Convention in Osaka, 2006

The above mentioned researchers observed that the flower color is mainly determined by anthocyanins. *Rosa hybrida* lacks violet to blue flower varieties due to the absence of delphinidin-based anthocyanins, usually the major constituents of violet and blue flowers, because roses do not possess flavonoid 3′,5′-hydoxylase (F3′5′H), a key enzyme for delphinidin biosynthesis. Other factors such as the presence of co-pigments and the vacuolar pH also affect flower color.

It was analyzed the flavonoid composition of hundreds of rose cultivars and measured the pH of their petal juice in order to select hosts of genetic transformation that would be suitable for the exclusive accumulation of delphinidin and the resulting color change toward blue. Expression of the viola *F3'5'H* gene in some of the selected cultivars resulted in the accumulation of a high percentage of delphinidin (up to 95%) and a novel bluish flower color.

For more exclusive and dominant accumulation of delphinidin irrespective of the hosts, the researchers down-regulated the endogenous dihydroflavonol 4-reductase (*DFR*) gene and over-expressed the *Iris*×*hollandica DFR* gene in addition to the viola *F3'5'H* gene in a rose cultivar. The resultant roses exclusively accumulated delphinidin in the petals, and the flowers had blue hues not achieved by hybridization breeding. Moreover, the ability for exclusive accumulation of delphinidin was inherited by the next generations. (Yukihisa Katsumoto, 2007).

9. Conclusions

1. The scientific progress could be not stopped by anyone and by nothing. It is very important to use the scientific progress for the whole humanity and to be in

concordance with the actual and the future generations' interest, without supporting the interests of only certain groups. This was the starting point of sustainability strategy which, in its essence, stipulate that the development is made for people and is realized by people.

2. The ecologic, geographic and antropic assemblies realize the landscape, with very important functions of general interest at the cultural, ecological, social level, this landscape being an important resource of the human economic activities. In this context, it should be mentioned that we don't know yet all the potential risks that the GMO could have, by long term accumulation, upon the environment.
3. The achievements of the genetic engineering have nowadays considerable benefits, but now we don't know the price we, or the future generations, will have to pay in the future for this benefits. The long term risks of the GMO are not entirely known today.

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Biosafety and Detection of Genetically Modified Organisms

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1. Introduction

Biosafety is a set of actions focused on preventing, minimizing and eliminating risks associated with research, production, teaching, use, technology development and services related to genetically modified organisms (GMOs) with the aims of protecting human and animal health and environmental preservation.

Transgenic organisms, or GMOs, are organisms in which genetic material has been altered by recombinant DNA technology. Biotechnology allows the insertion of one or more genes into the genome of an organism from a different organism or species (e.g., animals, plants, viruses, bacteria); the expression of the introduced gene results in a new feature in the phenotype of the modified organism. A shortened definition of genes is that they are DNA sequences that contain the necessary information to affect phenotypic expression in an organism, such as the shape of a seed or resistance to a specific pest. The information encoded by the gene is expressed through two principal steps: transcription, in which the coding region of the DNA is copied into single-stranded RNA; and translation, in which the amino acid sequence encoded by mRNA is assembled and translated into protein. Thus, for the creation of a GMO, it is necessary to introduce the gene responsible for a particular trait into the genome of the target organism through recombinant DNA techniques.

Several products derived from recombinant DNA technology are commercially available worldwide. GMO products already on the market include human insulin, somatropin and transgenic varieties of crops, such as maize, soybeans, cotton and common beans. The United States, Brazil and Argentina are among the principal countries engaged in the commercial production and marketing of GMOs (James, 2010).

The emergence of genetic engineering in the early 1970s in California, USA, with the isolation, introduction, and expression of the insulin gene in *Escherichia coli* provoked a strong reaction from the scientific community all over the world, which led to the Asilomar Conference in 1974. At that time, the scientific community proposed a moratorium on genetic engineering. They argued that rules and safeguards should be established to ensure

the use of genetic engineering techniques without risking human life and the environment. In a relatively short period of time, biosafety regulations were developed for the appropriate use of these technologies in the laboratory. After over 35 years of research on and commercial use of biotechnology, there have yet to be any reports about the adverse effects of the use of genetic engineering on human and animal health or the environment. Therefore, to ensure the appropriate generation and utilization of this technology, biosafety regulations and monitoring mechanisms have been developed in different countries around the world. Several field tests with transgenic varieties have been performed in the USA, Argentina, Bolivia and Chile since 1991. However, in Brazil, these tests only began in 1997.

2. Transgenic plants and their advantages

In the future, there will be difficulties in meeting the food demand in developing countries due the increasing trends of food prices and population growth. Therefore, it is necessary to employ new technologies, such as the use of transgenic varieties, to increase the productivity per unit area, especially in the developing nations. Qaim and Zilberman (2003) reported that in developing nations (i.e., China, India and Sub-Saharan Africa) farmers can achieve a greater than 60% grain yield advantage by using transgenic plants modified with the *Bt* gene instead of conventional varieties. In their work, these authors showed that the yield advantage comes solely from the impact of the *Bt* gene on the control of insect pests.

Biotechnology research currently plays a key role in food production because it helps to increase productivity, improve the nutritional quality of agricultural products and reduce production costs. Qaim and Zilberman (2003) reported that using the *Bt* gene for the control of insect pests gave a US $ 30 per ha advantage over conventional cotton. It is also advantageous because it allows reduction of the use of highly hazardous chemicals, such as organophosphates, carbamates, and synthetic pyrethroids, which belong to international toxicity classes I and II.

The commercialization of transgenic plants in Brazil has also strongly affected the agrochemical sector, which has annual profits of approximately 20 billion US dollars. Of this, approximately 8 billion US dollars per year corresponds to pesticides used for the control of diseases, insects and weeds. In some cases, the cost of pesticides in relation to the total cost of production reaches approximately 40%, as in cotton. However, varieties developed by genetic engineering that are tolerant to herbicides and resistant to insects, fungi, bacteria and viruses have led to reductions in the cost of agricultural production and, consequently, reduction of the impact of agrochemical wastes that have an adverse effect on the environment and human health.

The findings described above, obtained in developed and developing nations, demonstrate the contribution of transgenic plants to increasing the productivity per unit area to fulfill the increasing demand for agricultural products to feed the growing population of the world.

GMO technology is currently widely employed throughout the world. The global area of biotechnology crop coverage in 2010 reached approximately 148 million ha in 29 countries on five continents. The major biotechnology crops cultivated worldwide are maize, soybean, canola, cotton, sugar beet, alfalfa and papaya (James, 2010). The geographic distribution of biotech crops throughout the world is presented in Table 1.

3. Food biosafety

The food safety of transgenic plants is assessed in accordance with risk analysis. This methodology was initially developed with the aim of assessing deleterious effects on human health arising from potentially toxic chemicals present in food, pesticide residues, contaminants and food additives and was subsequently applied in assessing the food safety of GM plants.

One of the main foundations of risk analysis methodology is that transgenic plants are not inherently more dangerous than conventional crops; i.e., the potential health risks that may be associated with a transgenic variety are not because it is GM but rather are related to the possible chemical changes that may result from genetic modification (Konig et al., 2004). For example, a genetically modified common bean expressing an allergenic protein from the allergen Brazil Nut (*Bertholletia excelsa*) was not prohibited from being produced because it was obtained by genetic engineering, but because the genetic modification was incorporated in a gene that promotes the synthesis of an allergenic protein in this variety.

Order	Country	Area (millions of hectares)
1st	USA	66.8
2nd	Brazil	25.4
3rd	Argentina	22.9
4th	India	9.4
5th	Canada	8.8
6th	China	3.5
7th	Paraguay	2.6
8th	Pakistan	2.4
9th	South Africa	2.2
10th	Uruguay	1.1

Table 1. The ten major producers of transgenic crops in the world (adopted from James, 2010)

In general, most transgenic plants are modified to synthesize proteins that are absent in conventional varieties. These proteins are encoded by a transgene and introduced precisely for the purpose of conferring the desired trait. However, beyond this difference, other biochemical changes may result from the introduction of a transgene, and all of this is investigated during risk analysis.

In the case of transgenic plants, risk analysis is performed by comparing them with their non-GM counterparts, which are considered to be safe on the basis of their usage records. In risk analysis, instead of attempting to identify every hazard associated with the GM variety, one can seek to identify only new hazards that are not present in the traditional variety.

This type of comparative study is referred to as substantial equivalence analysis and is based on comparison of the biochemical profile of the transgenic variety with the conventional variety. The GM variety can be classified as substantially equivalent or substantially non-equivalent.

At this point, it should be noted that food security assessment of a GM plant is not restricted to applying the concept of substantial equivalence. This constitutes only the starting point for this assessment, and it aims to identify differences that will be analyzed later. Further analyses include allergenicity and toxicity tests performed *in silico*, *in vitro* and *in vivo* in animal models (i.e., rodents, birds, fish, and other species) to assess toxicity levels. In these tests, the LD_{50} (lethal dose in 50% of cases) is generally determined as an indicator of acute (i.e., short-term) toxicity.

The risk assessments are performed in three steps (Borém and Gomes, 2009):

Step 1. Risk assessment: This step can be defined as the evaluation of the probability of adverse health effects arising from human or animal exposure to a hazard. Risk assessment consists of four segments:

i. Hazard identification, which entails the identification of biological, chemical and physical hazards found in food that may cause adverse health effects;
ii. Hazard characterization, which entails an evaluation of an identified hazard in qualitative and quantitative terms and often involves the establishment of a dose-response relationship due to the magnitude of exposure (dose) to a physical, chemical, or biological hazard and the severity of adverse health effects;
iii. Exposure assessment, which entails a quantitative and qualitative assessment of the likelihood of ingestion of physical, chemical and biological agents through food;
iv. Risk characterization, which entails a qualitative and quantitative estimation of the likelihood and severity of an adverse effect on health based on identification and hazard characterization and on exposure assessment.

Step 2. Risk management: Risk management is measured from the results of risk assessment and other legitimate factors to reduce risks to the health of consumers. Measure of risk management may include labeling, imposition of conditions for marketing approval and post-trade monitoring.

Step 3. Risk communication: Risk communication includes the information exchange that must occur between all stakeholders, including the government, industry, the scientific community, media and consumers. It should occur throughout the assessment and risk management processes and should include an explanation to the public of the decisions made, ensuring access to documents obtained from the risk assessment and, at the same time, respecting the right to safeguard the confidentiality of industrial information.

Food biosafety analyses performed by different national and international organizations, such as the World Health Organization, the International Council for Science, the United Nations Food and Agriculture Organization, the Royal Society of London and the National Academies of Sciences from Brazil, Mexico, India, the United States, Australia, and Italy have demonstrated that transgenic varieties can be considered safe for human consumption.

4. Environmental biosafety

Similar to food risk assessment, environmental risk assessment considers three important points: the possibility, probability and consequences of a hazard, which should always be assessed on a case-by-case basis. This means that, following the identification of a possible

danger, you should consider whether that danger is possible, if it is likely and, if it were to occur, what the result would be (Conner et al., 2003).

In the specific case of risk assessment for GM plants, a fourth point should also be considered: the risks of non-adoption of this technology.

An essential element in any risk assessment is the establishment of correct benchmarks. As described for the assessment of food security, a GM crop plant is compared with its non-GM counterpart. Similarly, the environmental impact of transgenic plants should be evaluated in relation to the impact caused by conventional varieties.

These principles are essential for providing guidance regarding which tests should be conducted and what questions should be answered to generate information that will assist in making the decision to use or not use a specific transgenic variety. Failure to follow these principles can result in unnecessary and unhelpful evaluations in risk assessments.

For example, the cultivation of insect-resistant transgenic cotton in Brazil has raised concerns about gene escape, i.e., the possibility of the transgenic variety crossing with wild species of the genus *Gossypium* that are native in Brazil and thus sexually compatible with cultivated cotton (Freire and Brandão, 2006). The main issue is the possibility of the pollen of transgenic cotton plants fertilizing wild cotton. The offspring of such crosses could have consequences for the maintenance of genetic diversity, although this point remains very controversial, as several research groups do not believe that the introduced gene would produce any adaptive advantage when exposed to the natural environment.

Gene escape from transgenic plants can occur in three main ways:

i. When the transgenic plant becomes a weed or an invasive species (e.g., for crops with weed-like characteristics, such as sunflower, canola, and rice), the transgenic gene found in the transgenic plant may allow the crop to become weedier and more invasive;
ii. Intraspecific and interspecific hybridization, such as when transgenic DNA is transferred by crossing to other varieties of cultivated species and wild species, respectively;
iii. When transgenic DNA is asexually transmitted to other species and organisms.

For a gene to escape and be transferred to different species, certain conditions are necessary:

i. The two parental individuals must be sexually compatible;
ii. They must be located in neighboring areas and with flowering overlap between the two parental types;
iii. A sufficient amount of viable pollen must be present and transferred between individuals;
iv. The resulting progeny should be fertile and ecologically adapted to environmental conditions where the parents are located.

To avert gene escape from transgenic varieties to conventional varieties, isolation distance should be maintained. For example, maize is a wind-pollinated species, and the distances that pollen can travel depend on the wind pattern, humidity and temperature. In general, fields with transgenic varieties should be isolated from other conventional varieties with a distance of at least 200 m (Weeks et al., 2007). The risk of gene escape from soybeans and maize to wild relatives in Brazil is considered by most scientists to be small or nonexistent.

The risk of gene escape associated with transgenic soybeans in China and maize in Mexico to their wild relatives are different because China and Mexico are the centers of diversity of the respective species.

Additionally, transgenic crops may have an effect on a non-target organism. Evidence has shown that the lethal dose (LD_{50}) of *Bt* varieties for beneficial insects, such as bees and ladybugs, is far higher when such insects are exposed to fields of these transgenic varieties. Some studies have also reported the safety levels of *Bt* varieties for the monarch butterfly (Tabashnik, 1994; Tang, 1996).

A study under the auspices of the European Union addressing the environmental impacts caused by cultivation of GM crops was conducted for 15 years (1985-2000) involving 400 public research institutions and reached the following conclusion: "Our research shows that, according to standard risk assessments, GM organisms and their products do not present risks to human health or the environment. In fact, the use of more precise technology and conducting the most accurate analysis possible during the regulatory process associated with these varieties make these products even more secure than their conventional forms (European Union, 1999 – available at
http://bio4eu.jrc.ec.europa.eu/documents/FINALGMcropsintheEUBIO4EU.pdf).

5. Biosafety regulations

The need for official regulation of genetically modified organisms became more evident in the mid-1980s, when biotechnology companies sought permission to perform research on genetically modified organisms.

Currently, implementation of biosafety rules is determined on a case-by-case basis around the world based on technical and scientific data, with transparent decision-making and consistency, building public confidence. There is no international standard established, and each country is responsible for creating its own regulations for research, trade, production, transport, storage and disposal.

5.1 Regulatory agencies

Field testing of the first transgenic plants began in the early 1980s. To date, there have been more than 25,000 field tests performed worldwide, half of which have been in the United States and Canada. In South America, the greatest number of releases occurred in Argentina. The commercialization of GM crops began in 1994, with tomatoes genetically engineered by Calgene. Transgenic varieties of soybean, maize, cotton, canola and papaya, among other crops, already represent a significant share of agriculture in the United States, Brazil, Canada, and Argentina. These varieties have been modified for resistance to insects and viruses and tolerance to herbicides. Field testing and laboratory evaluation of GMOs have been performed by regulatory agencies in each country to evaluate the risks of the GMOs to human and animal health and the environment.

In the United States, the agencies that examine the safety of genetically modified varieties include the Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), and the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA).

The USDA-APHIS regulates field tests of both plants and genetically modified microorganisms. This is the agency that reviews the licensing procedures for field testing by industry, universities and nongovernmental organizations (NGOs). The processes related to the agricultural and environmental safety of herbicide organisms, such as Roundup Ready™ (RR) soybeans, are also reviewed by USDA-APHIS.

The FDA evaluates the safety and nutritional aspects of genetically modified varieties that are used for human food and feed for animals. The FDA guidelines are based on the fact that food derived from GMOs must meet the same rigorous safety standards required for conventional foods.

The EPA is responsible for ensuring the safety of GMOs and varieties that produce pesticide elements and chemical and biological substances for distribution, consumption and trade. Under U.S. law, the jurisdiction of the EPA is limited to pesticides. For example, a plant that has been genetically modified to resist insects falls within its jurisdiction, but not a plant modified to resist drought. Plant resistance to a pest is under the authority of the EPA because the plant produces a substance that acts as a pesticide. In contrast, drought resistance may be due to factors such as deeper roots, and this transgenic plant would be subject to regulation by the USDA-APHIS.

With respect to pest-resistant varieties, the EPA has four categories of analysis: product characterization, toxicology, effects on non-target organisms and disposal in the environment. The characterization of a product includes a review of its origin and how the transgene is expressed in living organisms, the nature of the pesticide, the modifications introduced to the trait (compared with what is found in nature) and the biology of the receiving plant. To analyze the toxicology, the level of acute oral toxicity of the pesticide substance is evaluated in rats. For proteins toxic to insects, the EPA also requires a digestibility test, which evaluates the time required for the protein to be digested by gastric and intestinal juices. The EPA also analyzes the allergenicity of the protein. With regard to environmental impacts, the agency examines the exposure and toxicity of the transgenic plant to non-target insects and beneficial insects.

The regulation of biosafety is governed through local agencies in each country, such as Health Canada and the Canadian Food Inspection Agency in Canada; the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Health and Labour in Japan; and Conabia in Argentina.

Several countries in Latin America, including Brazil, Argentina, Chile, Mexico and Venezuela, have established biosafety rules through specific legislation to regulate the use of genetic engineering and the release of the products of this technology into the environment. In Brazil, these rules are guided by Federal Law 11,105, enacted on March 24, 2005. This law also created the National Board of GM Biosafety (CTNBio), the National Biosafety Council (CNS), also known as the Council of Ministers, and the Biosafety Information Service (SIB). Fontes (2003) discussed the regulatory and legal concerns in Brazil in detail.

CTNBio is composed of 27 members, and their backups appointed by the scientific community, have deep scientific knowledge in the areas of biotechnology associated with humans, animals, plants and the environment, along with representatives of the several Ministries. CTNBio has developed a set of Normative Resolutions that now regulate most

aspects of modern biotechnology in the country. To date, the Board has authorized the commercial release of 31 GM events for use in agriculture and vaccines for husbandry in Brazilian territory.

CTNBio analyzes the requests that are forwarded to it by issuing opinions that are specific to each transgenic target of evaluation. Before any GMO product is released for planting, trade or use, it must have been subjected to analysis of possible risks to humans, animals and the environment. The results of these tests are evaluated by CTNBio, which then makes a recommendation for release of GMOs that do not pose a risk to human or animal health or the environment. Genetically modified products suspected to have any adverse effect on human or animal health or the environment are banned from commercial use by CTNBio.

5.2 Other recently developed techniques to avoid environmental and human health concerns

5.2.1 Chloroplast engineering

The chloroplast is the principal organelle of plant cells and eukaryotic algae where photosynthesis is carried out. This is the site where food production starts. Therefore, incorporation of genes in the chloroplast genome and their expression provide a relative advantage compared with expression in the nucleus (Wang et al. 2009). The existence of many copies of chloroplast plastids, in some cases up to 10 thousand per cell, mean that chloroplast DNA comprises approximately 10-20% of cellular DNA. The other advantage of chloroplast engineering is that it is associated with greater visibility of the effects of the transgene on the production of proteins and carbohydrates. In addition, a chloroplast transgene has an advantage over a nuclear DNA transgene because it reduces the impact of contamination of the transgene via pollen to wild relatives and other similar species. This reduces the risk of developing weeds resistant to toxins and herbicides. Furthermore, the expression of a transgene in chloroplast-transgenic plants is more stable than a nuclear transformant because transgenes integrate into the chloroplast genome by homologous recombination (Elizabeth, 2005). The other advantage of chloroplast transformation is the possibility of transforming several transgenes under the control of one promoter. Chloroplast transformation has been used by different researchers to develop cultivars resistant to diseases and pests, weeds, and abiotic stresses (Wang et al., 2009, Wani et al., 2010).

Chloroplast engineering has several applications in the fields of medicine, biology and agriculture. This technique is used for improvement of plant traits, such as resistance to biotic and abiotic stress (Rhodes and Hanson, 1993), introduction of insect-resistant transgenes into crop plants (Dufourmantel et al., 2005), biopharmaceutical production (Daniell et al., 2001), metabolic pathway engineering (Lossl et al., 2005), and research on RNA editing (Hayes et al., 2006). A detailed review on chloroplast engineering was presented by Wang et al. (2009).

5.2.2 Producing marker-free transgenic plants

The objective of developing marker-free transgenic plants is of great urgency, as most of the transgenic plants developed contain the marker gene used during the selection phase. Selection genes include resistance to ampicillin (or other antimicrobials) and herbicides. The existence of this type of gene in the environment has raised a great deal of concern from

environmentalists and consumer protection groups, as this might have unpredictable consequences for human and animal health. In specific cases, an herbicide-resistant gene used in the selection process could pass to weeds, resulting in the development of weeds that are resistant to herbicides. With respect to consumers, if a gene resistant to antibiotics is present in food products, it could spread to the human population, though there is no evidence for this at the moment. Therefore, developing appropriate methods of selection for transgenes without transferring the selectable marker to the environment will increase the commercialization of transgenic plants in the world by reducing the cost of developing and commercializing genetically modified crops and will make consumers more comfortable.

The currently available transformation techniques are not efficient in transforming a number of genes responsible for quantitative traits and other important agronomic traits in a single transformation. This makes joint gene transformation in a target organism impossible and increases the cost of the transformation and development of transgenic organisms. According to Puchta (2003), there are four possible ways to avoid marker genes from genetically modified crops: avoiding the use of selectable marker genes; employing marker genes with no harmful effects; joint transformation of the target trait gene and marker gene, followed by their segregation; and removing the selectable marker gene from the gene of interest (the transgene) through successful site-specific recombination or homologous recombination. Among the above four techniques used to eliminate marker genes from transgenic plants, the fourth one has recently received more attention because of its efficiency and acceptance.

The specific-site recombination system involves the Cre protein and two lox sites within the transgene construct. This system is used in a number of different types of genome manipulations. Two lox sites in direct orientation are required for the excision of the marker gene (Russell et al., 1992), and excision is performed with the expression of Cre. Elimination of the marker gene from the target gene in the transgenic plant occurs through site-specific recombination, in which the two lox sites are required for removing the marker gene from the plant genome by the use of the Cre recombinase. This technique has two principal advantages: it requires only a single round of genome manipulation; and it reduces the time required to obtain a marker-free transgenic plant (Thomason et al., 2001).

6. Detection of genetically modified organisms

There are several reasons to support detailed research on transgenic organisms, especially to allow their easy and quick identification among conventional organisms. The ability to identify a GMO is strongly related to animal, human and environmental biosafety, and it is within the rights of the consumer to know what he/she is eating. In the case of Brazil, which is the second largest transgenic producer, local legislation specifies that all food containing more than 1% material derived from GMOs should have a label indicating the presence of a GMO in its composition. The presence of genetically modified seeds in conventional seed samples has become a growing problem for international trade and may result in severe consequences for food exporters, such as Brazil. For compliance with laws and GMO regulatory measures to be effective, it is necessary to apply techniques that enable the sensitive, reliable detection and quantification of GMOs. The following section will describe the techniques used for the detection of GMOs.

6.1 Techniques for GMO detection

The genetic modifications introduced into an organism should be well known to better understand what techniques can be employed in the detection of GMOs. The basic structure of an exogenous DNA sequence inserted into a GMO is composed of three main elements, as described by Conceição and co-workers (2004): the promoter region, which is responsible for gene transcription; the gene itself, which defines the desired characteristic; and the terminator region, which is responsible for transcription termination. All detection systems are based on the elements present in the DNA sequence inserted into the GMO, either through direct detection of an exogenous DNA molecule inserted in the genome or indirectly through the protein product and by-products resulting from expression of the DNA insert.

6.1.1 Direct assays: Detection of the presence of exogenous DNA

6.1.1.1 PCR

The polymerase chain reaction (PCR) was developed by Mullis and Faloona in 1987, and it is the main technique used in molecular biology laboratories to detect GMOs. The technique is based on the replication of specific sequences of exogenous DNA. For this purpose, small pieces of DNA known as primers bind to exogenous DNA present in the GMO, and during the PCR amplification process, which is catalyzed by the DNA polymerase enzyme, thousands copies of the specific sequence are produced. Copies of DNA produced by PCR are easily visualized by electrophoresis in agarose gels. A DNA intercalating agent (e.g., ethidium bromide) is used for visualization of DNA bands present in the gel. If the same primers are used in a non-transgenic organism, the band will not displayed in an agarose gel, as there will be no detection of copies of exogenous DNA because it is not present in the wild or conventional organism.

The PCR technique is very specific, sensitive and safe and is able to detect both events of genetic modification (Bertheau et al., 2002; Giovannini and Concillo, 2002) and distinguish events associated with different gene constructs expressing the same protein (Yamaguchi et al., 2003). However, this technique also presents some limitations, such as: 1) the difficulty involved in designing primers, as it is necessary to know the genetic sequence of the DNA introduced into the GMO, and this information is usually confidential (Holst-Jensen et al., 2003); 2) the need for appropriate equipment and trained personnel; 3) the relatively high cost because the test is specific for each genetic alteration introduced; and 4) the special care required to avoid sample contamination (Miraglia et al., 2004, Yamaguchi et al., 2003).

The quality of DNA extracted is crucial to the success of the PCR method. There are several procedures described in the literature for DNA extraction from leaves, seeds, and even processed foods. The CTAB (cetyltrimethylammonium bromide) method is widely used in molecular biology laboratories. There are also several commercial kits that employ silica resin with high affinity for DNA molecules. Poor quality, degraded or low purity DNA can negatively influence the success of PCR, preventing the identification of foreign DNA in the sample. This low purity DNA can occur from the presence of inhibitors in DNA extracts (e.g., proteins, polysaccharides, and polyphenols) that hinder the annealing of primers to target DNA and/or inhibit the activity of the DNA polymerase enzyme (Ahmed, 2002). Consequently, the reaction will occur with a low efficiency or may not occur at all. For quality control in PCR, it is always necessary to use a standard reaction (control) that

evaluates the quality of the extracted DNA and avoids false-negative results. The default reaction may be performed using specific primers for any known endogenous gene.

The limit of detection for PCR is between 20 picograms and 10 nanograms of exogenous DNA. Thus, it is possible to detect a single genetically modified seed among 1,000 to 10,000 conventional seeds (Luthy, 1999).

6.1.1.2 Real-time PCR

Various PCR techniques are used in molecular analysis of transgenic events for different purposes. The real-time qPCR (quantitative PCR) technique was developed and has been used in the identification of GMO events and products. Real-time qPCR is used not only for the detection of specific DNA but also for the quantification of copy number of a particular target DNA sequence inserted in a GMO. The difference between conventional PCR and qPCR is the specificity and sensitivity of the latter method. In real-time qPCR, equipment capable of detecting the fluorescence emitted by the reaction during each cycle of amplification of the target DNA molecule is used whereas in conventional PCR, the result is visualized in an agarose gel after 30-45 cycles of amplification. Real-time qPCR is monitored from the first cycle of amplification until the last one by detecting the fluorescence emitted. Then, it is possible to recognize the exact time (i.e., cycle) at which the amplification of the target molecule can be detected (Figure 1). These data allow inference of the number of copies of the transgene present in the GMO based on an endogenous control reaction. Basically, when the number of amplification cycles required to detect the emitted fluorescence is reduced, the copy number of the transgene inserted in the GMO is higher (an inverse correlation). The sensitivity of the method is based not only on the uptake of the fluorescent signal but also on how fluorescence is emitted during the reaction. Most real-time PCR applications require only one fluorescent agent for double-stranded DNA. However, some applications require greater specificity, such as the TaqMan® system.

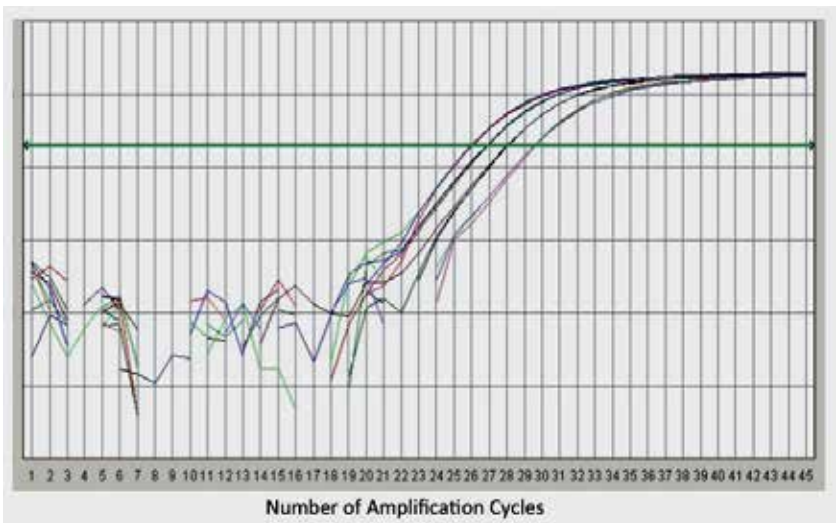

Fig. 1. Real-time qPCR. The curves in the graph show the progress of target molecule amplification. Capture of the fluorescent signal occurs throughout all PCR cycles. Source: Camargo (2009).

Real-time qPCR using the TaqMan® detection system employs a pair of primers and a probe labeled with a fluorophore (a marker that emits fluorescence when stimulated). These three oligonucleotides (two primers and a probe) are specific to the target sequence, thereby contributing to the greater specificity of the technique. In the process of primer amplification; the probe binds to the target DNA molecule, and a DNA polymerase enzyme initiates polymerization of the new molecule. When the enzyme meets the probe linked to the target DNA, it severs the probe and allows fluorescence to be emitted and then detected by the equipment. Therefore, the process of fluorescence emission is dependent on the joint action of four elements: two primers, one probe and the DNA polymerase enzyme.

There are factors related to amplification conditions that may adversely affect the reliability of the results obtained, such as the use of inappropriate temperatures for primer and probe annealing; low specificity of the primers and probe with respect to annealing to the DNA template; and inappropriate conditions for the activity of the polymerase enzyme (e.g., unadjusted salt concentration or pH of the buffer).

6.1.1.3 Southern blotting

The Southern blot technique, described by Southern in 1975, is also frequently used in laboratories to detect specific fragments of exogenous DNA integrated into the genomic DNA of a transgenic organism and its products. This technique essentially consists of five steps: 1) extraction and digestion of genomic DNA with one or more restriction enzymes; 2) separation of DNA fragments by electrophoresis in an agarose gel; 3) transfer and fixation of DNA present in the gel to a nitrocellulose or nylon membrane; 4) hybridization of DNA present in the membrane against a DNA probe that has sequence homology to the target DNA; and 5) visualization by autoradiography or colorimetry.

Southern blot analysis is very reliable and is considered molecular evidence of the integration of exogenous elements in a GMO genome. In addition, it is also possible to estimate the number of copies that were introduced into the genome of the recipient organism with this method (Figure 2).

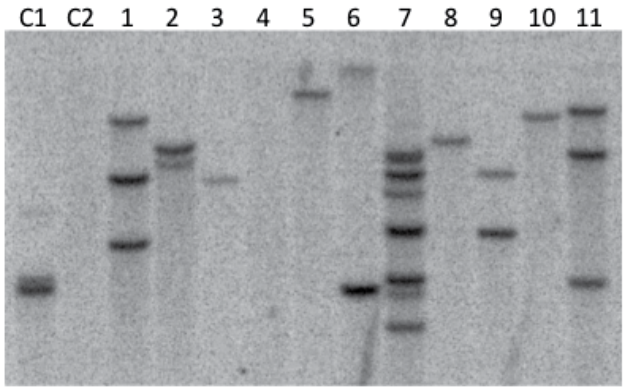

Fig. 2. Southern blot analysis. Analysis of transgenic events using the Southern blot technique. C1) Positive control (transgenic event); C2) negative control (wild plant); 1 to 11) transgenic events in the analysis. In this case, the number of bands refers to the copy number of target DNA sequences present in the genome of each event analyzed. Source: Camargo (2009).

However, this technique is associated with the limitation of requiring a large amount of genomic DNA (between 20 to 40 micrograms), which is sometimes difficult to obtain depending on the type of sample used. Other limitations to this technique are its high cost, operational complexity, long period required to perform the experiment and obtain results, the use of radioactive probes, and that it requires an appropriate infrastructure and adequate training for the handling and storage of radioactive products and waste disposal.

6.1.2 Indirect assays: Detection based on the presence of RNA

The genetic information in DNA must be translated into protein to be effective and have effects in an organism. The translation of information from DNA to protein occurs only because of the previous transcription of DNA into molecules of messenger RNA (mRNA). mRNA synthesis can be considered as the intermediate stage of the process of transferring the information contained in DNA and reflects the level of transcription activity, as the presence of mRNA is directly related to gene expression.

There are different molecular techniques that can be used in studies of gene expression. These include the northern blot and RT-PCR (reverse transcription - polymerase chain reaction) techniques. These techniques can be employed to determine the gene expression in different tissues and/or different stages of development of the organism under study and to monitor the gene expression of exogenous DNA in GMOs.

6.1.2.1 Northern blotting

The northern blot technique, also referred to as an RNA blot, was developed to study gene expression through the detection of RNA molecules present in a sample (Alwine et al., 1977). The execution of northern blotting is very similar to the Southern blot technique and basically consists of 5 steps: 1) total RNA extraction, 2) separation of RNA fragments by electrophoresis in an agarose gel, 3) transfer and fixation of the RNA present in the gel to a nitrocellulose or nylon membrane, 4) hybridization of the RNA present in the membrane against a DNA or RNA probe with homology to the target RNA sequence, and 5) revelation by autoradiography.

One of the most important steps in this technique is the extraction of total RNA from the sample because to obtain reliable results, it is necessary to obtain intact and pure RNA. Exercising care during the technique is much more critical than in Southern blotting because RNA degrades easily. To prevent its degradation, it is necessary to treat all objects with specific solutions to eliminate or minimize the presence of RNases, which are enzymes that are very effective in degrading RNA molecules.

6.1.2.2 RT-PCR

RT-PCR is widely used to verify gene expression by detecting mRNA molecules. This technique is based on reverse transcription of mRNA followed by PCR amplification. The reverse transcription reaction is based on the synthesis of complementary DNA (cDNA) from an mRNA molecule template by the reverse transcriptase enzyme. The product of this amplification is visualized in an agarose gel. The intensity of the bands visualized in the gel provides some indication of the amount of target mRNA present in the sample.

Quantitative RT-PCR (qRT-PCR) is a modern method based on the principles of RT-PCR (i.e., cDNA production followed by PCR). Therefore, qRT-PCR is more robust, specific and

sensitive; consequently, it provides better quantitative results. The amplification progress of the target molecule is displayed in real time by capturing a fluorescent signal in more sophisticated thermal cyclers, such as those described for the qPCR technique.

6.1.3 Indirect assays: Detection based on the presence of protein

6.1.3.1 Bioassays

In most of the GM varieties commercialized to date, genes have been introduced conferring tolerance to herbicides and/or resistance to viruses, fungi or insects (Borém and Almeida, 2011). The bioassay technique for the detection of GMOs in these cases is relatively simple, inexpensive and easy to establish in both laboratories and greenhouses, but a relatively long time is required to obtain results (the results are usually obtained after a week).

Bioassays for herbicide tolerance can be conducted using a plant or even seeds. In case of plants, a dose of the herbicide is sprayed on the leaves. Then, the plants are monitored daily to verify the presence or absence of any phenotype (i.e., symptoms) resulting from the application of the herbicide (Figure 3). The leaves of plants with no tolerance to the herbicide initially become yellow and then dry (i.e., the tissue undergoes necrosis). The leaves of herbicide-tolerant plants exhibit no or few symptoms of necrosis, and the plants continue to look as healthy as before the herbicide application. In the case of bioassays performed with seeds, the seeds are germinated in a medium containing a diluted solution of the herbicide. If the seeds are tolerant to the herbicide, germination will occur, and the plant will develop normally, as is seen for the seeds of transgenic Liberty Link™ corn and Roundup Ready™ soybeans, which are tolerant to glyphosate. If the seeds are sensitive to the herbicide, no germination will be observed. Currently, bioassays for herbicide tolerance are commonly used by companies that export seeds and grains to prove the authenticity and quality of their products (Torres et al., 2003).

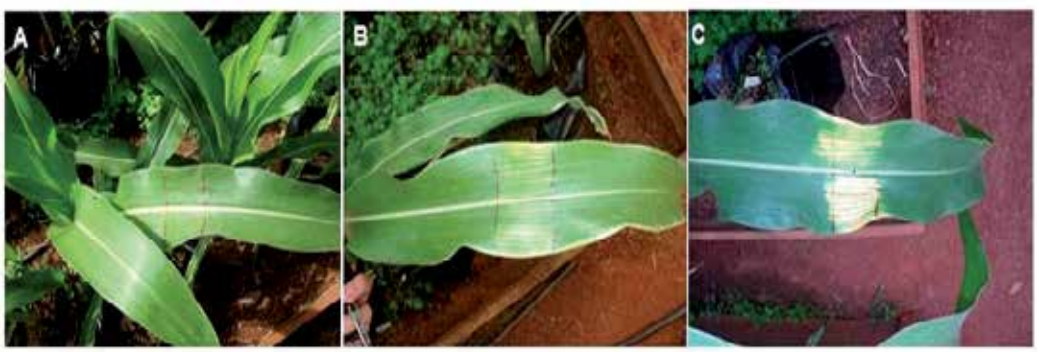

Fig. 3. Bioassay for herbicide tolerance in genetically modified maize plants overexpressing the *bar* gene. This gene encodes the enzyme phosphinothricin-N-acetyltransferase (PAT), which confers tolerance to the herbicide ammonium gluphosinate (PPT). a) Leaf of a genetically modified maize plant showing PPT tolerance after 10 days of herbicide application; b) leaf of a genetically modified maize plant showing mild PPT susceptibility after 10 days of application; c) leaf of an unmodified maize plant (negative control) showing an intense PPT susceptibility after 10 days of application. Source: Camargo (2009).

Insect-resistant plants can also be analyzed by bioassays. In this case, the bioassay can be performed by placing insects or their larval form, according to the cycle of the insect that attacks the plant, on the plants to be analyzed or even on leaf discs from the plants. Two types of information can be obtained from these experiments: 1) the mortality rate of the pest; and 2) the damage caused by the pests to the analyzed tissue. Analysis of these data will indicate whether or not the plant is resistant to the insect under investigation.

Such bioassays are based on the gene expression and phenotype of interest presented by the GMO. However, a disadvantage of this method is that the results of bioassays are generally not sufficiently definitive proof of the integration of exogenous DNA into the genome of a transgenic organism, and other evidence (i.e., results from other techniques) is necessary for its verification.

6.1.3.2 Immunoassays

Immunoassays are ideal methods for the qualitative and/or quantitative detection of specific proteins produced from an exogenous DNA sequence introduced into a GMO. The main immunoassays used for the detection and quantification of target proteins present in GMOs are the enzyme-linked immunosorbent assay (ELISA), western blotting and the lateral flow immunoassay (LFI).

6.1.3.2.1 ELISA

An ELISA identifies a target protein present in a protein extract containing a population of other proteins by using specific antibodies that bind to the target protein. The antigen-antibody reaction identifies the presence of exogenous protein in a qualitative and even quantitative form.

There are several types of ELISA, including direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA. The sandwich ELISA method, which uses two specific antibodies (Abs) for a target protein, is the most sensitive of these techniques and is used for the detection of GMOs (Yates, 1999). In this type of immunoassay, two types of antibodies specific for a target protein are used: a primary (i.e., capture) Ab, which is used to sensitize a plate to capture the target protein (i.e., the antigen, Ag) present in the sample; and a secondary (i.e., combined) Ab, usually in conjunction with an enzyme (e.g., peroxidase or alkaline phosphatase) that acts on a given substrate to produce color (in a colorimetric assay) or fluorescence (in a fluorimetric assay). The intensity of the color produced is directly related to the amount of antigen present in the sample, as the color will only occur when the target protein binds to the capture antibody on the plate, followed by binding of the enzyme-conjugated antibody to the immobilized target protein (Figure 4). Free antibodies and protein that did not form Ab-Ag-Ab complexes are discarded during microplate washing steps. Thus, there is little possibility for false-positive results to occur. For quantitative assays, a standard curve of protein at known concentrations is used.

Direct and indirect ELISA assays are similar to the sandwich ELISA, but with some modified steps. A direct ELISA assay uses only a specific antibody conjugated to an enzyme (i.e., the "secondary Ab"). Protein extract is added to a plate followed by the conjugated antibody. In the next step, the appropriate substrate is added; the enzyme then acts on it, and the reaction is revealed. An indirect ELISA assay uses a primary Ab specific for a target protein and an enzyme-conjugated Ab that is specific to the primary Ab (anti-IgG of the

organism in which the primary Ab was produced, usually rabbit or mouse). In this case, protein extract is added to a plate, followed by the primary Ab. The secondary Ab is added to the reaction, recognizing the primary Ab, and then binding to the Ab-Ag complex. The development stage occurs in the same way as in the direct ELISA. In many cases, the sandwich ELISA assay is two to five times more sensitive than the direct or indirect ELISA.

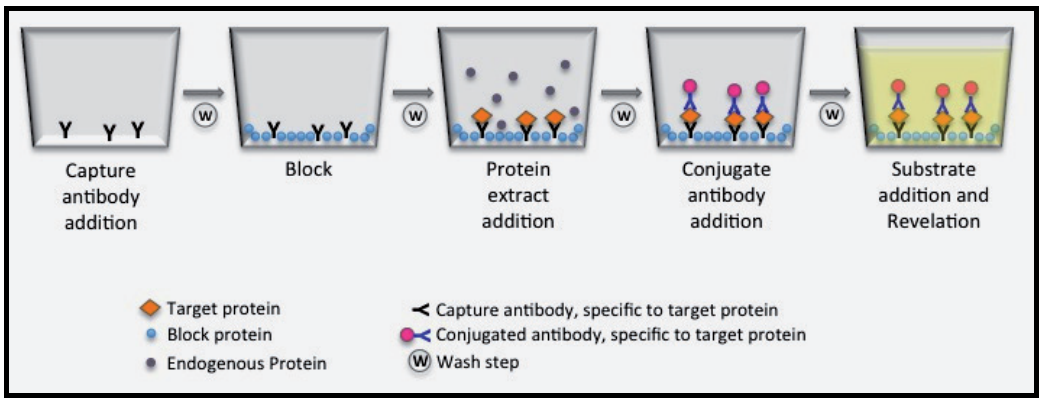

Fig. 4. Steps of the sandwich ELISA assay. The assay starts with sensitization of the plate with the capture antibody. After washing, the plate is blocked. Then, the protein extract is added. The plate is washed again, and conjugated antibody is added. After incubation, the plate is washed and then the protein present is revealed by adding the substrate. Source: Camargo (2009).

Another kind of ELISA is the competitive assay, in which the target protein in the sample and a standard protein conjugated to an enzyme compete for the binding of a capture antibody. In this type of test, the amount of target protein is inversely proportional to the colorimetric intensity produced. The more standard protein binds to the antibody, the more intense the color produced by the reaction. Some drawbacks of competitive ELISA are its restriction for use with only one specific antibody each time it is used and that it is less sensitive than the sandwich method.

The type of antibody used in an immunoassay can also contribute to the greater sensitivity and specificity of the assay. Monoclonal antibodies contribute to increasing the specificity of this type of technique while polyclonal antibodies increase sensitivity because they can recognize different epitopes of a target protein (Ahmed, 2002).

ELISA is a very sensitive, specific, robust, safe and rapid technique for the detection of GMOs in the laboratory. Moreover, it is the ideal technique for simultaneous analysis of a large number of samples under routine diagnosis.

6.1.3.2.2 Western blotting

The western blot technique is based on the separation of proteins present in the protein extract from a sample in non-denaturing polyacrylamide gels and subsequent transfer to nitrocellulose or nylon membranes. Detection of the target protein is performed by means of specific antibodies that recognize epitopes of the protein of interest. Visualization of the assay occurs through a colorimetric reaction or radiographic detection. Thus, western blot

analysis combines the resolution of electrophoresis with the specificity of immunological detection (Brasileiro and Carneiro, 1998).

The electrophoretic separation of proteins in the sample usually occurs under denaturing conditions. Thus, problems of solubility, aggregation and co-precipitation of the target protein with other proteins present in the sample are eliminated (Sambrook and Russel, 2001). However, antibodies against conformational epitopes of the target protein may not recognize these epitopes when denatured.

The western blot technique is semiquantitative, specific and sufficiently sensitive to detect proteins (Brett et al., 1999). The limit of detection for the target protein depends on a number of factors, including the type of membrane and the detection system used. In most cases, this limit corresponds to approximately 20 femtomoles (10-15 moles). Thus, it is possible to detect approximately 1 nanogram of a protein with a molecular weight of 50 kDa (Brasileiro and Carneiro, 1998). In seed analysis, the minimum limit of detection is 0.25% (Yates, 1999). Western blot analysis is a laborious technique, and it is capable of analyzing only a few samples simultaneously. Therefore, western blotting is rarely used in routine analysis of GMOs. This technique is usually used to confirm preliminary results generated by other detection techniques.

6.1.3.2.3 Lateral flow immunoassay

The lateral flow immunoassay (LFI) is widely used for analysis of material still in field trials and product testing because it is practical, inexpensive and fast. Its results are obtained within 5 to 15 minutes. Other advantages of this method are that it does not require special equipment and trained personnel. However, this technique is not sufficiently robust for quantification of GM material present in a sample, but it is a very sensitive technique for the qualitative detection of GMOs (Urbanek et al., 2001).

The principle of the lateral flow immunoassay is similar to the sandwich ELISA. The detection antibody is located at the end of a strip that is inserted into a sample solution. The target protein present in the sample binds to antibody present in the strip, and the Ag-Ab complex then migrates by capillary action to the other end of the strip, where there are two capture zones. In these areas, there are specific antibodies to capture the target protein or detection antibody. When the Ag-Ab complex passes through the capture zones, there is a colorimetric reaction (Figure 5). The presence of two colored bands on the strip indicates that the test is positive (i.e., the transgenic protein is present in the sample). The presence of only one band indicates that the sample is negative (i.e., that it contains no traces of the transgenic protein, but the test was performed correctly) (Conceição et al., 2004).

IFL strips are produced commercially for the detection of a wide range of proteins used in the production of GMOs. Soybeans, maize, canola, cotton and sugar beets genetically modified to contain the endotoxin Cry (Ab) from *Bacillus thuringiensis* or the CP4-EPSPS protein from *Agrobacterium tumefaciens* can be easily analyzed using this technique (Lipton et al., 2000).

In addition to their usefulness in the detection of GMOs, immunoassays are powerful tools for assessing the expression of a transgene. It is possible to identify the location of transgene expression in a plant, in which tissues the protein is present (e.g., roots, leaves, seeds) and the ratio of expression among different tissues (i.e., tissues that show more or less expression of the transgene).

6.1.4 Alternative techniques for the detection of GMOs

Because of the increasing number of GMOs and the complexity of the genetic changes that are emerging, new techniques are being developed or improved with the goals of increasing sensitivity and reliability, lowering costs and allowing simultaneous analysis. DNA microarrays, chromatography and mass spectrometry are examples of other techniques for detecting GMOs.

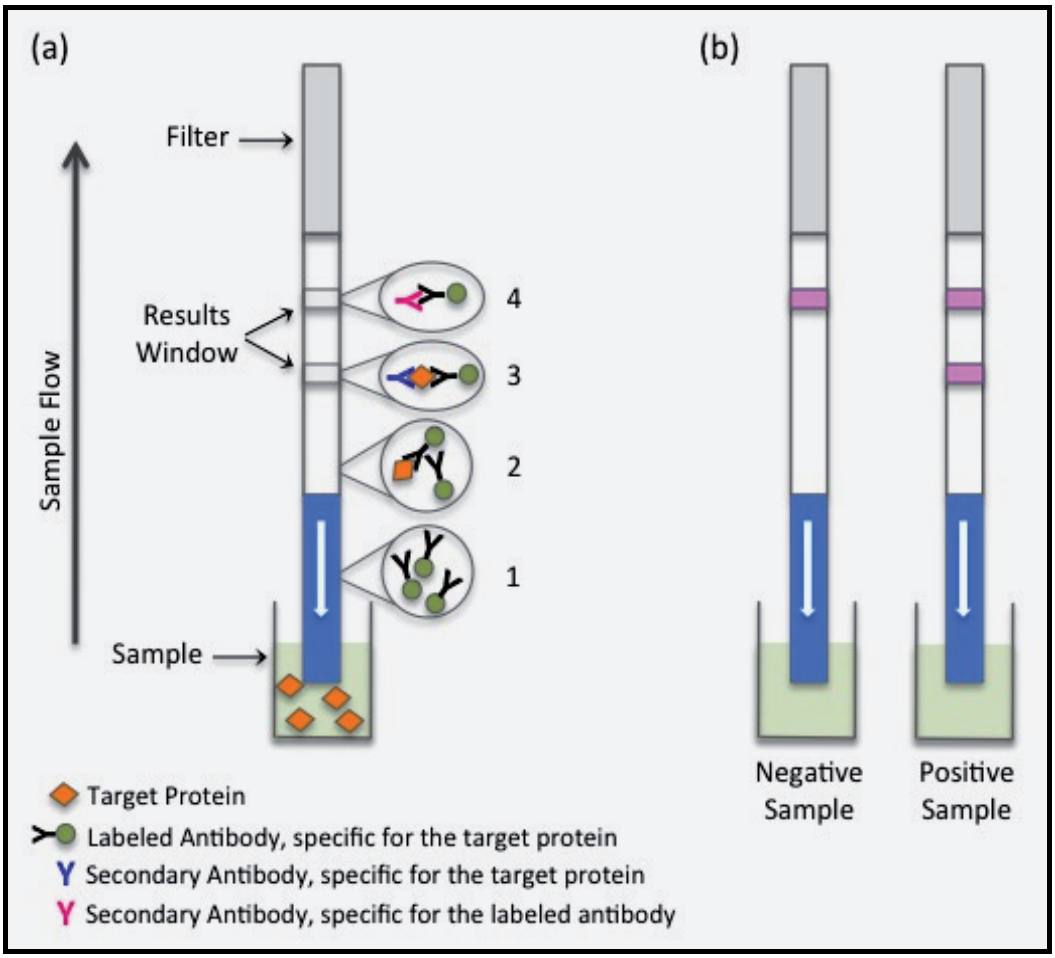

Fig. 5. Diagram of a lateral flow immunoassay (IFL). a) One end of the IFL strip is inserted into the sample. By capillarity, the sample travels up the strip toward the other end. During this run, the sample passes through a region where there are capture antibodies (1), and the target protein binds. Capture antibodies that are either complexed with the target protein or free (2) migrate and bind to specific antibodies for the target protein (3) or specific antibodies to the capture antibodies (4), respectively. b) A single band revealed in the results window indicates that the sample is negative (i.e., absence of the target protein), and two bands indicate that the sample is positive (i.e., presence of the target protein). Source: Camargo (2009).

7. Conclusions

Regulatory procedures for GM crops require extensive risk analysis on a case-by-case basis for modified organisms. Information regarding the number of copies of foreign DNA inserted into the genome, the expression level of the protein of interest, the parts of the plant in which the protein is present, the toxicity and allergenicity of the protein and its possible adverse effects for non-target organisms and for the environment is required. Thus, numerous techniques have been used to evaluate the biosafety of GMOs. The detection and identification of these organisms are also of great interest for identifying the purity of seed samples, labeling food, and trade reasons.

Knowledge regarding the genetic constitution of a GMO and the main features of each technique is essential for the implementation of these tests and to obtain accurate and reliable results with respect to detecting and evaluating a transgenic organism. In some cases, the simple integration of exogenous DNA into the host genome does not mean that the gene is being expressed and that the target protein is being produced. Thus, the combined use of more than one detection technique may be necessary for the complete assessment of GMOs.

8. Acknowledgements

The authors would like to thank CAPES (PNPD-CAPES-FINEP supply the fellowship for the first and last authors), FAPEMIG, CNPq, FINEP and EMBRAPA for financial support and researcherships.

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22

Elimination of Transgenic Sequences in Plants by *Cre* Gene Expression

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1. Introduction

The ability to insert foreign DNA into plant cells opened plenty opportunities for the development of new cell lines and improved varieties for agronomic and industrial purposes. Despite the great advances reached there are still some limitations in plant biotechnology based on genetic transformation. In most cases precise engineering of target genomic loci is difficult. Random DNA integration and multi-copy transgene insertions might result in unpredictable expression or gene silencing. Furthermore, commercial application of plant biotechnology products rises numerous regulatory and biosafety concerns about possible spread of the transgenes into the environment or the presence of selectable marker genes. One of the molecular tools that can help to overcome these limitations is site-specific recombination. Several site-specific recombination systems have been shown to be functional in plant cells: the Cre-*lox* system from bactreiophage P1 (Dale and Ow, 1990; Odell et al., 1990, Bayley et al., 1992), the FLP-*FRT* system from *Saccharomyces cerevisiae* (Lyznik et al., 1993; Lloyd and Davis, 1994; Kilby et al., 1995), the R-*RS* system from *Zygosaccharomyces rouxii* (Onouchi et al., 1991), the Gin-*gix* system from bacteriophage Mu (Maeser and Kahmann, 1991), the CinH-RS2 system from *Acetinetobacter* (Moon et al., 2011), the ParA system from a plasmid operon parCBA (Thomson et al., 2009) and the *Streptomyces* phage phiC31 system (Kittiwongwattana et al., 2007, Rubtsova et al., 2008). Currently, Cre-*lox* has become the most commonly employed site-specific recombination system. Although both types of recombination catalyzed by the Cre protein, site-specific integration and excision, found practical application (Ow, 2002; Gilbertson, 2003; Lyznik et al., 2003; Gidoni et al., 2008; Wang et al., 2011), the removal of *lox*-flanked sequences is the most widely used applications of Cre recombinase. The following technologies are based on excisional recombination: (i) regulation of gene expression, (ii) resolution of complex insertion sites to single copy structures, (iii) biological confinement, and (iv) elimination of selectable marker genes. Here we review the progress in the employment of Cre-mediated site-specific excisional recombination for applied plant biology and discuss in detail the advantages, limitations and potential improvements of technologies utilizing the Cre-*lox* system.

2. The Cre-*lox* site-specific recombination system: Structure, biological functions, mode of action

The Cre-*lox* site-specific recombination system from bacteriophage P1 belongs to the tyrosine integrase family whose members use a conserved tyrosine residue as catalytic nucleophile (Grindley et al., 2006). It performs at least two functions in the P1 life cycle: (i) it promotes the circularization of bacteriophage DNA after infection of bacteria (Segev and Cohen, 1981; Hochman et al., 1983), and (ii) it maintains the phage genome as unit-copy plasmid by resolving dimeric plasmids during bacterial division (Austin et al., 1981).

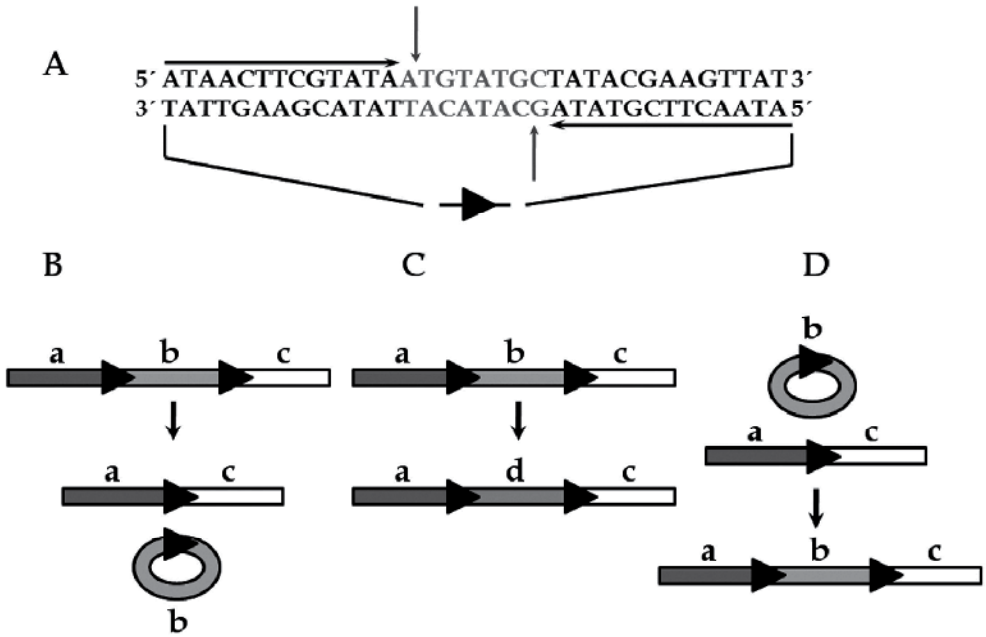

Fig. 1. The Cre-mediated recombination reaction. A: Schematic representation of the *lox* recombination site. The 13 bp inverted repeats are marked by large horizontal arrows. The points of the spacer region at which Cre cleaves the *lox* sites are denoted by small vertical arrows. Cre recombinase mediates inter- and intramolecular recombination leading to deletion (B), inversion (C) or integration (D) events.

The Cre-*lox* system consists of two short DNA recognition sequences known as *lox* (*locus of crossing-over*) and the recombinase protein Cre. Structural studies have revealed that a functional *lox* site is composed of two 13 bp inverted repeats flanking an 8 bp spacer region (Hoess et al., 1982; Hoess and Abremski, 1984) (Figure 1A). The inverted repeats and adjacing 4 bp of the spacer region compose a Cre binding domain. The asymmetry of the 8 bp spacer sequence determines the outcome of the recombination. The second component of the system, the 38 kDa Cre protein, includes two domains: a NH_2-terminal domain and a larger COOH-terminal domain, which contains the active site of the enzyme and major determinants for DNA binding specificity. The Cre recombinase does not require additional proteins or cofactors and performs enzymatic activity under a wide variety of cellular and non-cellular conditions. Crystallographic analysis of Cre-DNA complexes (Guo et al., 1997;

Guo et al., 1999) has revealed the recombination mechanisms. The process of site-specific recombination involves the formation and resolution of a Holliday junction intermediate, during which the DNA is transiently attached to the enzyme through a phosphotyrosine linkage. The reaction can result in integration, inversion or excision, depending on the position and orientation of the recombination sites. Recombination between two *lox* sites in direct orientation on the same DNA molecule results in excision of the *lox*-flanked DNA fragment (Figure 1B). In contrast, recombination between two *lox* sites in inverted repeat leads to inversion of the intervening DNA fragment (Figure 1C). Integration results from recombination between two *lox* sites situated on different DNA molecules (Figure 1D). The recombination reaction is reversible. Since intramolecular excision is kinetically favoured over bi-molecular integration, the excision reaction is essentially irreversible. In contrast, the insertion products are unstable in the presence of Cre recombinase.

3. Cre expression strategies: Efficiency and limitations

According to the presence of the *cre* sequence in the plant genome and the duration of *cre* expression, approaches to combine the *lox* recognition sequences and Cre protein can be grouped into three categories: (i) constitutive, (ii) transient and (iii) temporal expression. In the first group, the recombinase gene is stably integrated into the plant genome and expressed during the whole plant life. There are at least two main possibilities to integrate the *cre* gene into *lox*-containing plants: cross pollination and retransformation. To follow the crossing strategy, *cre* and *lox*-transgenic lines are developed and subsequently crossed (Bayley et al., 1992; Russell et al., 1992; Hoa et al., 2002). Applying the retransformation strategy, the *cre* gene is transformed into *lox*-lines (Odell et al., 1990; Dale and Ow, 1991; Zhang et al., 2003). Constitutive expression provides high recombination efficiencies in both model (Dale and Ow, 1991; Russell et al., 1992) and commercial crops (Hoa et al., 2002; Zhang et al., 2003). However, prolonged *cre* expression has some limitations. It is not optimal for plant species that are propagated by vegetative cuttings, since the crossing/segregation step for the *cre* gene can be problematic. Furthermore, additional time is required to perform a second round of transformation or cross pollination. A further strong argument against constitutive *cre* expression is the possible occurrence of genetic and phenotypic changes caused by the Cre recombinase, which were observed in plastid and nuclear genomes, respectively (Hajdukiewicz et al., 2001; Coppoolse et al., 2003).

Transient expression offers the possibility to reduce/avoid undesired side-effects caused by long-term persistence of the Cre protein. The following approaches have been described in the literature: application of the purified Cre protein and virus- or *Agrobacterium tumefaciens*-mediated *cre* expression.

Addition of Cre protein to induce site-specific recombination was initially demonstrated for animal cells (Baubonis and Sauer, 1993) and extended by Cao and co-workers (2006) to excise *lox*-flanked DNA fragments in plant culture. In theory, direct introduction of the recombinase protein into plant cells could be an elegant solution. In fact, the broad application of this method to commercial crops is highly problematic. Additional time and costs have to be invested to purify an enzymatically active Cre protein and to obtain optimal conditions for cell culture treatment. Reliable regeneration protocols from protoplasts are not available for several crops. Moreover, this regeneration step can introduce additional somaclonal variation.

The Cre function can be provided transiently by *Agrobacterium*-based vectors using T-DNA-independent and T-DNA-dependent expression. T-DNA-independent *Agrobacterium*-mediated *cre* expression is based on fusion of the Cre protein to the NH_2 –terminus of VirE2 and VirF proteins. *Agrobacterium* is able to transfer these fusions into *Arabidopsis* cells resulting in excision events, although detectable efficiency of the process was low (Vergunst et al., 2000). Therefore, this system might be used only for applications where rare recombination rates are essential. T-DNA dependent expression relies on the fact that non-integrated copies of T-DNA may persist in the nucleus for a period of time providing transient expression of genes from T-DNA. The Cre recombinase gene cloned between left and right T-DNA borders can be delivered into plant cells by the agro-inoculation technique and recombine *lox* sites in both nuclear (Gleave et al., 1999; Kopertekh and Schiemann, 2005) and plastid (Lutz et al., 2006) genomes as shown in tobacco. The principle of transient recombinase expression via *A. tumefaciens*-based vectors was proved only in model plant species yet.

Another possibility to deliver Cre protein without *cre* gene insertion into the plant genome is provided by the application of RNA viruses. Two Cre-virus vectors, PVX-Cre (Kopertekh et al., 2004a, 2004b) and TMV-Cre (Jia et al., 2006), have been shown to be functional in *lox*-target *N. benthamiana* and *N. tabacum* plants. In both vectors the *cre* gene was integrated between movement and coat protein genes. Recently, the application of PVX-Cre for marker gene elimination in potato has been demonstrated (Kopertekh et al., 2011). In comparison to the *A. tumefaciens* transient expression system, virus vectors were more efficient in generating recombination events. In general, *Agrobacterium*- and virus-based *cre* expression is mostly suitable for vegetatively propagated species. However, the necessity to develop efficient agroinfiltration methods or infectious Cre-virus vectors, as well as regeneration protocols for plant explants might hamper a broad application of these approaches.

To follow the temporal expression approach, a stably integrated *cre* gene is placed under the control of inducible or tissue specific promoters. To date, a regulated *cre* expression is usually combined with the autoexcision strategy. Self-excision plant transformation vectors contain two recognition sites and the *cre* gene on the same T-DNA molecule. Conditional expression of the *cre* gene results in simultaneous removal of all sequences situated between the *lox* sites. This autoexcision strategy provides several potential advantages. First, all components of the Cre-*lox* system can be incorporated into the plant genome in one transformation step. Second, this strategy could be employed for both generatively and vegetatively propagated species. Several inducible systems responsive to external stimuli have been reported for plants, e.g. heat-shock and β-estradiol regulated. The heat-shock regulated system seems to be the simplest and most familiar for use. Its function has been demonstrated as functional in *Arabidopsis* (Hoff et al., 2001), tobacco (Wang et al., 2005), potato (Cuellar et al., 2006), maize (Zhang et al., 2003), rice (Khattri et al., 2011) and aspen (Fladung and Becker, 2010). In the chemically regulated self-excision system developed by Zuo and associates (2001), the *cre* gene was combined with the XVE system which is induced by β-estradiol. The system was successfully applied to *Arabidopsis* (Zuo et al., 2001), rice (Sreekala et al., 2005) and tomato (Zhang et al., 2006; Zhang et al., 2009). Despite the great advantage of the temporally controlled recombinase expression, heat-shock and chemically regulated promoters require an external signal to be activated and the recombination frequencies are greatly dependent on the penetration of the signal into plant cells, respectively.

A promising alternative to the *cre* regulation described above is the use of developmentally inducible promoters. During the last few years a number of promoters active in different stages of plant development, namely in germline (Verweire et al., 2007; Van Ex et al., 2009), embryo (Li et al., 2007), microspore (Mlynarova et al., 2006; Luo et al., 2007), floral (Bai et al., 2008) and seed (Odell et al., 1994; Moravčíková, et al., 2008; Kopertekh et al., 2010) tissues have been tested to control *cre* expression. High efficiency of such promoters in *Arabidopsis* (Verweire et al., 2007), tobacco (Mlynarova et al., 2006), rice (Bai et al., 2008), soybean (Lie et al., 2007) and oilseed rape (Kopertekh et al., 2009) makes this approach universal for model and agronomically important species. In addition, the employment of germline-specific promoters allows a more efficient transmission of the recombined status to the progeny. The essential feature of conditional Cre systems is a careful regulation with respect to time and tissue. Background Cre activation was observed for heat-shock inducible (Hoff et al., 2001; Wang et al., 2005) and some seed-specific promoters (Odell et al., 1994; Moravčíková, et al., 2008), resulting in reduced efficiency of the systems.

In summary, methodological progress in *cre* gene expression strategies allows to modulate the recombinase activity in a temporal manner. The choice between the Cre expression systems depends mainly on the goals of the experiment, involved plant species, and finally available expertise.

4. Application of Cre-mediated excision in plant biotechnology

The removal of *lox*-flanked DNA fragments by Cre recombinase is broadly used in plant applied research. The applications described in the literature can be grouped into four categories: (i) regulation of gene activity, (ii) simplification of complex transgene structures, (iii) complete excision of a transgene to prevent gene flow, and (iv) marker gene removal.

4.1 Gene regulation

Cre-mediated site-specific recombination offers an effective way to turn on or off gene expression in transgenic plants by removing DNA fragments located between directly repeated recombination sites. What are the potential uses of this technology?

One example is the use of plants as bioreactors to produce recombinant proteins that are toxic to plant cells. Tremblay et al. (2007) designed transgenic *Arabidopsis* plants harbouring a *Turnip Mosaic Virus* (TuMV) amplicon in which a *lox*-flanked translational terminator integrated between the P1 and HCPro coding sequences prevented virus replication. After delivery of Cre recombinase by agroinfiltration, a PVX-Cre vector or a transgenic chemically inducible system, the intervening DNA fragment was eliminated resulting in virus accumulation.

The same strategy was used for conditional recombinase-mediated gene expression in plant cell culture (Joubes et al., 2004). In a plant transformation vector, excision of the *gfp* coding sequence by heat-shock and a dexamethasone inducible Cre recombinase lead to expression of the gene of interest. The system was tested in *N. tabacum* bright yellow-2 (B-2) cells and its efficiency was demonstrated for the *gus* reporter gene and a potent inhibitor of the cell cycle mutant allele of the A-type cyclin-dependent kinase (CDKA).

Another example of recombinase-mediated gene regulation is the restoration of pollen fertility. Transgenic tobacco plants containing a *lox*-flanked stilbene synthase (*sts*) gene under control of a tapetum-specific promoter displayed the male-sterile phenotype (Bayer and Hess, 2005). Pollen fertility was restored after crossing with *cre*-expressing tobacco lines. This method may provide a valuable strategy for the production of hybrid plants.

In contrast to animal systems the few reports describing Cre recombinase-mediated gene regulation in plant systems only demonstrate a proof of principle without practical application yet.

4.2 Generation of single copy transformants by Cre-*lox* recombination

During plant genetic transformation multiple T-DNA copies are often integrated at a single locus. Complex integration sites are commonly associated with intrachromosomal recombination (Srivastava et al., 1996) and transgene silencing (Wang and Waterhouse, 2000; De Buck et al., 2001). Moreover, a single integration pattern may simplify the functional and structural characterization of a transgene. Therefore, single copy transgenic plants are more desirable for commercial practice. Several approaches such as conventional screening amongst a large pool of transformants (De Buck and Depicker, 2004), agrolistics (Hansen and Chilton, 1996), niacinamide application (De Block et al., 1997) or use of Cre-mediated site-specific recombination (Srivastava et al., 1999) have been developed to select/generate single copy lines. The Cre-*lox*-based strategy is based on a transgene flanked by *lox* sites in opposite orientation. In case of tandem insertion of T-DNAs at a single locus, the Cre recombinase resolves multiple units to a single-copy insert.

The proof of concept and successful application of the Cre-*lox*-based strategy was reported for the first time by Srivastava et al. (1999). Four transgenic wheat *lox*-target lines, containing a DNA fragment flanked by recombination sites in inverted repeats, were generated by particle bombardment. The Cre recombinase was provided by crossing with *cre*-expressing plants. Cre-mediated resolution of the complex T-DNA structure was observed in T_2 progeny plants for all four lines investigated. However the authors reported (i) incomplete resolution of complex loci in 20-40% of the T_2 progenies from three lines and (ii) persistence of excised DNA fragment extrachromosomally in one plant.

The strategy described above was modified to generate single-copy maize plants more efficiently. In comparison to the original method, the *cre*-expressing construct was introduced into *lox*-transgenic maize cells transiently by particle bombardment (Srivastava and Ow, 2001). This modification was highly efficient: 85% of regenerated plants contained 1 to 2 copies of the introduced DNA, with 38% harbouring a single copy. In 23% of single copy lines recombination was performed by transient *cre* expression: they harboured only the *lox*-target construct.

The Cre-mediated resolution approach was also functional in *Arabidopsis*. In the *lox*-transformation vector two recombination sites in inverted repeat were cloned inside the T-DNA immediately adjacent to the left and right T-DNA border ends (De Buck et al., 2007). Seven transgenic lines with a complex integration locus were crossed with *cre*-transgenic plants. The progeny of two hybrids demonstrated a single-copy T-DNA status without integration of the released DNA fragment in the plant genome. In some transformants, the Cre-mediated resolution of complex loci increased the transgene expression at least tenfold.

Based on these results an alternative transformation system to generate single copy transformants has been developed and proved in *Arabidopsis* (De Pape et al., 2009). To omit the crossing step between *cre*- and *lox*-plants, a *lox*-target construct was transferred by floral deep transformation into *cre* expressing plants. 55% of primary transformants contained a single copy of the introduced T-DNA. However 73% showed inversion of the DNA fragment between the *lox* sites which can result in variable transgene expression. Further improvement was achieved by introducing only one *lox* site in the transformation vector: 70% of primary transformants harboured a single-copy of T–DNA without inversion.

In summary, the recombinase-based resolution strategy can efficiently resolve complex integration patterns in important agricultural crops, particularly wheat and maize, as well as in the model plant *A. thaliana*. However, the following potential limitations have to be envisaged for this strategy. First, this approach may not be suitable for multiple locus integration events since Cre-mediated resolution can cause chromosomal deletions. Second, incomplete resolution of the complex locus is possible. Finally, released DNA fragment may be present in the plant genome.

4.3 Transgene confinement

One concern related to genetically modified plants is the potential effects resulting from transgene transfer into the environment. To address this issue several biological confinement strategies have been proposed. Current technologies, namely male sterility, chloroplast transformation, cleistogamy and transgene removal from pollen or seeds, offer new possibilities for biological confinement (Daniell, 2002; Keenan and Stemmer, 2002; Moon et al., 2009). In this chapter we will mainly describe biological confinement strategies based on the Cre-*lox* recombination system. Here, all functional transgenes are flanked by two recognition sites in direct orientation. Upon expression of the *cre* gene driven by tightly regulated chemically induced or tissue specific promoters, the transgene sequences are removed leaving only a short recognition sequence in the genome. Since gene flow occurs most frequently via seed or pollen dispersal, transgene removal from seed or pollen by developmentally regulated *cre* recombinase could minimize transgene transfer.

The seed-sterile technology is based on two expression units: *cre*-expression unit and cytotoxic ribosome-inhibitor (RIP) gene expression unit (Daniell, 2002). The *cre* gene is linked with a repressor-operator (Tet) system which allows *cre* expression in the presence of tetracycline. In the second expression unit, a seed-specific late embryonic abundance (LEA) promoter and a *RIP* gene are separated by a *lox*-flanked "spacer sequence". Tetracycline induced *cre* expression results in the removal of the "spacer sequence" and the fusion of LEA promoter and *RIP* gene. The RIP protein destructs the seed tissue resulting in production of non-viable seeds. The following potential problems are linked with this strategy: (i) all three components of the system (Cre, RIP and Tet) should be present together in one plant, (ii) the repressor-operator (Tet) system should display high efficiency in crop plants and the chemical inducer should penetrate the plant tissue uniformly, (iii) the seed-specific LEA promoter can be subjected to silencing causing undesired transgene dispersal.

In the second advanced strategy developed by Mlynarova et al. (2006), a *lox*-embedded cassette includes (i) marker gene, (ii) gene of interest and (iii) *cre* gene driven by the NTM 19 microspore-specific promoter. This design allows autoexcision of all transgenes during

microsporogenesis without application of an additional induction factor. It was highly efficient in tobacco plants: only two out of 16800 seeds (0.024%) contained non-excised transgene sequences. Additionally, the authors did not observe premature activation or absence of activation for the tissue-specific Cre-system under laboratory stress conditions.

The efficiency and reliability of recombinase-mediated confinement methods was further improved by the application of pollen- and seed-specific promoters and hybrid *lox-FRT* recombination sites (Luo et al., 2007). The *lox-FRT* fusion sequences dramatically enhanced the excision frequency: analysis of 25000 progeny seedlings for several transgenic tobacco lines revealed that transgenes in pollen or seeds were excised with 100% efficiency. Despite simplicity and high efficiency of the developmentally regulated Cre-system to prevent gene flow, the need to maintain the hemizygous status may be a great disadvantage for transgenic crops multiplied by seeds.

It should be pointed out that all strategies presented in this section were only tested in model plants such as tobacco and *Arabidopsis*. Therefore, no data are available on the efficiency and stability of these systems in actual crop species under agronomic conditions.

4.4 Cre-mediated excision of marker genes

In most cases, plant transformation is inefficient and transgenic cells and regenerants must be selected from a great number of non-transformed cells via incorporation of selectable marker genes. Once plant transformation is completed, these marker genes can be eliminated. There are several reasons to produce marker-free plants (Hohn et al., 2001; Hare and Chua, 2002; Miki and McHugh, 2004; Goldstein et al., 2005): marker gene removal can prevent the movement of selectable markers within the environment, simplify the regulatory process and allow the reuse of the same marker. Different methods have been identified that enable marker gene removal: co-transformation (Komari et al., 1996), transposon-dependent repositioning (Goldsbrough et al., 1993), as well as homologous (Zubko et al., 2000) and site-specific recombination (Dale and Ow, 1991). Site-specific marker gene removal will be the main topic of this section. The plant material used has been ordered according to species, supposing that this structure of the chapter might help to compare the efficiency of different methods and to choose the optimal approach for the plant to be used. Table 1 provides summarised information about Cre-site-specific marker gene elimination systems and their efficiency in different plant species.

The theoretical concept of Cre-mediated marker gene excision was proved in tobacco about twenty years ago by two research groups (Dale and Ow, 1991; Russel et al., 1992). Marker-free plants were generated by applying the Cre recombinase constitutively either via cross-pollination or a second round of transformation. The authors reported that re-transformation provided much higher recombination efficiency. This principle was also functional in the plastid genome (Corneille et al., 2001). Both methods for constitutive *cre* expression were efficient in tobacco chloroplasts, but *Agrobacterium*-mediated Cre recombinase delivery caused plastid genome rearrangements.

Transient expression vectors - *Agrobacterium*- or virus, - worked efficiently in tobacco. Simple cocultivation of transgenic tobacco leaves harbouring the marker gene with *A. tumefaciens* containing a *cre*-plasmid led to the removal of the flanked region in 0.25% of the regenerants (Gleave et al., 1999). In comparison to cocultivation technique, the

agroinfiltration method greatly increased the recombination efficiency. Regenerants without marker genes were obtained with a frequency of about 34%. In 14% of plants site-specific recombination was performed without stable recombinase integration. Delivery of the Cre protein by agroinfiltration was also adopted to remove marker genes from the plastid genome (Lutz et al., 2006). Another option to perform transient *cre* expression is the use of Cre-virus vectors. The first plant Cre-virus vector was based on PVX and demonstrated high recombination rates (48-82%) in *N. benthamiana* (Kopertekh et al., 2004b). This vector was also suitable to generate marker-free tobacco plants without a regeneration step (Kopertekh et al., 2004a). The second Cre-virus vector described is based on TMV. It was functional in *N. tabacum* plants with an efficiency of about 34% (Jia et al., 2006).

Genotype	Induction factor, expression system/promoter	*cre* expression type	Excision rate	Gene of interest	Reference
Tobacco *N. tabacum*	Cross-pollination, retransformation, 35S promoter	Constitutive	ND	*luc*	Dale and Ow, 1991
Tobacco *N. tabacum*	Cross-pollination, retransformation, 35S promoter	Constitutive	95% (retransformation)	*gusA*	Russell et al., 1992
Tobacco *N. tabacum*	Cross-pollination, 35S promoter	Constitutive	19.2%	*ASAL*	Chakraborti et al., 2008
Tobacco *N. tabacum* (plastid genome)	Cross-pollination, retransformation, 35S promoter	Constitutive	ND	-	Corneille et al., 2001
Tobacco *N. benthamiana*	PVX-Cre expression vector	Transient	48-82%	*gfp*	Kopertekh et al., 2004
Tobacco *N. tabacum*	TMV-Cre expression vector	Transient	34%	*gusA*	Jia et al., 2006
Tobacco *N. tabacum*	*A. tumefaciens*-expression vector	Transient	0.25%	*gusA*	Gleave et al., 1999
Tobacco *N. tabacum* (plastid genome)	*A. tumefaciens*-expression vector	Transient	10%	*bar*	Lutz et al., 2006
Tobacco *N. benthamiana*	*A. tumefaciens*-expression vector	Transient	34%	*gfp*	Kopertekh et al., 2005
Tobacco *N. tabacum*	Heat-shock, HSP17.5E promoter from soybean	Temporal	30-80%	*gusA*	Wang et al., 2005
A. thaliana	Chemical induction, β-estradiol inducible transactivator XVE	Temporal	29-66%	*gfp*	Zuo et al., 2001
A. thaliana	Tissue-specific induction, *AP1* and *SDS* germline specific promoters	Temporal	83-100%	-	Verweire et al., 2007
Rice	Cross-pollination, 35S promoter	Constitutive	58%	*gusA*	Hoa et al., 2002
Rice	Co-cocultivation with a purified Cre-recombinase protein	Transient	26%	*gusA*	Cao et al., 2006

Rice	Heat-shock, HSP17.5E promoter from soybean	Temporal	16%	*gusA*	Khattri et al., 2011
Rice	Chemical induction, β-estradiol inducible transactivator XVE	Temporal	29.1%	*gfp*	Sreekala et al., 2005
Maize	Cross-pollination	Constitutive	ND	*cordapA*	Ow, 2007
Maize	Cross-pollination, 35S promoter Heat-shock, HSP17.5E promoter from soybean	Constitutive Temporal	ND	*gfp*	Zhang et al., 2003
Maize	Cross-pollination, Ubi promoter	Constitutive	ND	*gusA*	Kebrach et al., 2005
Wheat	Cross-pollination, 35S promoter	Constitutive	ND	-	Srivastava et al., 1999
Potato	Heat-shock, hsp70 promoter from *Drosophila melanogaster*	Temporal	4.7%	-	Cuellar et al., 2006
Potato	PVX-Cre expression vector	Transient	20-27%	*gfp*	Kopertekh et al., 2011
Brassica juncea	Cross-pollination, 35S promoter	Constitutive	ND	*gusA*	Arumugam et al., 2007
Brassica napus	Tissue-specific induction, seed-specific napin promoter from *B. napus*	Temporal	13-81%	*vst*I	Kopertekh et al., 2009
Soybean	Tissue-specific induction, *Arabidopsis app1* embryo-specific promoter	Temporal	13%	*gusA, gat*	Li et al., 2007
Tomato	Chemical induction, β-estradiol inducible transactivator XVE	Temporal	15%	*cry*IAc	Zhang et al., 2006
Tomato	Chemical induction, β-estradiol inducible transactivator XVE	Temporal	ND	*atlpk2β*	Zhang et al., 2009

ND, not determined
luc: luciferase gene
gfp: green fluorescent protein gene
atlpk2β: inositol polyphospate 6-/3-kinase gene
gus: beta-glucuronidase gene
*vst*I: stilbene synthase gene from *Vitis vinifera*
bar: phosphinothricin acetyltransferase gene
*cry*IAc: a synthetic *Bacillus thuringiensis* endotoxin gene
ASAL: allium sativum leaf agglutinin gene
gat: glyphosate acetyltransferase gene
cordapA: dihydrodipicolinate synthase gene

Table 1. Cre-based systems for marker gene elimination

Different promoters, including heat-shock and developmentally regulated ones, were tested in autoexcision vectors in tobacco. In the heat-shock inducible system, the Cre recombinase was more effective in somatic tissues in comparison to germline cells: 70-80% of the regenerants derived from heat-treated leaves lost *lox*-flanked DNA fragments, whereas only 30-40% of seeds after heat-shock gave rise to marker-free plants (Wang et al., 2005). A developmentally regulated Cre-*lox* system based on the seed-specific napin promoter was more efficient in *N. benthamiana* plants: genetic and molecular analysis of T1 progeny indicated DNA excision in all transgenic lines tested (Kopertekh et al., 2010).

Both tobacco and *Arabidopsis thaliana* served as model systems to test different gene elimination approaches. An elegant self-excision Cre-system regulated by β-estradiol was applied for the first time in *Arabidopsis* with an efficiency of 29-66% (Zuo et al., 2001). Furthermore, Verweire et al. (2007) reported an almost complete autoexcision driven by germline promoters.

Rice has been intensively studied for Cre-mediated marker gene excision. The efficiency of all three categories of methods, transient, constitutive and temporal expression, has been evaluated. In one of the first studies on the Cre-*lox* system in rice, *lox*- and *cre*-constructs were combined by cross-fertilization of transgenic plants (Hoa et al., 2002). In the Cre-*lox* hybrids from T_2 crosses a high marker gene deletion frequency of 58.3% was observed. Marker gene excision was also accomplished in transgenic rice cells by simple co-cultivation with a purified cell-permeable Cre recombinase protein (Cao et al., 2006). About 26% of regenerants derived from Cre-treated calli were scored as putative recombinants. However, no data are available about germinal inheritance of the recombined "footprint". Thus, it is difficult to assess the efficiency of this of this approach properly. Marker gene excision and inheritance of the excised locus were observed in one transgenic rice line containing a *lox*-target construct and a single copy of the *cre* gene under the control of the HSP17.5E heat-shock inducible promoter (Khattri et al., 2011). An obvious drawback of this co-transformation approach is the necessity to segregate the *cre*-construct after recombination. Sreekala et al. (2005) demonstrated the removal of the flanked fragment from the genome of transgenic rice in a single-step transformation by using the β–estradiol regulation of Cre. In total, 29% of transgenic T_0 plants were marker-free or could segregate marker-free progeny. In the Cre-*lox* system controlled by a floral specific promoter complete auto-excision was observed in three out of eight rice lines with an efficiency of 37.5% (Bai et al., 2008). This approach may be considered as the most promising for the removal of unnecessary sequences in rice since (i) Cre expression is restricted to a special tissue, (ii) recombined lines can be obtained without crossing or additional treatment and (iii) this one-step transformation approach provides high recombination frequencies.

Two strategies - cross-pollination and heat-shock inducible autoexcision - have been shown to be useful to develop transgenic maize plants harbouring only the trait gene. The crossing strategy worked with nearly 100% efficiency in several laboratories (Zahn et al., 2003; Kebrach et al., 2005). Moreover, commercial marker-free maize LYO38 was developed by Monsanto through sexual crossing between *lox*- and *cre*-plants following segregation of the *cre* gene in the next generation. A comparative study by Zhang et al. (2003) also demonstrated that autoexcision induced by heat-shock provided precise, complete and stable marker gene excision.

There is less information available on Cre-mediated marker gene elimination in wheat. Srivastava et al. (1999) combined two potential applications of site-specific recombination in

one plant vector. Transgenic wheat plants harbouring a DNA fragment between mutant *lox*511 sites in opposite orientation and a marker gene between wild type *lox* sites in direct orientation were crossed with a *cre*-transgenic line. Some T_1 plants without the selection marker and a reduced copy number were detected by PCR.

The feasibility of the Cre-*lox* system for the removal of marker genes in *Brassicaceae* was demonstrated in two studies. In the first one, the *lox* sites and *cre* gene under control of a constitutive promoter were combined by cross-pollination to produce marker-free *Brassica juncea* plants (Arumugam et al., 2007). The Cre recombinase displayed low activity in meristematic cells. Thus, an additional regeneration step from leaf explants was necessary to obtain *B. juncea* plants without marker genes. The application of seed-specific napin promoter from *B. napus* to control the *cre* gene seems to be more suitable to perform the germline transmission of the recombination event (Kopertekh et al., 2009). Marker-free *B. napus* plants could be generated with high efficiency (13-81%).

Two techniques, *cre* induction by heat-shock and PVX-Cre-expression have been optimized for vegetatively propagated potato. About 4% of regenerated shoots derived from heat treated internodes and tubes demonstrated the marker-free phenotype (Cuellar et al., 2007). Transient PVX-Cre-based expression resulted in a more efficient excision of the *npt*II gene cloned between recognition sites (Kopertekh et al., 2011). Excision rates of 20-27% were achieved by applying the particle bombardment infection method and the P19 silencing suppressor protein.

In the auto-excision Cre-system developed for soybean transformation, a selectable marker gene was expressed at an early transformation step and then removed by the Cre recombinase driven by *app1* embryo-specific promoter from *A. thaliana* (Li et al., 2007). This excision reaction led to the activation of the glyphosate acetyltransferase (*gat*) gene. It was shown that 13% of events exhibited complete excision of the marker gene.

The application of a chemically-regulated autoexcision Cre-system in tomato was reported by Zhang et al. (2006). β-estradiol treatment resulted in the excision of *cre* and marker genes and subsequently in the fusion of the endotoxin gene *cryIA* with the promoter sequence. 15% of T_1 progeny plants harboured a marker-free phenotype.

Generally, the newly designed Cre-systems have first been tested in tobacco and *Arabidopsis,* and subsequently extended to actual crops. It should be pointed out that the same Cre-systems demonstrate higher recombination efficiencies in model species in comparison to agricultural crops. For example, for the β-estradiol inducible self-excision Cre system in *Arabidopsis* 29-66% efficiency was observed, whereas in rice and tomato only 30% and 15%, respectively. Similarly, heat-shock induction resulted in 30-80% excision rates in tobacco and only 4% in potato. Recently, this tendency was also demonstrated for the transient PVX-Cre vector. In comparison to *N. benthamiana* (48-82%), lower excision rates of 20-27% were shown for potato (Kopertekh et al., 2011).

5. Conclusions

Since the initial work of Dale and Ow (1990) demonstrating the functionality of the Cre-*lox* system in plant cells, a number of technologies based on site-specific recombination have been developed, tested and implemented into transformation protocols. All these technologies rely on two basic genome modifications caused by Cre recombinase:

integration or removal of foreign DNA fragments. In this review we focused on the employment of Cre-mediated elimination of transgene sequences. The literature analysis indicates several current trends in optimizing the recombination strategies and their practical application. First, future employment of the Cre-*lox* system will likely incorporate more precise temporal and spatial control of *cre* expression. To this end a number of conditional and transient excisional Cre-systems have been designed and tested during the last decade (see the paragraph "Cre-expression strategies: efficiency and limitations"). Second, the methodological progress mentioned above allowed extending the recombination technology from model (tobacco and *Arabidopsis* plants) to actual crops, including generatively and vegetatively propagated species, monocots and dicots. Third, the excisional recombination method was combined with trait genes, illustrating the development from laboratory experiments to practical utilization; this tendency can mainly be observed for the marker gene elimination technology. Among commercial traits combined with the Cre-*lox* system are modification of protein composition (Ow, 2007), tolerance to environmental stress (Zhang et al., 2009) as well as herbicide (Lutz et al., 2006; Li et al., 2007) and insect (Chakraborti et al., 2008; Zhang et al., 2006) resistance. The first marker-free commercial maize event LY038, which received the US regulatory approval in 2006, provides higher lysine content (Ow, 2007).

However, the approval process for the commercial utilization of genetically modified plants based on the techniques described above might require additional regulatory costs. The first consideration could be connected to the possible reintegration/persistence of excised DNA fragments. Although it is generally assumed that the elimination product is lost upon cell division there is one report showing the presence of deleted DNA as an extra-chromosomal circle in wheat cells (Srivastava and Ow, 2003). The second consideration is linked with possible unintended effects which might be caused by the Cre-*lox* system. Numerous reports exist that demonstrate high specificity of Cre-mediated recombination. Nevertheless, several articles have described undesirable Cre-mediated changes in mammalian genomes (Schmidt et al., 2000; Thyagarajan et al., 2000; Loonstra et al., 2001; Silver and Livingston, 2001). The impact of Cre activity on the plant genome is not well studied. Two types of effects have been described: phenotypic abnormalities and DNA rearrangements in chloroplasts. In petunia, tomato, tobacco and *Arabidopsis* aberrant phenotypes such as leaf chlorosis, growth retardation and reduced fertility were associated with high levels of *cre* expression (Coppoolse et al., 2003). These phenotypic abnormalities were not connected with chromosomal rearrangements: they always co-segregated with the *cre* transgene. In contrast, non-specific DNA recombination products have been identified in the plastid genome by two research groups (Corneille et al., 2001; Hajdukiewicz et al., 2001). Temporal or developmental regulation of the Cre activity would decrease or eliminate these side-effects and subsequently simplify risk assessment process. Another concern related to the Cre-*lox* application is the presence of a *lox* recognition site in the final product. Theoretically, non-predicted recombination between this *lox* site and pseudo-*lox* sites in the genome can occur in the presence of the Cre protein. In fact, the probability of such an event is extremely low. First, the recombination reaction strongly depends on a sequence similarity between the introduced *lox* and genomic pseudo-*lox* sites. The recombination efficiency is greatly reduced when only a few nucleotides in the *lox* spacer region are different (Hoess et al., 1986). Second, the distance between recombination sites plays an important role: the recombination between *lox* sites located at unlinked chromosomes is less efficient (Qin et al., 1994).

Despite the regulatory issues described above, we expect that site-specific excisional recombination will become a routine method in plant biotechnology and find a broader application for the commercial use of crop plants.

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23

GMO Safety Assessment-Feasibility of Bioassay to Detect Allelopathy Using Handy Sandwich Method in Transgenic Plants

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1. Introduction

Recently, there are many reports on production and field test of transgenic plants including forest trees (Hinchee *et al*., 2011, Kole and Hall, 2008, Walter *et al*., 2004). However there are arguments and delicate matters about field release of transgenic trees concerning the influence to environment (McLean and Charrest, 2000). Regulations on recombinant DNA plant biotechnology were developed in USA, Canada, Europe, Oceania, China and Japan (Strauss, 2003, Kalaitzandonakes, 2004, Redenbaugh and McHughen, 2004, Lu & Hu, 2011, Watanabe et al., 2004) as well as in international agreement (Strauss *et al*., 2009). The Conference of the Parties 9 meeting held in Milan 2003 decided recognizing host parties evaluate risks associated with the use of genetically modified organisms by afforestaion and reforestation project activities. Risk assessments shall be carried out in a scientifically sound manner and taking into account recognized risk assessment techniques to identify and evaluate the possible adverse effects of living modified organisms on the conservation and sustainable use of biological diversity (Strauss *et al*., 2009). Environmental safety is considered in speed of degradation of introduced genes in the soil, impact on soil invertebrates like earthworms, impact on aquatic invertebrates like daphnia, impact on beneficial insects like ladybugs, impact on fish, birds, mammals and other plants. Eucalyptus species has in general allelopathy activity like suppression of growth and germination of understory weeds (Zeng *et al*., 2008). Poplar has also some allelopathy to the crops like wheat or mycorrhizal fungi in the field (Singh et al., 1993, Olsen *et al*., 1971).

Transgenic trees should be introduced into commerce after they have been critically evaluated for environmental safety. Allelopathy is one of the environmental effects of plants to other plants. It is important to elucidate the effects of the transformations on the allelopathy activity. Here we report the application of a handy sandwich-type bioassay method which was efficient with crop species (Fujii et al., 2003, Golisz et al., 2007) to assess the allelopathy of transgenic aspen trees containing anti-sense peroxidase (*prx*) (Yahong *et al*., 2001) and antibiotic (neomycin phosphotransferase ; *nptII*) (Bevan *et al*., 1983) gene, and eucalyptus trees containing transcriptional element (*Ntlim*1) (Kawaoka *et al*., 2000, 2006) and

antibiotic (*nptII*) gene. The purpose of this study is to offer possible screening method to eliminate high allelopathy transformants before field plantation.

This is the first report on application of the new bioassay of handy sandwich method to assess the allelopathy of transgenic aspen and eucalyptus.

2. Materials and methods

2.1 Transgenic trees and control

Transgenic aspen (*Populus sieboldii* x *grandidentata*)containing anti-sense *prx* and antibiotic (*nptII*) gene, and eucalyptus (*Eucalyptus camaldulensis*) (Fig. 1) containing transcriptional element (*Ntlim*1) and antibiotic (*nptII*) gene were used as experimental targets. Both transgenic approaches were intended to create less lignin wood for efficient pulping. Control tree of aspen was the same clone Y-63 which was transformed, and that of eucalyptus was seedling of same species.

Fig. 1. Transgenic *Eucalyptus camaldulensis*.

2.2 Confirmation of transcription of introduced genes

RNA was extracted from leaves and stems of individual transformants with a QIAGEN RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the protocol provided by the manufacturer using lysis buffers which contains guanidine thiocyanide and RNA adsorption membrane. One microgram of total RNA was reverse transcribed and then the cDNA amplified with a QIAGEN One Step RT-PCR Kit (QIAGEN, Hilden, Germany) according to the protocol provided by the manufacturer. The oligonucleotide primers used for RT-PCR were 5′- AAACAATTACCAACACTACC-3′ (forward) and 5′-ACCTGAAAGGGCAACCAGGT-3′ (reverse) with anti-peroxidase gene, and 5′-

GAGGCTATTCGGCTATGACT-3′ (forward) and 5′-AATCTCGTGATGGCAGGTTG-3′ (reverse) with *npt II* gene. Conditions for amplification were reverse transcription at 50 °C for 30 min, initial PCR activation step at 95 °C for 15 min followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The amplified fragments of cDNA was subjected to electrophoresis in a 2 % (w/v) agarose gel and detected with ethidium bromide.

2.3 Allelopathy level determination by sandwich method

The sandwich method assays the allelopathic activity of leaches from dried leaves on seed germination and growth of receptor plants such as lettuce (Fujii et al., 2003, 2004). Sandwich method was done according to that of Fujii et al. (2003) and Golisz et al. (2007). They used this bioassay method to screen large number plants to detect allelopathy in the 239 medicinal plant species. They demonstrated this method is a less time-consuming bioassay method and could be used to screen a large number of samples. In our experiment, one year old leaves of the transgenic and control trees of *E. camaludulensis* and *P. sieboldii x grandidentata* grown in the containment green house were collected and oven-dried at 60 °C for over night, and then 50 mg in dry weight of them sandwiched between the layers of 0.5 % low melting point (31 °C) agar in the multi-well dishes (6 wells whose diameter is 35 mm). Leaves of 1 year old *E. cinerea* was also used as reference in the experiment of aspen. Six replicated samples were used for each transformant and control.

Seeds of the lettuce of Great Lakes 366 were sown on the agar bed, and then germinated under constant temperature at 20 °C. Five seeds were sown in each well.After 60 hours incubation in the dark, the length of the hypocotyls and roots was measured respectively. The inhibition rate by comparing that of control (water) was considered as allelopathy level of each sample, i.e. less growth rate indicates the high allelopathy level. Buckwheat might be also used as an indicator plant of allelopathy like lettuce (Fig. 2).

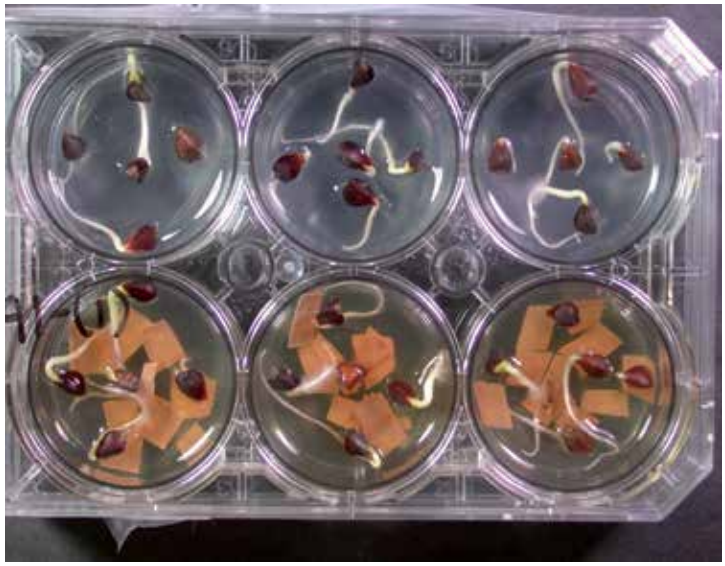


Fig. 2. Six-well multidish plastic plate used for sandwich method.

2.4 Statistical analysis

Analysis of variance (ANOVA) with allelopathy (indicated as growth rate of bioassay plant) was carried out by Fisher's test. Average values were compared using a two-sided t-test.

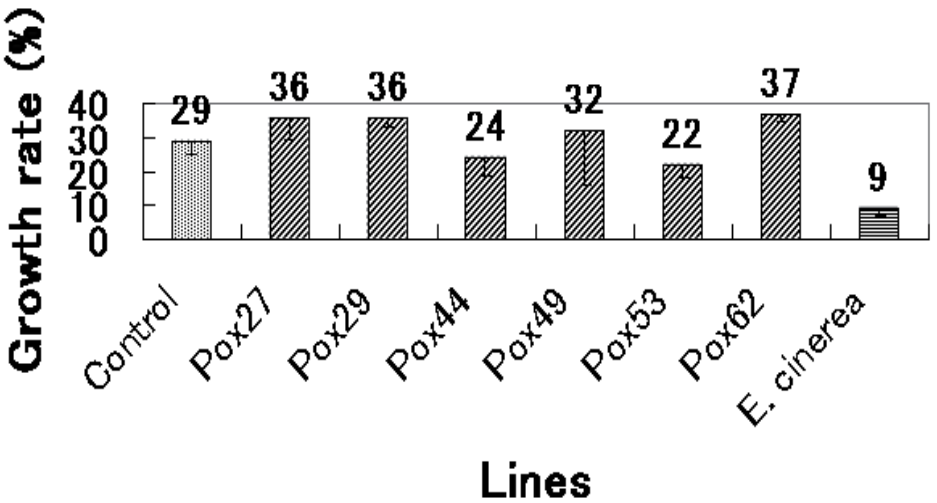
3. Results and discussion

It was checked by RT-PCR that transferred antibiotic genes were still expressed in the leaves and stems of transgenic trees. However, anti-sense gene of peroxidase was only expressed in the stems of transgenic trees. These results are explained that a promoter for antibiotic gene was 35S of cauliflower mosaic virus and that for peroxidase was a promoter from aspen peroxidase relating to xylogenesis.

There are slight variations of allelopathy level indicated by this bio-assay among different lines of transgenic trees. In the transgenic aspen, sandwich bio-assay evaluated by lettuce root length inhibition indicated that allelopathy of transgenic materials varied from 22 to 37 % while control was 29 % (Fig. 3). These may be of some effects of different production level of metabolic substances in the similar way like other transgenic aspen where the concentrations of total flavonoids, quercetin, kaempferol and myricetin derivatives in the leaves were different between control and transgenic trees (Haggman *et al*., 2003).However, we did not detect statistically significant differences among transgenic and control trees by analysis of variance ($P > 0.12$). In contrast, there was a statistically significant difference between species *Populus* and *Eucalyptus* with allelopathy ($P < 0.002$) which indicated the feasibility of the sandwich method to detect the different allelopathy level between species. *E. cinerea* had stronger allelopathy than *Populus* hybrid (Fig.3). There was similar tendency in assessing allelopathy level of transgenic and non-transgenic aspen using hypocotyl growth as an indicator (Fig.4). When cellulose rich transgenic white poplar (*Populus alba*) introduced with bacterial xyloglucanase gene was compared with non-transgenic control, sandwich method revealed no statistical difference between them (J-BCH, 2007).

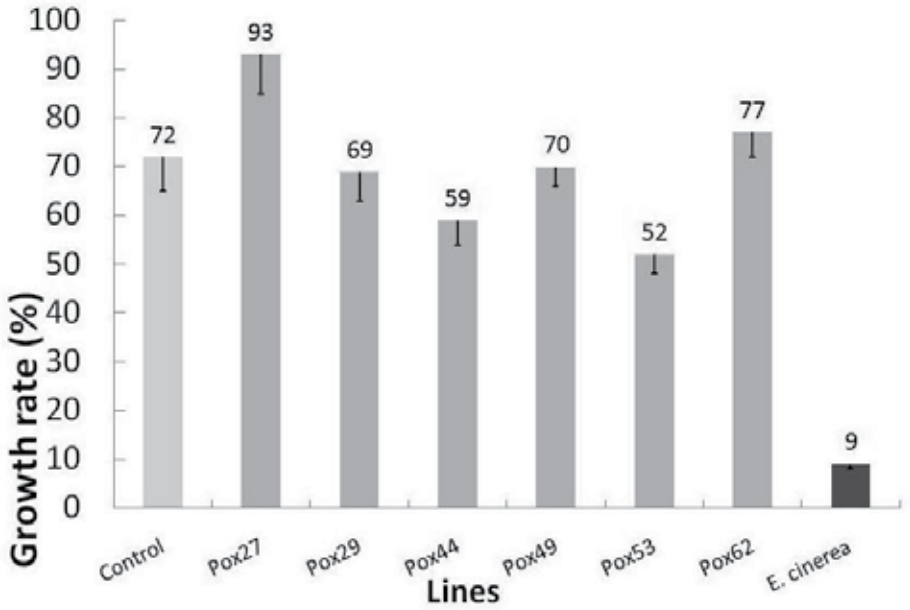
In the case of transgenic eucalyptus, allelopathy levels of transgenic materials were from 17 to 66 % while controls were from 17 to 26 % (Figs. 5).Among transgenic lines, (2)-4 was the lowest allelopathy tree which indicated the statistically significant difference from other lines and controls ($P < 0.01$). However, part of the variation in allelopathy level may be not only from transformation but also from different genetic background caused by seedling materials.Average allelopathy level of eucalyptus was higher than that of aspen as 28 % versus 31 % indicated as average growth rate to water culture. In the case of salt-tolerant transgenic *Eucalyptus camaldulensis*, there was no substantial variation between nontransgenic and transgenic lines with respect to the hypocotyl growth and root elongation of lettuce in sandwich method (Kikuchi *et al*., 2006, 2009).They also conducted the gas chromatographic and high-performance liquid chromatographic analysis to show no qualitative and quantitative difference between the transgenic and nongenetically modified genotypes of Eucalyptus. Bioassay method is superior as the primary assessment method for allellopathy in considering the simplicity, speed, low cost, and reproducibility to chemical instrumental methods.Sandwich method for allelopathy assessment might be useful as one of the criteria of environmental effects of any

transgenic platns.However, drying many samples at the same time is time consuming. So, for evaluating multiple leaves, modified sandwich method used homogenized fleshy leaf samples instead of drying them (Shimazaki et al., 2009). Chemical changes by modification or processing of some compounds may occur during the process of homogenization which leads enhancement of the effect of the transgenic and non-transgenic plant on the growth of germinating seeds. Even if the chemical changes were induced during sample preparation (drying or homogenization process), it would be thought to reflect the chemical differences between the transgenic and non-transgenic plants (Shimazaki, 2009). In the floricultural plants, the transgenic carnation (*Dianthus caryophyllus*) expressing flavonoid 3', 5'-hydroxylase gene (Moon series, www.florigene.com), assessing the allelopathic substances was done by lettuce seed germination bioassay tests in soil containing carnation debris (Kikuchi, 2008).For further study, toxicology and allergenicity testing of products from introduced genes should be carried out before commercialization of transgenic plants.



Pox 27, 29, 44, 49, 53 and 62; transgenic aspen
E; *Eucalyptus cinerea*
Bar; standard error

Fig. 3. Allelopathy level of transgenic *Populus sieboldii x grandidentata* (as % of lettuce root growth to water medium)



Pox 27, 29, 44, 49, 53 and 62; transgenic aspen
E; *Eucalyptus cinerea*
Bar; standard error

Fig. 4. Allelopathy level of transgenic Populus sieboldii x grandidentata (as % of lettuce hypocotyl growth to water medium)

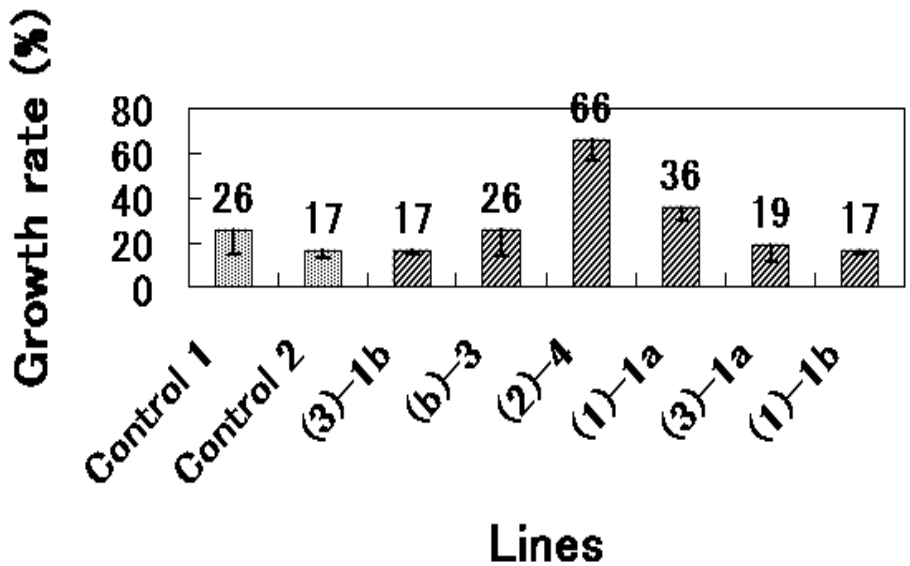


Fig. 5. Allelopathy level of transgenic *Eucalyptus camaludulensis* (as % of lettuce root growth to water medium)(3)-1b, (b)-3, (2)-4, (1)-1a, (3)-1a and (1)-1b; transgenic eucalyptus Bar; standard error

Trees have many characteristics that make them more difficult to assess them than those of agricultural crops: they have long life cycle, the production cycle may be 10 to 70 years, pollen moves over enormous distance, there are tremendous genetic and phenotypic variation and ecological complexity (McLean and Charrest, 2000). In the handling of transgenic plants, the key words are "familiarity" and "substantial equivalence". Familiarity is the knowledge of the characteristics of a plant species and the experience with the use of that plant species. Substantial equivalence is that of a novel trait within a particular plant species, in terms of its specific use and safety to the environment and human health, to those in that same species, that are in use and generally considered as safe based on valid scientific rationale. Checking of the allelopathy of transgenic trees is substantial equivalence matter. In the substantial equivalence, we must consider altered weediness potential, gene flow to related species, altered plant pest potential, potential impact on non-target organisms, and potential impact on biodiversity. Allelopathy of transgenic trees may have influence to other plants and biodiversity. The sandwich method can easily detect the high allelopathetic materials. We think, therefore, that it is useful to use this handy sandwich method as one of the criteria for assessing biosafety of plants including forest trees.

4. Conclusion

The Cartagena Protocol on Biosafety promulgated guidelines for evaluating the biosafety of living modified organisms (http://www.biodiv.org/biosafety).Transgenic plants should not be planted in the field without an environmental biosafety assessment. Commercial usage and environmental release can be permitted after evaluation in the containment growth room and field. The public concerns of environmental biosafety assessments are to define property of the transgenic plants and assess the influence of the plant on other organisms. Allelopathic influence of transgenic plants must be checked before field release.

Among the methods used for evaluating the allelopathic effects of plants are the dish pack,plant box, sandwich and soil mix methods (Shiomi et al.,1992; Yamaguchi et al., 1994; Sekine et al., 2007). Sandwich method might be one of the most handy and reliable methods for checking the transgenic plants.

5. Acknowledgment

We thank Dr. K. Fujii, National Institute for Agro-Environment Institute, for his introduction of sandwich method.We appreciate the kind offer of transgenic aspen from former Prof. N. Morohoshi, Tokyo University of Agriculture and Technology.

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Edited by Yelda Özden Çiftçi

Development of efficient transformation protocols is becoming a complementary strategy to conventional breeding techniques for the improvement of crops. Thus, Transgenic Plants - Advances and Limitations covers the recent advances carried on improvement of transformation methods together with assessment of the impact of genetically transformed crops on biosafety. Each chapter has been written by one or more experienced researchers in the field and then carefully edited to ensure throughness and consistency.

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