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Genetic Transformation

Edited by María Alejandra Alvarez

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Meet the editor

Dr. María Alejandra Alvarez is a member of the National Council of Research (CONICET- Argentina) in Argentina. She worked at the University of Buenos Aires where she has began her studies on In vitro plant cell cultures for secondary metabolism production in 1986. Since 2001 she has conducted research on molecular farming for producing biopharmaceuticals. In 2005 she moved to the Instituto de Ciencia y Tecnología Dr. César Milstein (CONICET) where she is currently the director of The Plant Biotechnology group.

Contents

Preface

It has been more than twenty five years now since the first transformed plant was reported. Plant transgenesis has evolved since those first attempts; the advances have led to the elucidation of numerous aspects of plant biology, physiology, and Agrobacterium biology. Even more spectacular is the impact of plant transformation on crop improvement. To this date, transgenic crops represent a 10 % of the 1.5 billion hectares of cropland worldwide, and constitute one of the main sources of incomes for countries like the USA (66.8 million hectares), Brazil (25.4), Argentina (22.9), India (9.4), Canada (8.8), China (3.5), Paraguay (2.6), Pakistan (2.4), South Africa (2.2) and Uruguay with 1.1 million hectares. Also, plant transformation had influence on fruit and forest improvement and the development of desirable traits for ornamental plant breeders. Moreover, the significant advances made in the use of transformed plants for the production of recombinant proteins and for the engineering of secondary metabolism pathways have made a new easy-scalable and economical rendering platform available to the pharmaceutical industry.

Chapters in this book represent selected examples of the advances that are currently undergoing in this field. In the first section, the history and progress of Agrobacterium utilization as a transformation vector is presented along with a chapter dedicated to the analysis of the related events from a structure-functional analysis. Also the physiological and molecular background of phytoremediation is analyzed.

In the second section, the subject of plant improvement is widely covered in the chapters related to the amelioration of crops, fruits and flowers with a couple of chapters dedicated to the last advances in molecular farming and RNAi technology.

Finally, in the last section the paramount contribution that plant transformation has made on secondary metabolism is reviewed.

I would like to thank each of the authors for their great efforts in producing their articles. I am sure the readers will appreciate the contribution each of the researchers has made and will recognize the value of each chapter.

Finally, I would like to thank the staff of the InTech Open Access Publisher for their invaluable support along the process of publishing this book, particularly Ms. Dragana Manestar and Ms. Natalia Reinic.

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Part 1

Agrobacterium:
New Insights into a Natural Engineer

1

Agrobacterium-Mediated Genetic Transformation: History and Progress

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1. Introduction

Agrobacterium tumefaciens is a Gram-negative soil phytopathogenic bacterium that causes the crown gall disease of dicotyledonous plants, which is characterized by a tumorous phenotype. It induces the tumor by transferring a segment of its Ti plasmid DNA (transferred DNA, or T-DNA) into the host genome and genetically transforming the host. One century has past after *A. tumefaciens* was firstly identified as the causal agent of crown gall disease (Smith & Townsend, 1907). However, *A. tumefaciens* is still central to diverse fields of biological and biotechnological research, ranging from its use in plant genetic engineering to representing a model system for studies of a wide variety of biological processes, including bacterial detection of host signaling chemicals, intercellular transfer of macromolecules, importing of nucleoprotein into plant nuclei, and interbacterial chemical signaling via autoinducer-type quorum sensing (McCullen & Binns, 2006; Newton & Fray, 2004; Pitzschke & Hirt, 2010). Therefore, the molecular mechanism underlying the genetic transformation has been the focus of research for a wide spectrum of biologists, from bacteriologists to molecular biologists to botanists.

1.1 History of *Agrobacterium tumefaciens* research

A. tumefaciens is capable of inducing tumors at wound sites of hundreds of dicotyledonous plants, and some monocots and gymnosperms (De Cleene and De Ley, 1976), which may happen on the stems, crowns and roots of the host. At the beginning of the last century, crown gall disease was considered a major problem in horticultural production. This disease caused significant loss of crop yield in many perennial horticultural crops (Kennedy, 1980), such as cherry (Lopatin, 1939), apple (Ricker et al., 1959), and grape (Schroth et al., 1988). All these horticultural crops are woody species and propagated by grafting scions onto rootstocks. The grafting wounds are usually covered by soil and thus provide an excellent infection point for the soil-borne *A. tumefaciens*. In 1941, it was proved that crown gall tumor tissue could be permanently transformed by only transient exposure to the pathogen of *A. tumefaciens* (White and Braun, 1941). Thereafter, a 'tumor-inducing capacity' was proposed to be transmitted from *A. tumefaciens* to plant tissue (Braun, 1947; Braun and Mandle, 1948). Twenty years late, molecular techniques provided the first evidence that crown gall tumors,

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cultured axenically, contained DNA of *A. tumefaciens* origin, which implied that host cells were genetically transformed by *Agrobacterium* (Schilperoort et al., 1967). In 1974, the tumor-inducing (Ti) plasmid was identified to be essential for the crown gall-inducing ability (Van Larebeke et al., 1974; Zaenen et al., 1974). Southern hybridization turned out to prove that the bacterial DNA transferred to host cells originates from the Ti plasmid and ultimately resulted in the discovery of T-DNA (transferred DNA), specific segments transferred from *A. tumefaciens* to plant cells (Chilton et al., 1977; Chilton et al., 1978; Depicker et al., 1978). The T-DNA is referred to as the T-region when located on the Ti-plasmid. The T-region is delimited by 25-bp directly repeated sequences, which are called T-DNA border sequences. The T-DNAs, when transferred to plant cells, encode enzymes for the synthesis of (1) the plant hormones auxin and cytokinin and (2) strain-specific low molecular weight amino acid and sugar phosphate derivatives called opines. The massive accumulation of auxin and cytokinin in transformed plant cells causes uncontrolled cell proliferation and the synthesis of nutritive opines that can be metabolized specifically by the infecting *A. tumefaciens* strain. Thus, the opine-producing tumor effectively creates an ecological niche specifically suited to the infecting *A. tumefaciens* strain (Escobar & Dandekar, 2003; Gelvin, 2003). Besides the T-DNAs, Ti-plasmid also contains most of the genes that are required for the transfer of the T-DNAs from *A. tumefaciens* to the plant cell.

Initial study of these plant tumors was intended to reveal the molecular mechanism that may be relevant to animal neoplasia. Although no relationship was found between animal and plant tumors, *A. tumefaciens* and plant tumor were proved to be of intrinsic interest because the tumorous growth was shown to result from the transfer of T-DNA from bacterial Ti-plasmid to the plant cell and the stable integration of the T-DNA to plant genome. The demonstration that wild-type T-DNA coding region can be replaced by any DNA sequence without any effect on its transfer from *A. tumefaciens* to the plant inspired the promise that *A. tumefaciens* might be used as gene vector to deliver genetic material into plants. In the early of 1980's, two events about *A. tumefaciens* mediated genetic transformation signaled the beginning of the era of plant genetic engineering. First, *A. tumefaciens* and its Ti-plasmid were used as a gene vector system to produce the first transgenic plant (Zambryski et al., 1983). The healthy transgenic plants had the ability to transmit the disarmed T-DNA, including the foreign genes, to their progeny. Second, non-plant antibiotic-resistance genes, for example, a bacterial kanamycin-resistance gene, could be instructed to function efficiently in plant cells by splicing a plant-active promoter to the coding region of the bacterial genes. This enabled accurate selection of transformed plant cells (Beven, 1984). The eventual success of using *A. tumefaciens* as a gene vector to create transgenic plants was viewed as a prospect and a "wish". The future of *A. tumefaciens* as a gene vector for crop improvement began to look bright. During the 1990's, maize, a monocot plant species that was thought to be outside the *A. tumefaciens* "normal host range", was successfully transformed by *A. tumefaciens* (Chilton, 1993). Today, many agronomically and horticulturally important plant species are routinely transformed by *A. tumefaciens*, and the list of plant species that can be genetically transformed by *A. tumefaciens* seems to grow daily (Gelvin, 2003). At present, many economically important crops, such as corn, soybean, cotton, canola, potatoes, and tomatoes, were improved by *A. tumefaciens*–mediated genetic transformation and these transgenic varieties are growing worldwide (Valentine, 2003). By now, the species that are susceptible to *A. tumefaciens*–mediated transformation were broadened to yeast, fungi, and mammalian cells (Lacroix et al., 2006b).

In the new century, intrests of most *Agrobacterium* community shifted to the transfer channel and host. Most recent important articles on *Agrobacterium*-mediated T-DNA transfer are to explore the molecular mechanism of T-complex targeting to plant nucleus. Recent progresses of these aspects of *Agrobacterium*-mediated genetic transformation will be the emphases of this chapter and be discussed in the following related sections.

1.2 Basic process of *A. tumefaciens*–mediated genetic transformation

The process of *A. tumefaciens*–mediated genetic transformation is a long journey. For the sake of description, many authors divided this process into several steps (Guo et al., 2009a; Guo, 2010; McCullen & Binns, 2006; Pitzschke & Hirt, 2010). Here, we arbitrarily and simply split it into five steps: (1) Sensing of plant chemical signals and inducing of virulence (vir) proteins. The chemical signals released by wounded plant are perceived by a VirA/VirG two-component system of *A. tumefaciens*, which leads to the transcription of virulence (vir) gene promoters and thus the expression of vir proteins. (2) T-DNA processing. T-DNA is nicked by VirD2/VirD1 from the T-region of Ti plasmid and forms a single-stranded linear T-strand with one VirD2 molecule covalently attached to the 5′ end of the T-strand. (3) Attaching of *A. tumefaciens* to plant and transferring of T-complex to plant cell. *A. tumefaciens* cell attaches to plant and transfers the T-complex from *A. tumefaciens* to plant cell by a VirD4/B T4SS transport system. (4) Targeting of T-complex to plant cell nucleus and integrating of T-DNA into plant genome. The T-complex is transported into the nucleoplasm under the assistance of some host proteins and then integrated into plant genomic DNA. (5) Expressing of T-DNA in plant cell and inducing of plant tumor. The T-DNA genes encode phytohormone synthases that lead to the uncontrolled proliferation of plant cell and opine synthases that provide nutritive compounds to infecting bacteria.

2. Events happening in *Agrobacteriun*

A. tumefaciens can perceive the signal molecules from plants and recognize the competent hosts. To fulfill the infection, *Agrobacterium* must respond to the signal molecules. The respondence occuring in *Agrobacterium* includes host recognition, virulence gene expression, and T-DNA processing.

2.1 Sensing of plant signal molecules and *vir* gene induction

Many genes are involved in *A. tumefaciens*-mediated T-DNA transfer, but most of the genes required for T-DNA transfer are found on the *vir* region of Ti plasmid. This *vir* region comprises at least six essential operons (*virA*, *virB*, *virC*, *virD*, *virE*, and *virG*,) and two non-essential operons (*virF* and *virH*) encoding approximate 25 proteins (Gelvin, 2000; Zhu et al., 2000; Ziemienowicz, 2001). These proteins are termed virulence (vir) proteins and required for the sensing of plant signal molecules as well as the processing, transfer, and nuclear localization of T-DNA, and the integration of T-DNA into the plant genome. The protein number encoded by each operon differs; *virA*, *virG* and *virF* encode only one protein; *virE*, *virC*, and *virH* encode two proteins; *virD* encodes four proteins and virB encodes eleven proteins. Only *virA* and *virG* are constitutively transcripted. The transcription of all other *vir* operons in *vir* region is coordinately induced during infection by a family of host-released phenolic compounds in combination with some monosaccharides and extracellular acidity in the range of pH 5.0 to 5.8. Virtually all of the genes in the *vir* region are tightly regulated by two proteins VirA and VirG encoded by *virA* operon and *virG* operon (Lin et al., 2008).

The inducible expression of *vir* operons was first found by using the cocultivation of *A. tumerfaciens* with mesophyll protoplasts, isolated plant cells or cultured tissues (Stachel et al., 1986). In vegetatively growing bacteria, only *virA* and *virG* are expressed at significant level. However, when *Agrobacteria* are cocultivated with the susceptible plant cells, the expression of *virB*, *virC*, *virD*, *virE* and *virG* are induced to high levels (Engstrom et al., 1987). The partially purified extracts of conditioned media from root cultures can also induce the expression of *vir* operons, demonstrating that the *vir*-inducing factors are some diffusible plant cell metabolites. By screening 40 plant-derived chemicals, Bolton et al. (1986) identified seven simple plant phenolic compounds that possess the *vir*-inducing activity. Most of these *vir*-inducing phenolic compounds are needed to make lignin, a plant cell wall polymer. The best characterized and most effective *vir* gene inducers are acetosyringone (AS) and hydroxy-acetosyringone from tobacco cells or roots (Stachel et al., 1985). The specific composition of phenolic compounds secreted by wounded plants is thought to underlie the host specificity of some *Agrobacterium* strains. Besides phenolic compounds, other inducing factors include aldose monosaccharides, low pH, and low phosphate (Brencic & Winans, 2005; McCullen & Binns, 2006; Palmer et al., 2004). However, phenols are indispensable for *vir* gene induction, whereas the other inducing factors sensitise *Agrobacteria* to phenols.

2.2 Regulation of *vir* gene induction

The regulatory pathway for *vir* gene induction by phenolic compounds is mediated by the VirA/VirG two-component system, which has structural and functional similarities to other already described for other cellular regulation mechanisms (Bourret et al., 1991; Nixon et al., 1986). Two component regulatory systems comprise two core components, a sensor kinase and an intracellular response regulator. The sensor kinase responds to signal input and mediates the activation of the intracellular response regulator by controlling the latter's phoshporylation status (Brencic & Winans, 2005; McCullen & Binns, 2006). For the *Agrobacterium* VirA/VirG two-component system, VirA is a membrane-bound sensor kinase. The presence of acidic environment and phenolic compounds at a plant wound site may directly or indirectly induce autophosphorylation of VirA. The phosphorylated VirA can transfer its phosphate to the cytoplasmic VirG to activate VirG. The activated VirG binds to the specific 12bp DNA sequences called *vir* box enhancer elements that are found in the promoters of the *virA*, *virB*, *virC*, *virD*, *virE* and *virG* operons, and then upregulates the transcription of these operons (Winans, 1992).

Octopine-type Ti plasmid encoded VirA protein has 829 amino acids. VirA is a member of the histidine protein kinase class and able to autophosphorylate. When VirA autophosphorylates *in vitro*, the phosphate was found to bind to histidine residue 474, a histidine residue that is absolutely conserved among homologous proteins (Jin et al., 1990). VirA protein can be structurally divided into a number of domains. In an order from N-terminus to C-terminus, these domains are defined as transmembrane domain 1 (TM1), periplasmic domain, transmembrane domain 2 (TM2), linker domain, kinase domain and receiver domain (Lee et al., 1996). The periplasmic domain is required for the interaction with ChvE, the sugar-binding protein that responds to the *vir*-inducing sugars. The linker domain is located on the region of amino acid 280~414, which was supposed to interact with the *vir* gene inducing phenolic compounds (Chang & Winans 1992). A highly amphipathic helix sequence of 11 amino acids was identified in the region of amino acid 278-288. This amphipathic sequence is highly

conserved in a large number of chemoreceptor proteins and thus was supposed to be the receptor site for phenolic inducers (Turk et al., 1994). However, it is unclear whether the phenolic inducers interact with VirA directly or indirectly. The kinase domain is a highly conserved domain that presents in the family of the sensor proteins and contains the conserved histidine residue 474 that is the autophosphorylation site. Site-directing mutation of this His 474 results in avirulence and the lost of *vir* gene inducing expression in the presence of plant signal molecules (Jin et al., 1990). The receiver domain shows an unusual feature that is homologous to a region of VirG. Similar receiver domains are present in a small number of homologous histidine protein kinases, but the function of this domain is unclear.

VirG is a transcriptional activator of 241 amino acid residues. It is composed of two main domains, N-terminal domain and C-terminal domain. The aspartic acid 52 in the N-terminal domain of VirG can be phosphorylated by the phosphorylated VirA (Jin et al, 1993). The phosphorylation of N-terminal domain is thought to induce the conformation change of C-terminal domain. The C-terminal domain of VirG possesses the DNA-binding function, resulting in VirG specifically binding to the *vir* box sequence that is found within 80 nucleotides upstream from the transcription initiation sites of *vir* genes. Phosphorylation is required for the transcriptional activation function of VirG, but how phosphorylation modulates the properties of VirG is unknown. Some models suggested that phosphorylation might increase the affinity of VirG to its binding sites or promote the ability of VirG to contact RNA polymerase (Lin et al., 2008; McCullen & Binns, 2006; Wang et al., 2002).

2.3 T-DNA processing

The activation of *vir* genes initiates a cascade of events. Following the expression of *vir* genes, some Vir proteins produce the transfer intermediate, a linear single stranded (ss) DNA called T-DNA or T-strand that is derived from the bottom (coding) strand of the T-region of the Ti plasmid. T-region is flanked by two 25 bp long imperfect direct repeats, termed border sequences. VirD2/VirD1 is able to recognize the border sequences and cleave the bottom strand of T-region at identical positions between bp 3 and 4 from the left end of each border (Sheng & Citovsky, 1996). Upon the cleavage of T-DNA border sequence, VirD2 remains covalently associated with the 5´-end of the ssT-strand *via* tyrosine residue 29 (Vogel & Das, 1992). The excised ssT-strand is removed, and the resulting single-stranded gap in the T-region is repaired, most likely replaced by a newly synthesizing DNA strand. The association of VirD2 with the 5´-end of the ssT-strand is believed to prevent the exonucleolytic attack to the 5´-end of the ssT-strand (Durrenberger et al., 1989) and to distinguish the 5´-end as the leading end of the T-DNA complex during transfer.

One report indicated that VirD1 possesses a topoisomerase-like activity (Ghai and Das, 1989). VirD1 appears to be a type I DNA topoisomerase that do not require ATP for activity. However, a late study (Scheiffele et al., 1995) contradicted this conclusion. The VirD1 protein purified by Scheiffele et al. (1995) never showed any topoisomerase activity. It was speculated that the topoisomerase activity observed by Ghai and Das (1989) might originate from VirD2. Mutational study of VirD1 showed that a region from amino acids 45~60 is important for VirD1 activity. Sequence comparison of this fragment with the functionally analogous proteins of conjugatable bacterial plasmids showed that this region is a potential DNA-binding domain (Vogel & Das, 1994).

The nopaline Ti plasmid encoded VirD2 consists of 447 amino acids with a molecular weight of 49.7 kDa. Deletion analysis of VirD2 demonstrated that the C-terminal 50% of VirD2 could be deleted or replaced without affecting its endonuclease activity. Sequence

comparison of VirD2 from different *Agrobacterium* species shows that the N-terminus is highly conserved with 90% homology, whereas only 26% homology is found in the C-terminus (Wang et al., 1990). A sequence comparison of VirD2 protein with its functionally homologous proteins in bacterial conjugation and in rolling circle replication revealed that a conserved 14-residue motif lies in the residues 126~139 of VirD2. This motif contains the consensus sequence HxDxD(H/N)uHuHuuuN (invariant residues in capital letters; x, any amino acid; u, hydrophobic residue) (Ilyina & Koonin, 1992). Mutational analysis indicated that all the invariant residues except for the last asparagine (N) in this motif are important for the endonuclease activity of VirD2. The second aspartic acid (D) and three nonconserved residues in this motif are also essential for the endonuclease activity of VirD2 (Vogel et al., 1995). This motif is believed to coordinate the essential cofactor Mg^{2+} by the two histidines in the hydrophobic region of the motif (Ilyina & Koonin, 1992). The poorly conserved C-terminal halves of VirD2 from different *Agrobacterium* species displayed a very similar hydropathy profile (Wang et al., 1990). The C-terminal domain of VirD2 is thought to guide the T-complex to the plant nucleus. The sequence characterization and function of this region of VirD2 will be discussed in a late section of this chapter.

3. Contact of *Agrobacterium* with plant and transfer of *Agrobacterial* molecules to plant

3.1 Chemotaxis of *A. tumefaciens*

A. tumefaciens is a motile organism, with peritrichous flagellae, that possesses a highly sensitive chemotaxis system. It could respond to a range of sugars and amino acids and be attracted to these sugars and amino acids (Loake et al., 1988). *A. tumefaciens* mutants deficient in motility and in chemotaxis were fully virulent when inoculated directly. However, when used to inoculate soil, which was air-dried and then used to grow plants, these mutants were completely avirulent. These results indicated that the motility and chemotaxis are critical to *A. tumefaciens* infection under natural conditions (Hawes & Smith, 1989). Wild-type *A. tumefaciens* strains both containing and lacking Ti plasmid exhibited chemotaxis toward excised root tips from all plant species tested and toward root cap cells of pea and maize, suggesting that the majority of chemotactic responses in *A. tumefaciens* appear to be chromosomally encoded (Loake et al., 1988; Parke et al., 1987). However, the chemotactic response to some phenolic compounds, for example acetosyringone, which were identified as strong *vir* gene inducers, is controversial. Some reports showed that chemotaxis toward acetosyringone requires the presence of a Ti plasmid, specifically the regulatory genes *virA* and *virG*, and occurs with a threshold sensitivity of $< 10^{-8}$ M, some 1000-fold below the maximal *vir*-inducing concentration (Ashby et al., 1988; Shaw et al., 1989). Whereas, reports from other groups indicated that acetosyringone did not elicit chemotaxis at any concentration (Hawes & Smith, 1989) and chemotaxis toward related compounds did not require the Ti plasmid (Park et al., 1987). So, it does seem difficult to rationalize a role for acetosyringone and the regulatory genes *virA* and *virG* in chemotaxis.

3.2 Attachment of *A. tumefaciens* to plant

It is reasonable that an intimate association between pathogen and host cells is required for the transfer of T-DNA and virulence proteins from *A. tumefaciens* to plant cells. *A. tumefaciens* can efficiently attach to both plant tissues and abiotic surfaces, and establish

complex biofilms at colonization sites. Microscopic observation of bacteria interacting with the plant cells demonstrates a significant propensity to attach in a polar fashion (Smith & Hindley, 1978; Tomlinson & Fuqua, 2009). All *Agrobacterium* mutants deficient in attachment to plant cells are either avirulent or extremely attenuated in virulence (Cangelosi et al., 1989; Douglas et al., 1982, 1985; Matthysse & McMahan, 2001; O'Connell & Handelsman, 1989). Although obviously critical, the attachment process is one of the least-characterized sets of cellular processes in the entire interaction. Little progress on this area was made in recent years (Tomlinson & Fuqua, 2009).

3.2.1 Bacterial genes involved in the attachment of *A. tumefaciens* to plant

The binding of *A. tumefaciens* to host plant cells seems to require the participation of specific receptors that may exist on the bacterial and plant cell surface because the binding of *A. tumefaciens* to host plant cells is saturable and unrelated bacteria fail to inhibit the binding of *A. tumefaciens* to host plant cells (B.B. Lippincot & J.A. Lippincot, 1969). A number of *A. tumefaciens* mutants reported to affect the attachment of bacteria to plant cells have been isolated. Some related genes are identified and sequenced (Matthysse et al., 2000; Reuhs et al., 1997). However, it is surprising that a large number of genes are involved in the bacterial attachment to host cells and the actual functions of most genes are unclear (Matthysse et al., 2000). All the genes reported to affect the bacterial attachment to host cells are chromosomal genes.The genes involved in the binding of bacteria to host plant cells are identified to mainly locate on two regions of the bacterial chromosome.

The binding of bacteria to host cells is thought to be a two-step process (Matthysse & McMahan, 1998). The binding in the first step is loose and reversible because the bound bacteria are easy to being washed from the binding sites by shear forces, such as water washing or vortexing of tissue culture cells. Genes involved in this step are identified to locate on the *att* gene region (more than 20 kb in size) of the bacterial chromosome. Gene mutations in this region abolish virulence. The mutants in the *att* gene region can be divided into two groups. The first group can be restored to attachment and virulence by the addition of conditioned medium. This group appears to be altered in signal exchange between the bacterium and the host. Mutations in this group of mutants occur in the genes homologous to ABC transporters and transcriptional regulator as well as some closely linked downstream genes (Matthysse et al., 2000; Matthysse & McMahan, 1998; Reuhs et al., 1997). The second group of mutants in the *att* gene region is not affected by the presence of conditioned medium. This mutant group appears to affect the synthesis of surface molecules, which may play a role in the bacterial attachment to the host. This group includes mutants in the genes homologous to transcriptional regulator and ATPase as well as a number of biosynthetic genes, which include the transacetylase required for the formation of an acetylated capsular polysaccharide. The acetylated capsular polysaccharide is required for the bacterial attachment to some plants because the production of the acetylated capsular polysaccharide is correlated to the attachment of wild-type strain C58 to the host cells and the purified acetylated capsular polysaccharide from wild-type strains blocks the binding of the bacteria to some host cells (Matthysse et al., 2000; Matthysse & McMahan, 1998, 2001; Reuhs et al., 1997).

The second step in the bacterial attachment to the host results in tight binding of the bacteria to the plant cell surface because the bound bacteria can no longer be removed

from the plant cell surface by shear forces. This step requires the synthesis of cellulose fibrils by the bacteria, which recruits larger numbers of bacteria to the wound sites. Cellulose-minus bacterial mutants show reduced virulence (Minnemeyer et al., 1991). The genes required for the synthesis of bacterial cellulose fibrils (*cel* genes) are identified to locate on the bacterial chromosome near, but not contiguous with the *att* gene region (Robertson et al., 1988).

Some other chromosomal virulence genes *chvA*, *chvB*, and *pscA* (*exoC*) are believed to be involved indirectly in bacterial attachment to host (Cangelosi et al., 1987; Douglas et al., 1982; O'Connell & Handelsman, 1989). These genes are involved in the synthesis, processing, and export of a cyclic β-1,2-glucan, which has been implicated in the bacterial binding to plant cells. Mutations in *chvA*, *chvB*, and *pscA* (*exoC*) cause a 10-fold decrease in binding of bacteria to zinnea mesophyll cells and strongly attenuate virulence (Douglas et al., 1985; Kamoun et al., 1989; Thomashow et al., 1987). ChvB is believed to be involved in the synthesis of the cyclic β-1,2-glucan (Zorreguieta & Ugalde, 1986). ChvA is homologous to a family of membrane-bound ATPases and appears to be involved in the export of the cyclic β-1,2-glucan from the cytoplasm to the periplasm and extracellular fluid (Cangelosi et al., 1989; De Iannino & Ugalde, 1989). However, the virulence of *chvB* mutants is temperature sensitive (Banta et al., 1998). At lower temperature (16 ^{o}C), *chvB* mutants became virulent and were able to attach to plant roots (Bash & Matthysse, 2002).

3.2.2 Plant factors involved in the attachment of *A. tumefaciens* to plant

In addition to bacterial factors, some plant factors are essential for the attachment of *A. tumefaciens* to plant cells. Two plant cell wall proteins: a vitronectin-like protein (Wagner & Matthysse, 1992) and a rhicadhesin-binding protein (Swart et al., 1994) have been proposed to mediate the bacterial attachment to plant cells. Vitronectin is an animal receptor that is specifically utilized by different pathogenic bacteria (Burridge et al., 1988). A plant vitronectin-like protein is reported to occur in several *A. tumefaciens* host plant (Sanders et al., 1991). Human vitronectin and antivitronectin antibodies were shown to inhibit the binding of *A. tumefaciens* to plant tissues. Nonattaching *A. tumefaciens* mutants, such as *chvB*, *pscA* and *att* mutants, showed a reduction in the ability to bind vitronectin. Therefore, the plant vitronectin-like protein was proposed to play a role in *A. tumefaciens* attachment to its host cells (Wagner & Matthysse, 1992). However, a recent report argues against the role of the vitronectinlike protein in bacterial attachment and *Agrobacterium*-mediated transformation (Clauce-Coupel et al., 2008).

Genetic studies showed that additional plant cell-surface proteins might play a role in *A. tumefaciens* attachment. Two *Arabidopsis* ecotypes, B1-1 and Petergof, which are highly recalcitrant to *Agrobacterium*-mediated transformation, were proposed to be blocked at an early step of the binding (Nam et al., 1997). Two *Arabidopsis* T-DNA insertion mutants of the ecotype Ws, *rat1* and *rat3*, which are resistant to *Agrobacterium* transformation (*rat* mutants), are deficient in *A. tumefaciens* binding to cut root surfaces (Nam et al., 1999). DNA sequence analysis indicated that *rat1* and *rat3* mutations affect an arabinogalactan protein (AGP) and a potential cell-wall protein, respectively. AGPs were confirmed to be involved in *A. tumefaciens* transformation (Nam et al., 1999). Interestingly, AGP17 (*rat1* mutant) appears to be involved in host defense reactions and signaling (Gaspar et al., 2004; Gelvin, 2010a). Other two *rat* mutans, *rat*T8 and *rat*T9, were identified to be mutated in the genes coding for receptor-like protein kinases (Zhu et al., 2003).

3.3 Transfer of *Agrobacterial* molecules to plant

Following the production of T-DNA and attachment to the host cells, *Agrobacterium* transports T-DNA and virulence proteins into the host. The transportation must cross the bacterial cell membrane and wall, as well as host cell membrane and wall.

3.3.1 Transfer apparatus

A. tumefaciens uses type IV secretion system (T4SS) to transfer T-DNA and effector proteins to its host cells (Cascales & Christie, 2003, 2004). The T4SS was initially defined to be a class of DNA transporters whose components are highly homologous to the conjugal transfer (tra) system of the conjugative IncN plasmid pKM101 and the *A. tumefaciens* T-DNA transfer system (Burns, 2003; Christie & Vogel, 2000). T4SS, also known as the mating pair formation (Mpf) apparatus, is a cell envelope-spanning complex (composed of 11-13 core proteins) that is believed to form a pore or channel through which DNA and/or protein is delivered from the donor cell to the recipient cell. Recently the members of T4SS have steady increased, with the identification of additional systems involved in DNA and protein translocation (Alvarez-Martinez & Christie, 2009; Cascales & Christie, 2003; Christie & Vogel, 2000; Gillespie, 2010). However, the best-studied T4SS member is the VirB/D4 transporter of *A. tumefaciens*. In the past decade, much of the research on *Agrobacterium*-mediated T-DNA transfer focused on the *vir*-specific T4SS, the T-complex transporter. Therefore, the *A. tumefaciens* T-complex transporter has become a paradigm of T4SS (Alvarez-Martinez & Christie, 2009; Cascales & Christie, 2003).

The VirB/D4 T4SS is assembled from 11 proteins (VirB1 to VirB11) encoded by the *virB* operon, and VirD4. At least 10 of the 11 VirB proteins are believed to be the structural subunits of the T-pilin and associated transport apparatus that spans from the cytoplasm of the cell, through the inner membrane, periplasmic space and outer membrane, to the outside of the cell. In the past few years, work in identifying the interactions among the VirB protein subunits and defining the steps in the transporter assembly pathway has extended our knowledge of the structure of the VirB transport apparatus. To demonstrate the architecture of the VirB/D4 transporter, a model that depicts the subcellular locations and interactions of the VirB and VirD4 subunits of the *A. tumefaciens* VirB/D4 T4SS was proposed (Alvarez-Martinez & Christie, 2009; Cascales & Christie, 2004). Recently, VirB7, VirB9, and VirB10 homologs from the pKM101 T4SS were purified and the cryoEM structure of a core complex composed of pKM101 VirB7-like TraN, VirB9-like TraO, and VirB10-like TraF was revealed (Fronzes et al., 2009).

Agrobacterium-mediated T-DNA transfer to plant shows striking similarities to the plasmid interbacterial conjugation (Ream, 1989; Stachel & Zambryski, 1986). Bacterial conjugation can be visualized as the merging of two ancient bacterial systems: the DNA rolling-circle replication system and type IV secretion system (T4SS) (Llosa et al., 2002). The DNA rolling-circle replication system in plasmid conjugation was also known as the DNA transfer and replication (Dtr) system. The Dtr system corresponds to the T-DNA relaxase nucleoprotein complex. The T4SS responding for the plasmid conjugation was initially called mating pair formation (Mpf) system. In order to recognize these two systems and link them, a protein is normally required for many conjugal plasmids to couple the Dtr to the Mpf. This protein was called coupling protein as its function (Gomis-Ruth et al., 2002).

VirD4 is a homologue of coupling protein family and is believed to be the coupling protein that links the transferred molecules and T4SS transporter. VirD4 is an inner membrane

protein with potential DNA binding ability and ATPase activity. Membrane topology analysis of VirD4 revealed that VirD4 contains an N-terminal-proximal region, which includes two transmembrane helices and a small periplasmic domain, and a large C-terminal cytoplasmic domain (Cascales & Christie, 2003; Das & Xie, 1998). VirD4 localizes to the cell pole. The polar location of VirD4 was not dependent on T-DNA processing, the assembly of T4SS transporter and the expression of other Vir proteins. Both the small periplasmic domain and the cytoplasmic nucleotide-binding domain are required for the polar localization of VirD4 and essential for T-DNA transfer. VirD4 forms a large oligomeric complex (Kumar & Das, 2002). VirD4 can recruit VirE2 to the cell poles (Atmakuri et al., 2003) and weakly interact with VirD2-T-strand complex (Cascales & Christie, 2004). Although VirD4 is essential for coordinating the T4SS to drive T-DNA transfer, it has been unclear whether VirD4 physically/directly interacts with the T4SS transporter. However, the interaction between VirD4 homologues and the protein components of Dtr system exhibits specificity. It was supposed that VirD4 protein might recruit T-complex to the T4SS transporter through contacts with the T-complex protein and then through the contacts with VirB10 coordinate the passage of T-complex through the T4SS channel (Cascales & Christie, 2003; Llosa et al., 2003). However, it should be pointed out that the recruitment of T-complex might be much more difficult than the recruitment of single VirE2 molecule due to the difference of molecular size between T-complex and VirE2. Recently, two cytoplasmic proteins, VBP (VirD2-binding protein) (Guo et al., 2007a, 2007b) and VirC1 (Atmakuri et al., 2007) were reported to be involved in the recruitment of the T-complex to T4SS. Genome-wide sequence analysis showed that *A. tumefaciens* contains three *vbp* homologous genes. Reverse genetic study showed that mutatons of three *vbp* genes highly attenuated the bacterial ability to cause tumors on plants (Guo et al., 2007a, 2009b).

3.3.2 *Agrobacterial* molecules transported to plant

Agrobacterial molecules transported into host cells by VirB/D4 T4SS include the VirD2-T-strand complex, VirE2, VirE3, VirF, and VirD5. VirD2 is covalently bound to the 5′ end of the T-strand. The bound VirD2, probably in conjunction with other protein components, such as VBP (Guo et al., 2007a, 2007b) and VirC1 (Atmakuri et al., 2007), confers recognition of the VirD2-T-strand complex by the VirB/D4 T4SS. VirD2 also "pilots" the T-strand through the translocation channel. It was supposed that the VirB/D4 T4SS is actually a protein transporter and the T-strand is the "hitchhiker" (Cascales & Christie, 2004).

VirE2 is a single-stranded DNA-binding protein (Christie et al., 1988; Citovsky et al., 1988) that can bind single-stranded DNA without sequence specificity, and is supposed to protect the T-strand from the nucleolytic degradation because single-stranded T-DNA is believed to be susceptible to nucleases. The binding of VirE2 to single-stranded DNA is strong and co-operative, suggesting that VirE2 coats the T-strand along its length (Citovsky et al., 1989). Another possible function of VirE2 is to guide the nuclear import of T-DNA (Ziemienowicz et al., 1999, 2001). This will be discussed in the following section of this chapter. Induced *Agrobacterium* cell can produce sufficient VirE2 to bind all intracellular single-stranded T-DNA. When bound to single-stranded DNA, VirE2 can alter the ssDNA from a random-coil conformation to a telephone cord-like coiled structure and increases the relative rigidity (Citovsky et al., 1997). Initial hypothesis is that the protective role of VirE2 is required to function in both bacteria and plant cells. So, the prevailing view on the T-DNA transfer is that a packaged nucleoprotein complex, the T-complex, composed of the T-strand DNA

containing the 5´-associated VirD2 and coated with VirE2 along its length, is the transfer intermediate (Howard & Citovsky, 1990; Zupan & Zambryski, 1997). This T-complex structure model implies that both VirD2 and VirE2 together with the T-strand are transported into plant cell in the same time. This idea makes biological sense because it is likely that VirE2 with a high affinity to ssDNA may form a complex with the T-strand already inside *Agrobacterium* cell, especially if both VirE2 and the T-strand are transported through the same channel (Binns et al., 1995). Indeed, the T-complex, which contains T-strand, VirD2 and VirE2, was observed in the crude extracts from *vir*-induced *Agrobacterium* by using anti-VirE2 antibodies to co-immunoprecipitate both T-strand and VirE2 (Christie et al., 1988).

However, two kinds of evidence argued against that the protective role of VirE2 is required to function inside bacterial cells. The first is the observation that a strain expressing *virE2* but lacking T-DNA can complement a *virE2* mutant in a tumor formation assay (Otten et al., 1984) and the T-strand accumulates to wild-type levels in *virE2* mutants (Stachel et al., 1987; Veluthambi et al., 1988). The second kind of evidence is that *virE2* expression in transgenic tobacco plants restores the infectivity of a VirE2-deficient *Agrobacterium* strain (Citovsky et al., 1992). In addition, the observation that *virE2* mutants can transfer T-DNA into plant cells (Yusibov et al., 1994) also proved that VirE2 is not essential for the export of T-DNA. All these data appear to support that T-DNA may not be packaged by VirE2 in the bacterial cells, at least, the packaging of T-DNA inside bacterial cells by VirE2 is not necessary for the tumor formation. VirE2 can be transported independently, but the transportation of VirE2 requires the activities of VirE1. VirE1 is a chaperone and is necessary for VirE2 translation and stability but not essential for the recognition of the translocation signal of ViE2 by the transport machinery and the subsequent translocation of VirE2 into plant cells, indicating that the role of VirE1 playing in the export process of VirE2 seems restricted to the stabilization of VirE2 by preventing VirE2 from the premature interactions in the bacterial cell before translocation into plant cells (Vergunst et al., 2003).

Like VirD2 and VirE2, agrobacterial protein VirF can also be exported to plant cell (Vergunst et al., 2000). *virF* gene is found only in the octopine-specific Ti plasmid. It is not essential for T-DNA transfer. Initially, VirF is thought to be a host-range factor of *Agrobacterium* (Regensburg-Tuink & Hooykaas, 1993). A more recent report showed that VirF interacts with an *Arabidopsis* Skp1 protein (Schrammeijer et al., 2001). Yeast Skp1 protein and its animal and plant homologs are subunits of the complexes involved in targeted proteolysis. This targeted proteolysis can regulate the plant cell cycle. So, it was suggested that VirF may function in setting the plant cell cycle to effect better transformation (Gelvin, 2003; Tzfira & Citovsky, 2002).

Protein truncation and fusion of T4SS substrates demonstrated that certain C-terminal motifs were required for the export of targeted substrates. The C-terminal 37 amino acids of VirF and the C-terminal 50 amino acids of VirE2 and VirE3 are sufficient to mediate transport of these fusion proteins to plants (Vergunst et al., 2000, 2003). The minimal size of VirF required to direct the translocation of VirF-fusion protein to plants is the C-terminal 10 amino acids. Site-directed mutations showed that several arginines within this region are required for transport (Vergunst et al. 2005). These export signals mediate the recognition of substrates by the VirB/D4 T4SS. A possible consensus sequence R-x(7)-R-x-R-x-R (x, any amino acid) was identified in the C termini of substrates secreted by the VirB/D4 T4SS.

4. Events happening in host

Following the entry of agrobacterial molecules in plant cell cytoplasm, the VirD2-T-strand interacts with VirE2 and plant proteins, likely forming "super-T-complex", which is responsible for subcellular travelling of T-strand from cytoplasm through nuclear membrame into nucleus, and to the chromatin, thus facilitating T-DNA integration into host genome. All these biological processes occurring in host cells require the involvement of many host factors.

4.1 Nuclear targeting of T-complex

The dense structure of the cytoplasm, which greatly restricts the free diffusion of macromolecules, and the size of the "super-T-complex", which far exceeds the 60 kDa size-exclusion limit of the nuclear pore (Lacroix et al., 2006a), indicate that active transport processes are required for the nuclear import of T-complex. As a rule, active nuclear import of proteins requires a specific nuclear localization signal (NLS). Typical nuclear localization signals are short regions rich in basic amino acids (Silver, 1991).

4.1.1 Nuclear localization signals in *Agrobacterial* molecules

Because T-strand is presumed to be completely coated with proteins inside plant cells it is impossible for T-strand itself to carry NLSs. Thus, the NLSs that guide T-complex nuclear import most likely reside in its associated proteins, VirD2 and VirE2. Sequence analysis reveals that both VirD2 and VirE2 contain NLSs. Two NLSs are found in VirD2. One is the typical bipartite NLS that resides in residues 396~413. The nuclear localizing function of this bipartite NLS was confirmed by the observation that VirD2-GUS fusion protein, when expressed in tobacco protoplasts, can target to plant cell nuclei (Howard et al., 1992; Tinland et al., 1992). However, mutations that destroy this bipartite NLS attenuate, and do not abolish tumorigenesis, indicating that although this NLS plays a role in T-DNA transfer, it is not essential (Rossi et al., 1993; Shurvinton et al., 1992). Another NLS in VirD2 is found in residues 32~35, adjacent to the active site in the endonuclease domain (Tinland et al., 1992). This NLS is a monopartite NLS. GUS proteins fused with this NLS accumulate in plant nuclei, but this NLS does not play a role in T-DNA nuclear localization (Shurvinton et al., 1992). The sequences of residues 419~423 at the C-terminus of VirD2, known as the ω domain, are important for tumorigenesis, but do not contribute to nuclear localization activity despite its proximity to the bipartite NLS. The ω domain was supposed to be involved in T-DNA integration (Mysore et al., 1998).

VirE2, the most abundant protein component of the T-complex, contains two bipartite NLSs in its central region (residues 205~221, and residues 273~287). When fused to GUS, each VirE2 NLS is capable of directing the fusion protein to the nucleus of a plant cell, but the maximum accumulation in the nucleus requires both VirE2 NLSs (Citovsky et al., 1992). Because these two NLSs overlap with the DNA binding domains, mutations of *virE2* that abolish the activity of one of these NLSs will also eliminate the DNA binding activity. So, no genetic evidence can be provided to verify the function of these two VirE2 NLSs in T-complex nuclear localization. When VirE2 binds to T-strand, the NLSs of VirE2 may be occluded and inactive. It has been observed that for the nuclear import of short ssDNA, VirD2 was sufficient, whereas import of long ssDNA additionally required VirE2 (Ziemienowicz et al., 2001). Although predominantly nuclear localization of VirE2 was

observed in earlier studies, several recent reports demonstrated the cytoplasmic localization of VirE2 (Bhattacharjee et al., 2008; Grange et al., 2008). Indeed, results from different research groups indicate that VirE2 localizes to different subcellular compartments in different tissues (Gelvin, 2010b). All the evidence argued against the function of VirE2 in T-complex nuclear localization. RecA, a NLS-lacking ssDNA binding protein, could substitute for VirE2 in the nuclear import of T-strand, further demonstrating that VirE2 functions not in the nuclear localization, possibly in mediating the passage of T-strand through the nuclear pore (Ziemienowicz et al., 2001). VirE2 was assumed to shape the T-complex such that it is accepted for translocation into the nucleus.

4.1.2 Plant proteins involved in T-complex nuclear targeting

Besides the agrobacterial proteins VirD2 and VirE2, some plant proteins were supposed to be involved in the T-complex nuclear translocation. Early yeast two-hybrid screen identified an *A. thaliana* importin-α (AtKAP, now known as importin-α1) that interacts with VirD2 (Ballas & Citovsky, 1997). Importin-α proteins interact with NLS-containing proteins and guide the nuclear translocation of these proteins. Importin-α proteins constitute a protein family and *Arabidopsis* encodes at least nine of these proteins (Gelvin, 2003). Interaction between VirD2 and importin-α1 was verified to be VirD2 NLS dependent (Ballas & Citovsky, 1997). The importance of importin-α proteins in the T-complex transfer process was confirmed by the genetic evidence that a T-DNA insertion into the importin-α7 gene, or antisense inhibition of expression of the importin-α1 gene, highly reduces the transformation efficiency (Gelvin, 2003). Importin-α1, as well as all other investigated importin-α family members, also interacts with VirE2 (Bhattacharjee et al., 2008) and VirE3 (Garcia-Rodriguez et al., 2006).

Other plant proteins that were identified to interact with VirD2 include several cyclophilins, a kinase CAK2M, and a protein phosphatase 2C (PP2C). Deng et al. (1998) showed that an *Arabdopsis* cyclophilin interacted strongly with VirD2. They further characterized the interaction domain of VirD2 and found that a central domain of VirD2 (residues 274~337) was involved in the interaction with cyclophilin. No previous function of VirD2 had been ascribed to this central region. Cyclosporin A, an inhibitor of VirD2-cyclophilin interaction, inhibits *Agrobacterium*-mediated transformation of *Arabidopsis* and tobacco (Deng et al., 1998). Cyclophilin were presumed to serve as a molecular chaperone to help in T-complex trafficking within the plant cell. Cyclin-dependent kinase-activating kinase CAK2M interacts with VirD2 and catalyzes the phosphorylation of VirD2 *in vivo*. CAK2M may target VirD2 to the C-terminal regulatory domain of RNA polymerase II large subunit (RNApolII CTD) (Pitzschke & Hirt, 2010). A tomato type 2C protein phosphatase (PP2C) that was identified to interact with VirD2 can catalyze the dephosphorylation of VirD2. This phosphatase was assumed to be involved in the phosphorylation and dephosphorylation of a serine residue near the C-terminal NLS in the VirD2. Overexpression of this phosphatase decreased the nuclear targeting of a GUS-VirD2-NLS fusion protein, suggesting that phosphorylation of the C-terminal NLS region may affect the nuclear targeting function of VirD2 (Tao et al, 2004).

Two VirE2-interacting proteins were designated VIP1 and VIP2. VIP1 was showed to facilitate the VirE2 nuclear import in yeast and mammalian cells. Tobacco VIP1 antisense plants were highly resistant to *A. tumefaciens* infection (Tzfira et al., 2001), whereas,

transgenic plants that overexpress VIP1 are hypersusceptible to *A. tumefaciens* transformation (Tzfira et al., 2002). VIP1 is a basic leucine zipper (bZIP) motif protein and shows no significant homology to known animal or yeast proteins (Tzfira et al., 2001). So, how VIP1 facilitates the nuclear import of VirE2 remains unclear. Unlike VIP1, VIP2 was unable to mediate VirE2 into the yeast cell nucleus. However, VIP1 and VIP2 interacted with each other. Thus, VIP1, VIP2 and VirE2 were assumed to function in a multiprotein complex (Tzfira & Citovsky, 2000, 2002). A recent paper showed that VIP1 is phosphorylated by the mitogenactivated protein kinase MPK3 and the VIP1 phosphorylation affects both nuclear localization of VIP1 and *Agrobacterium*-mediated transformation, implying that VIP1 phosphorylation is important for super-T-complex nuclear targeting (Djamei et al., 2007).

4.2 Integration of T-DNA into plant genome

The integration of the incoming ssT-strand of the T-complex into plant genome is the final step of the *Agrobacterium*-mediated genetic transformation. Whether or not the host can be successfully transformed is highly dependent on whether the T-DNA could be integrated into the suitable sites of the host genome.

4.2.1 Integration site

The DNA sequence analysis of several T-DNA host DNA junctions revealed that these junctions, in general, appear more variable than the junctions created by insertions of transposons, retroviruses, or retrotransposons (Gheysen et al., 1987). A statistical analysis of 88,000 T-DNA genome-wild insertions of *Arabidopsis* revealed the existence of a large integration site bias at both the chromosome and gene levels (Alonso et al., 2003). At the chromosomal level, fewer T-DNA insertions were found at the centromeric region. At the gene level, insertions within promotor and coding exons make up nearly 50% of all insertion sites. However, these statistical results may be skewed by the antibiotic resistance selection of transformed plants (T1 plants) because only the T1 plants with transcriptionally active T-DNA insertions can be selected (Alonso et al., 2003; Valentine, 2003). Recently, a genome-wide analysis of T-DNA-integration sites in *Arabidopsis* performed under non-selective conditions showed that T-DNA integration occurs rather randomly (Kim et al., 2007). Another statistical analysis of 9000 flanking sequence tags characterizing transferred DNA (T-DNA) transformants in *Arabidopsis* showed that there are microsimilarities involved in the integration of both the right and left borders of the T-DNA insertions. These microsimilarities occur only in a stretch of 3 to 5 bp and can be between any T-DNA and genomic sequence. This mini-match of 3 to 5 bp basically allows T-DNA to integrate at any locus in the genome. It was also showed that T-DNA integration is favored in plant DNA regions with an A-T-rich content (Brunaud et al., 2002).

4.2.2 Integration mechanism

The observation of the random, as opposed to targeted, nature of T-DNA integration indicated that the integration occurs in illegitimate recombination. To date, it has not been possible to target T-DNA to any particular locus in the genome with any great efficiency. So, the T-DNA integration has been one of the motives of intense investigation of *A. tumefaciens*. But, the molecular mechanism of the T-DNA integration remains largely unknown. Two major models for T-DNA integration have been proposed: single-strand-gap repair model

and double-strand-break repair model (Gelvin, 2010a; Mayerhofer et al., 1991; Tzfira et al., 2004). In the single-strand-gap repair model, VirD2-T-strands invade regions of microhomology between T-DNA and plant DNA sequences and partially anneal to the microhomologous regions. VirD2 on the 5′-end of the T-strand causes a nick in one strand of plant DNA and ligates the T-strand to the nick. Following the ligation of the T-strand to the target DNA, a nick is introduced in the second strand of the target DNA and extended to a gap by exonucleases. During the gap repairing, the complementary strand of T-DNA is synthesized, resulting in incorporation of a double-strand copy of the T-strand into the plant genome. The double-strand-break repair model hypothesizes that single-strand T-strands are replicated in the plant nucleus to a double-strand form and then the double-strand T-DNA is integrated into double-strand breaks in the target DNA. The double-strand-break repair model requires the T-DNA to be converted to a double-strand form before its integration into the double-strand breaks. However, there are more results that strongly support the double-strand-break repair model (Lacroix et al., 2006a). It seems that double-stranded T-DNA integration is the native model of T-DNA integration.

4.2.3 Plant proteins involved in the T-DNA integration

The plant proteins that may be involved in the T-DNA integration process are only now beginning to be defined. As mentioned before, CAK2M phosphorylates VirD2 and targets VirD2 to the C-terminal regulatory domain of RNA polymerase II large subunit (RNApolII CTD), a factor that is responsible for recruiting TATA-box binding proteins (TBP) to actively transcribed regions. CAK2M can also phosphorylate CTD. By associating with VirD2, TBP may guide T-strands to transcriptionally active regions of chromatin for integration. It was supposed that TBP or CAK2M may target VirD2 to the CTD, thereby controlling T-DNA integration (Bako et al., 2003). These nuclear VirD2-binding factors provide a link between T-DNA integration and transcription-coupled repair, suggesting that transcription and transcription-coupled repair may play a role in T-DNA integration (Bako et al., 2003).

To integrate into plant chromosomal DNA, T-DNA must interact with chromatin. More than 109 chromatin genes of 15 gene families were identified to be related to the transformation susceptibility (Crane and Gelvin, 2007). As T-DNA integrates into the plant genome by illegitimate recombination (Mayerhofer et al, 1991), proteins involved in DNA repair and recombination should also be involved in T-DNA integration. Non-homologous end-joining proteins, including Ku70, Ku80, Rad50, Mre11, Xrs2, and Sir4, were identified to be required for T-DNA intigration (Gelvin, 2010a; Tzfira and Citovsky, 2006; Van Attikum et al., 2001).

5. Conclusions

Several decades of intensive studies on *Agrobacterium* make the transformation of many plant and non-plant species by *Agrobacterium*-mediated transformation protocols become routine. The ability of *Agrobacterium* to genetically transform a wide variety of plant and non-plant species has earned it an honour of "nature's genetic engineer" and placed it at the forefront of future biotechnological applications (Rao et al., 2009). However, the *Agrobacterium*-mediated genetic transformation is still an extremely inefficient process, in which only few of the host cells can be infected, and T-DNA integration and stable expression occur in an even smaller fraction of the infected cells. Out of question, a better understanding of the fundamental mechanisms of *Agrobacterium*-mediated genetic

transformation is essential for improving the biotechnological applications of this bacterium as a gene vector for genetic transformation of plant and non-plant species. In addition, *Agrobacterium*-mediated genetic transformation serves as an important model system for studying host-pathogen recognition and delivery of macromolecules into target cells and thus the in-depth study and molecular analysis of *Agrobacterium*-mediated transformation will also add to our understanding of all the biological processes involved in the *Agrobacterium*-mediated genetic transformation.

Agrobacterium-mediated genetic transformation is a complex process that involves *Agrobacterium* reactions to wounded plant, T-DNA transfer in both bacteria and host cells, host reactions to *Agrobacterium* infection, and genetic transformation of host cells. This complex process requires the concerted function of both *Agrobacterium* and host. The golden period of *Agrobacterium* research led us to understand many of the *Agrobacterium*'s biological processes and mechanisms, such as virulence protein inducing, T-DNA processing, and macromolecule exporting by T4SS. However, many key steps of *Agrobacterium*-mediated genetic transformation still remain poorly understood and require further investigation. Particularly the events happening in the host infected by *Agrobacterium* are relatively more poorly understood.

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7. References

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., & Barajas, P. et al. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 301: 653-657.

Alvarez-Martinez, C.E., & Christie, P.J. (2009) Biological diversity of prokaryotic type IV secretion systems. *Microbiol Mol Biol Rev* 73(4): 775-808.

Ashby, A.M., Watson, M.D., Loake, G.Y. & Shaw, C.H. (1988) Ti plasmid-specified chemotaxis of *Agrobacterium tumefaciens* C58C[1] toward *vir*-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. *J Bacteriol* 170: 4181-4187.

Atmakuri, K., Cascales, E., Burton, O.T., Banta, L.M., & Christie, P.J. (2007) *Agrobacterium* ParA/MinD-like VirC1 spatially coordinates early conjugative DNA transfer reactions. *EMBO J.* 26: 2540–2551.

Atmakuri, K., Ding, Z., & Christie, P.J. (2003) VirE2, a type IV secretion substrate, interacts with the VirD4 transfer protein at cell poles of *Agrobacterium tumefaciens*. *Mol Microbiol* 49: 1699-1713.

Bako, L., Umeda, M., Tiburcio, A.F., Schell, J., & Koncz, C. (2003) The VirD2 pilot protein of *Agrobacterium*-transferred DNA interacts with the TATA box-binding protein and a nuclear protein kinase in plants. *Proc Natl Acad Sci USA* 100: 10108-10113.

Ballas, N., & Citovsky, V. (1997) Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein. *Proc Natl Acad Sci USA* 94: 10723-10728.

Banta, L.M., Bohne, J., Lovejoy, S.D., & Dostal, K. (1998) Stability of the *Agrobacterium tumefaciens* VirB10 protein is modulated by growth temperature and periplasmic osmoadaption. *J Bacteriol* 180: 6597-6606.

Bash, R., & Matthysse, A.G. (2002) Attachment to roots and virulence of a *chvB* mutant of *Agrobacterium tumefaciens* are temperature sensitive. *Mol Plant-Microbe Interact* 15: 160-163.

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res* 12: 8711-8721.

Bhattacharjee, S., Lee, L.-Y., Oltmanns, H., Cao, H., Veena, Cuperus, J. & Gelvin, SB. (2008) AtImpa-4, an *Arabidopsis* importin α isoform, is preferentially involved in *Agrobacterium*-mediated plant transformation. *Plant Cell* 20: 2661-2680.

Binns, A.N., Beaupre, C.E., & Dale, E.M. (1995) Inhibition of VirB-mediated transfer of diverse substrates from *Agrobacterium tumefaciens* by the InQ plasmid RSF1010. *J Bacteriol* 177: 4890-4899.

Bolton, G.W., Nester, E.W., & Gordon, M.P. (1986) Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. *Science* 232: 983-985.

Bourret, R., Borkovich, K., & Simon, M. (1991) Signal transduction pathways involving protein phosphorylation in prokaryotes. *Annu Rev Biochem* 60: 401-441.

Braun, A.C. (1947) Thermal studies on the factors responsible for the tumor initiation in crown gall. *Am J Bot* 34: 234-240.

Braun, A.C., & Mandle, R.J. (1948) Studies on the inactivation of the tumor-inducing principle in crown gall. *Growth* 12: 255-269.

Brencic A., & Winans S.C. (2005) Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. *Microbiol Mol Biol Rev* 69: 155–194.

Brunaud, V., Balzeergue, S., Dubreucq, B., Aubourg, S., Samson, F., Chauvin, S., Bechtold, N. Cruaud, C., & DeRose, R. et al. (2002) T-DNA integration into the *Arabidopsis* genome depends on sequences of pre-insertion sites. *EMBO Rep* 3: 1152-1157.

Burns, D.L. (2003) Type IV transporters of pathogenic bacteria. *Current Opinion in Microbiology* 6: 29-34.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., & Turner, C. (1988) Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu Rev Cell Biol* 4: 487-525.

Cangelosi, G.A., Hung, L., Puvanesarajah, V., Stacey, G., Ozga, D.A., Leigh, J.A., & Nester, E.W. (1987) Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* exopolysaccharide synthesis and their roles in plant interactions. *J Bacteriol* 169: 2086-2091.

Cangelosi, G.A., Martinetti, A.G., Leigh, J.A., Lee, C.C., Theines, C., & Nester, E.W. (1989) Role of *Agrobacterium tumefaciens* ChvA protein in export of β-1,2-glucan. *J Bacteriol* 171: 1609-1615.

Cascales, E., & Christie, P.J. (2003) The versatile bacterial type IV secretion system. *Nature Rev Microbiol* 1: 137-149.

Cascales, E., & Christie, P.J. (2004) Definition of a bacterial type IV secretion pathway for a DNA substrate. *Science* 304: 1170-1173.

Chang, C.-H., & Winans, S.C. (1992) Functional roles assigned to the periplasmic, linker, and receiver domains of the *Agrobacterium tumefaciens* VirA protein. *J Bacteriol* 174: 7033-7039.

Chilton, M.-D. (1993) *Agrobacterium* gene transfer: Progress on a "poor man's vector" for maize. *Proc Natl Acad Sci USA* 90: 3119-3120.

Chilton, M.-D., Drummond, M.H., Merlo, D.J., & Sciaky, D. (1978) Highly conserved DNA of Ti plasmids overlaps T-DNA maintained in plant tumors. *Nature* 275: 147-149.

Chilton, M.-D., Drummond, M.H., Merlo, D.J., Sciaky, D., Montoya, A.L., Gordon, M.P., & Nester, E.W. (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* 11: 263-271.

Christie, P.J., & Vogel, J.P. (2000) Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends in Microbiology* 8: 354-360.

Christie, P.J., Ward, J.E., Winans, S.C., & Nester, E.W. (1988) The *Agrobacterium tumefaciens virE2* gene product is a single-stranded-DNA-binding protein that associates with T-DNA. *J Bacteriol* 170: 2659-2667.

Citovsky, V., De Vos, G., & Zambryski, P. (1988) Single-stranded DNA binding protein encoded by the *virE* locus of *Agrobacterium tumefaciens*. *Science* 240: 501-504.

Citovsky, V., Guralnick, B., Simon, M.N., & Wall, J.S. (1997) The molecular structure of *Agrobacterium* VirE2-single stranded DNA complexes involved in nuclear import. *J Mol Biol* 271: 718-727.

Citovsky, V., Kozlovsky, S.V., Lacriox, B., Zaltsman, A., Dafny-Yelin, M., Vyas, S., Tovkach, A. & Tzfira, T. (2007) Biological systems of the host cell involved in *Agrobacterium infection*. *Cell Microbiol*, 9: 9-20.

Citovsky, V., Wong, M.L., & Zambryski, P. (1989) Cooperative interaction of *Agrobacterium* VirE2 protein with single stranded DNA: implications for the T-DNA transfer process. *Proc Natl Acad Sci USA* 86: 1193-1197.

Citovsky, V., Zupan, J., Warnick, D., & Zambryski, P. (1992) Nuclear localization of *Agrobacterium* VirE2 protein in plant cells. *Science* 256: 1802-1805.

Clauce-Coupel, H., Chateau, S., Ducrocq, C., Niot, V. & Kaveri, S., et al. (2008) Role of vitronectin-like protein in *Agrobacterium* attachment and transformation of *Arabidopsis* cells. *Protoplasma* 234: 65075

Crane, Y.M. & Gelvin, S.B. (2007) RNAi-mediated gene silencing reveals involvement of *Arabidopsis* chromatin-related genes in *Agrobacterium*-mediated root transformation. *Proc Natl Acad Sci USA* 104: 15156–15161.

Dafny-Yelin, M., Levy, A. & Tzfira, T. (2008) The ongoing saga of *Agrobacterium*–host interactions. *Trends Plant Sci* 13: 102-105.

Das, A. & Xie, Y.-H. (1998) Construction of transposon *Tn3phoA*: Its application in defining the membrane topology of the *Agrobacterium tumefaciens* DNA transfer proteins. *Mol Microbiol* 27: 405-414.

De Cleene, M., & De Ley, J. (1976) The host range of crown gall. *Bot Rev* 42: 389-466.

De Iannino, N.I., & Ugalde, R.A. (1989) Biochemical characterization of avirulent *Agrobacterium tumefaciens chvA* mutants: synthesis and excretion of β-(1,2)glucan. *J Bacteriol* 171: 2842-2849.

Deng, W., Chen, L., Peng, W.-T., Liang, X., Sekiguchi, S., Gordon, M.P., Comai, L., & Nester, E.W. (1999) VirE1 is a specific molecular chaperone for the exported single-stranded-DNA-binding protein VirE2 in *Agrobacterium*. *Mol Microbiol* 31: 1795-1807.

Deng, W., Chen, L., Wood, D.W., Metcalfe, T., Liang, X., Gordon, M.P., Comai, L., & Nester, E.W. (1998) *Agrobacterium* VirD2 protein interacts with plant host cyclophilins. *Proc Natl Acad Sci USA* 95: 7040-7045.

Depicker, A., Van Montagu, M., & Schell, J. (1978) Homologous sequences in different Ti plasmids are essential for oncogenicity. *Nature* 275:150-152.

Djamei, A., Pitzschke, A., Nakagami, H., Rajh, I., & Hirt, H. (2007) Trojan horse strategy in *Agrobacterium* transformation: abusing MAPK defense signaling. *Science* 318: 453-456.

Douglas, C.J., Halperin, W., & Nester, E.W. (1982) *Agrobacterium tumefaciens* mutants affected in attachment to plant cells. *J Bacteriol* 152: 1265-1275.

Douglas, C.J., Staneloni, R.J., Rubin, R.A., & Nester, E.W. (1985) Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence gene. *J Bacteriol* 161: 850-860.

Durrenberger, F., Crameri, A., Hohn, B., & Koukolikova-Nicola, Z. (1989) Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. *Proc Natl Acad Sci USA* 86: 9154-9158.

Engstrom, P., Zambryski, P., Montagu, M.V., & Stachel, S. (1987) Charaterization of *Agrobacterium tumefaciens* virulence proteins induced by the plant factor acetosyringone. *J Mol Biol* 197: 635-645.

Escobar, M.A., & Dandekar, A.M. (2003) *Agrobacterium tumefaciens* as an agent of disease. *TRENDS in Plant Science* 8: 380-386.

Fronzes, R., Schafer, E., Wang, L., Saibil, H. R., Orlova, E. V., & Waksman, G. (2009) Structure of a type IV secretion system core complex. *Science* 323: 266–268.

Garcia-Rodriguez, F.M., Schrammeijer, B. & Hooykaas, P.J.J. (2006) The *Agrobacterium* VirE3 effector protein: a potential plant transcriptional activator. *Nucl Acids Res* 34: 6496–504.

Gaspar, Y.M., Nam, J., Schultz, C.J., Lee, L.-Y. & Gilson, P.R., et al. (2004). Characterization of the *Arabidopsis* lysine-rich arabinogalactan-protein AtAGP17 mutant (*rat1*) that results in a decreased efficiency of *Agrobacterium* transformation. *Plant Physiol*. 135: 2162–2171.

Gelvin, S.B. (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annu Rev Plant Physiol Plant Mol Biol* 51: 223-256.

Gelvin, S.B. (2003) *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiol Mol Biol Rev* 67: 16-37.

Gelvin, S. B. (2010a) Plant proteins involved in *Agrobacterium*-mediated genetic transformation. *Annu Rev Phytopathol* 48: 45–68.

Gelvin, S. B. (2010b) Finding a way to the nucleus. *Current Opinion in Microbiology*. 13: 53–58.

Ghai, J., & Das, A. (1989) The *virD* operon of *Agrobacterium tumefaciens* Ti plasmid encodes a DNA-relaxing enzyme. *Proc Natl Acad Sci USA 86:* 3109-3113.

Gheysen, G., Van Montagu, M., & Zambryski, P. (1987). Integration of *Agrobacterium tumefaciens* transfer DNA (T-DNA) involves rearrangements of target plant DNA sequences. *Proc Natl Acad Sci USA* 84: 6169-6173.

Gillespie, J.J., Brayton, K.A., Williams, K.P., Quevedo Diaz, M.A., Brown, W.C., Azad, A.F., & Sobral, B.W. (2010). Phylogenomics reveals a diverse *Rickettsiales* type IV secretion system. *Infect Immun* 78: 1809–1823.

Gomis-Ruth, F.X., Moncalian, G., de la Cruz, F., & Coll, M. (2002) Conjugative plasmid protein TrwB, an integral membrane type IV secretion system coupling protein. Detailed structural features and mapping of the active site cleft. *J Biol Chem* 277: 7556-7566.

Grange, W., Duckely, M., Husale, S., Jacob, S., Engel, A. & Hegner, M. (2008) VirE2: a unique ssDNA-compacting molecular machine. *PloS Biol*, 6: 343-351.

Guo, M., Hou, Q., Hew, C. L., & Pan, S. Q. (2007a) *Agrobacterium* VirD2-binding protein is involved in tumorigenesis and redundantly encoded in conjugative transfer gene clusters. *Mol Plant-Microbe Interact* 20: 1201-1212.

Guo, M., Jin, S., Sun, D., Hew, C. L., & Pan, S. Q. (2007b) Recruitment of conjugative DNA transfer substrate to *Agrobacterium* type IV secretion apparatus. *Proc Natl Acad Sci USA* 104: 20019-20024.

Guo, M., Gao, D., & Jin, Y. (2009a) Progress in the formation and transfer of *Agrobacterium* T-complex. *Pro Biochem Biophys* 36: 1408-1414.

Guo, M., Zhu, Q., & Gao, D. (2009b) Development and optimization of method for generating unmarked *A. tumefaciens* mutants. *Pro Biochem Biophys* 36: 556-565.

Guo, M. (2010) Discovery and biologocal function identification of *Agrobacterium* T-complex recruiting protein. *Acta Agriculturea Universitatis Jiangxiensis* 32: 992-996.

Hawes, M.C., & Smith, L.Y. (1989) Requirement for chemotaxis in pathogenicity of *Agrobacterium tumefaciens* on roots of soil-grown pea plants. *J Bacteriol* 171: 5668-5671.

Howard, E., & Citovsky, V. (1990) The emerging structure of the *Agrobacterium* T-DNA tansfer complex. *Bioessays* 12: 103-108.

Howard, E.A., Zupan, J.R., Citovsky, V., & Zambryski, P.C. (1992) The VirD2 protein of *Agrobacterium tumefaciens* contains a C-terminal bipartite nuclear localization signal: Implications for nuclear uptake of DNA in plant cells. *Cell* 68: 109-118.

Ilyina, T., & Koonin, E. (1992) Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. *Nucleic Acids Res* 20: 3279-3285.

Jasper, F., Koncz, C., Schell, J., & Steinbiss, H-H. (1994) *Agrobacterium* T-strand production *in vitro*: sequence-specific cleavage and 5′ protection of single-stranded DNA templates by purified VirD2 protein. *Proc Natl Acad Sci USA* 91: 694-698.

Jin, S., Roitsch, T., Ankenbauer, R.G., Gordon, M.P., & Nester, E.W. (1990) The VirA protein of *Agrobacterium tumefaciens* is autophosphorylated and essential for *vir* gene induction. *J Bacteriol* 172: 525-530.

Jin, S., Song, Y., Pan, S.Q., & Nester, N.W. (1993) Characterization of a *virG* mutation that confers constitutive virulence gene expression in *Agrobacterium tumefaciens*. *Mol Microbiol* 7: 555-562.

Kamoun, S., Cooley, M.B., Rogowsky, U.M., & Kado, C.I. (1989) Two chromosomal loci involved in production of exopolysaccharide in *Agrobacterium tumefaciens*. *J Bacteriol* 171: 1755-1759.

Kennedy, B.W. (1980) Estimates of U.S. crop losses to prokaryote plant pathogens. *Plant Dis* 64: 674-676.

Kim, S.I., Veena, J.H. & Gelvin, S.B. (2007) Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the *Arabidopsis* genome generated under non-selective conditions. *Plant J*. 51: 779–791.

Kumar, R.B. & Das, A. (2002) Polar location and functional domains of the *Agrobacterium tumefaciens* DNA transfer protein VirD4. *Mol Microbiol* 43: 1523-1532.

Lacroix, B., Li, J., Tzfira, T., & Citovsky, V. (2006a) Will you let me use your nucleus? How *Agrobacterium* gets its T-DNA expressed in the host plant cell. *Can J Physiol Pharmacol* 84: 333-345.

Lacroix, B., Tzfira, T., Vainstein, A., & Citovsky, V. (2006) A case of promiscuity: *Agrobacterium*'s endless hunt for new partners. *TRENDS in Genetics* 22: 29-37.

Lee, Y., Jin, S., Sim, W., & Nester, E.W. (1996) The sensing of plant signal molecules by *Agrobacterium*: genetic evidence for direct recognition of phenolic inducers by the VirA protein. *Gene* 179: 83-88.

Lin, Yi-Han, Gao, R, Binns A.N. & Lynn D.G. (2008) Capturing the VirA/VirG TCS of *Agrobacterium tumefaciens*. *Adv Exp Med Biol* 631: 161-177.

Lippincott, B.B., & Lippincott, J.A. (1969) Bacterial attachment to a specific wound site as an essential stage in tumor initiation by *Agrobacterium tumefaciens*. *J Bacteriol* 97: 620-628.

Llosa, M., Gomis-Ruth, F.X., Coll, M., & Cruz, F. (2002) Bacterial conjugation: a two-step mechanism for DNA transport. *Mol Microbiol* 45: 1-8.

Llosa, M., Zunzunegui, S., & Cruz, F. (2003) Conjugative coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes. *Proc Natl Acad Sci USA* 100: 10465-10470.

Loake, G.J., Ashby, A.M., & Shaw, C.M. (1988) Attraction of *Agrobacterium tumefaciens* C58C[1] towards sugars involves a highly sensitive chemotaxis system. *J Gen Microbiol* 134: 1427-1432.

Lopatin, M.I. (1939) Influence of bacterial root canter on the development of the cherry tree in the orchard. *Plant Prot* 18:167-173.

Matthysse, A.G., & McMahan, S. (1998) Root colonization by *Agrobacterium tumefaciens* is reduced in *cel*, *attB*, *attD*, and *attR* mutants. *Appl Environ Microb* 64: 2341-2345.

Matthysse, A.G., & McMahan, S. (2001) The effect of the *Agrobacterium tumefaciens attR* mutation on attachment and root colonization differs between legumes and other dicots. *Appl Environ Microb* 67: 1070-1075.

Matthysse, A.G., Yarnall, H., Boles, S.B., & McMahan, S. (2000) A region of the *Agrobacterium tumefaciens* chromosome containing genes required for virulence and attachment to host cells. *Biochimica et Biophysica Acta* 1490: 208-212.

Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Bakkeren, G., Crameri, A., Angelis, K., Redei, G.P., Schell, J., Hohn, B., & Koncz, C. (1991) T-DNA integration: A model of illegitimate recombination in plants. *EMBO J* 10: 697-704.

McCullen C.A., & Binns A.N. *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. *Annu Rev Cell Dev Biol*, 2006, 22: 101-127.

Minnemeyer, S.L., Lightfoot, L.R., & Matthysse, A.G. (1991) A semi-quantitative bioassay for relative virulence of *Agrobacterium tumefaciens* strains on *Bryophyllum daigremontiana*. *J Bacteriol*. 173: 7723-7724.

Mysore, K.S., Bassuner, B., Deng, X., Darbinian, N.S., Motchoulski, A., Ream, W., & Gelvin, S.B. (1998) Role of the *Agrobacterium tumefaciens* VirD2 protein in T-DNA transfer and integration. *Mol Plant-Microbe Interact* 11: 668-683.

Nam, J., Matthysse, A.G., & Gelvin, S.B., (1997) Differences in susceptibility of *Arabidopsis* ecotypes to crown gall disease may result from a deficiency in T-DNA integration. *Plant Cell* 9: 317-333.

Nam, J., Mysore, K.S., Zheng, C., Knue, M.K., & Matthysse, A.G., *et al*. (1999) Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium*. *Mol Gen Genet* 261: 429-438.

Newton, J.A., & Fray, R.G. (2004) Integration of environmental and host-derived signals with quorum sensing during plant-microbe interactions. *Cellular Microbiology* 6: 213-224.

Nixon, B.T., Ronson, C.W., & Ausubel, F.M. (1986) Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes *ntrB* and *ntrC*. *Proc Nat Acad Sci USA* 83: 7850-7854.

O'Connell, K.P., & Handelsman, J. (1989) *chvA* locus may be involved in export of neutral cyclic β-1,2 linked D-glucan from *Agrobacterium tumefaciens*. *Mol Plant-Microbe Interact* 2: 11-16.

Otten, L., DeGreve, H., Leemans, J., Hain, R., Hooykass, P., & Schell, J. (1984) Restoration of virulence of *vir* region mutants of *A. tumefaciens* strain B6S3 by coinfection with normal and mutant *Agrobacterium* strains. *Mol Gen Genet* 195: 159-163.

Palmer, A.G., Gao, R., Maresh, J., Erbiol, W.K. & Lynn, D.G. 2004. Chemical biology of multihost/pathogen interactions: chemical perception and metabolic complementation. *Annu Rev Phytopathol* 42: 439–64.

Pansegrau, W., Schoumacher, F., Hohn, B., & Lanka, E. (1993) Site-specific cleavage and joining of single-stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: analogy to bacterial conjugation. *Proc Natl Acad Sci USA* 90: 11538-11542.

Parke, D., Ornston, L.N., & Nester, E.W. (1987) Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in *Agrobacterium tumefaciens*. *J Bacteriol* 169: 5336-5338.

Pitzschke1, A., & Hirt, H. (2010) New insights into an old story: *Agrobacterium*-induced tumour formation in plants by plant transformation. *EMBO J* 29: 1021-1032.

Rao, A. Q., Bakhsh, A., Kiani, S., Shahzad, K., Shahid, A. A., Husnain, T., & Riazuddin, S. (2009). The myth of plant transformation. *Biotechnology Advances*. 27: 753-763.

Ream, W. (1989) *Agrobacterium tumefaciens* and interkingdom genetic exchange. *Annu Rev Phytopathol* 27: 583-618.

Regensburg-Tuink, A.J., & Hooykaas, P.J. (1993) Transgenic *N. glauca* plants expressing bacterial virulence gene *virF* are converted into hosts for nopaline strains of *A. tumefaciens*. *Nature* 363: 69-71.

Reuhs, B.L., Kim, J.S., & Matthysse, A.G. (1997) Attachment of *Agrobacterium tumefacins* to carrot cells and *Arabidopsis* wound sites is correlated with the presence of a cell-associated, acidic polysaccharide. *J Bacteriol* 179: 5372-5379.

Ricker, A.J., Berbee, J.G., & Smalley, E.B. (1959) Effects of crown gall and hairy root on the growth of apple trees. *Phytopathology* 49: 88-90.

Robertson, J.L., Holliday, T., & Matthysse, A.G. (1988) Mapping of *Agrobacterium tumefaciens* chromosomal genes affecting cellulose synthesis and bacterial attachment to host cells. *J Bacteriol*. 170: 1408-1411.

Rossi, L., Hohn, B., & Tinland, B. (1993) The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plants. *Mol Gen Genet* 239: 345-353.

Salman, H., Abu-Arish, A., Oliel, S., Loyter, A., & Klafter, J., et al. (2005) Nuclear localization signal peptides induce molecular delivery along microtubules. *Biophys. J.* 89, 2134–2145

Sanders, L., Wang, C.-O., Walling, L., & Lord, E. (1991) A homolog of the substrate adhesion molecule vitronectin occurs in four species of flowering plants. *Plant Cell* 3: 629-635.

Scheiffele, P., Pansegrau, W., & Lanka, E. (1995) Initiation of *Agrobacterium tumefaciens* T-DNA processing: purified protein VirD1 and VirD2 catalyze site- and strand-specific cleavage of superhelical T-border DNA in vitro. *J Biol Chem* 270: 1269-1276.

Schilperoort, R.A., Veldstra, H., Warnaar, S.Q. Mulder, G., & Cohen, J.A. (1967) Formation of complexes between DNA isolated from tobacco crown gall tumours and complementary to *Agrobacterium tumefaciens* DNA. *Biochim Biophys Acta* 145: 523-525.

Schrammeijer, B., Dulk-Ras, A., Vergunst, A.C., Jacome, E.J., & Hooykaas, P.J.J. (2003) Analysis of Vir protein translocation from *Agrobacterium tumefaciens* using *Saccharomyces cerevisiae* as a model: evidence for transport of a novel effector protein VirE3. *Nucleic Acids Research* 31: 860-868.

Schrammeijer, B., Risseeuw, E., Pansegrau, W., Regensburg-Tuink, T.J.G., Crosby, W.L., & Hooykaas, P.J.J. (2001) Interaction of the virulence protein VirF of *Agrobacterium tumefaciens* with plant homologs of the yeast Skp1 protein. *Curr Biol* 11: 258-262.

Schroth, M.N., Mccain, A.H., Foott, J.H., & Huisman, O.C. (1988) Reduction in yield and vigor of grapevine caused by crown gall disease. *Plant Dis* 72: 241-246.

Shaw, C.H.J., Ashby, A.M., Brown, A., Royal, C., Loake, G.J., & Shaw, C.H. (1989) VirA and G are necessary for acetosyringone chemotaxis in *Agrobacterium tumefaciens*. *Mol Microbiol* 2: 413-417.

Sheng, J., & Citovsky, V. (1996) *Agrobacterium*-plant cell DNA transport: Have virulence proteins, will travel. *The Plant Cell* 8: 1699-1710.

Shurvinton, C.E., Hodge, L., & Ream, W. (1992) A nuclear localization signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. *Proc Natl Acad Sci USA* 89: 11837-11841.

Silver, P.A. (1991) How proteins enter the nucleus. *Cell* 64: 489-497.

Smith, E.F., & Townsend, C.O. (1907) A plant-tumor of bacterial origin. *Scince* 24: 671-673.

Smith, V.A. & Hindley, J. (1978) Effect of agrocin 84 on attachment of *Agrobacterium* tumefaciens to cultured tobacco cells. *Nature* 276:498–500.

Stachel, S.E., Messens, E., Van Montagu, M., & Zambryski, P. (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* 318: 624-629.

Stachel, S.E., Nester, E.W., & Zambryski, P.C. (1986) A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. *Proc Natl Acad Sci USA* 83: 379-383.

Stachel, S.E., Timmerman, B., & Zambryski, P. (1987) Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: Requirement of 5′ virD gene products. *EMBO J* 6: 857-863.

Stachel, S.E., & Zambryski, P.C. (1986) *Agrobacterium tumefaciens* and the susceptible plant cell: a novel adaptation of extracellular recognition and DNA conjugation. *Cell* 47: 155-157.

Sundberg, C., Meek, L., Carroll, K., Das, A., & Ream, W. (1996) VirE1 protein mediates export of the single-stranded DNA binding protein VirE2 from *Agrobacterium tumefaciens* into plant cells. *J Bacteriol* 178: 1207-1212.

Sundberg, C.D., & Ream, W. (1999) The *Agrobacterium tumefaciens* chaperone-like protein, VirE1, interacts with VirE2 at domains required for single-stranded DNA binding and cooperative interaction. *J Bacteriol* 181: 6850-6855.

Swart, S., Logman, T.J., Smit, G., Lugtenberg, B.J., & Kijne, J.W. (1994) Purification and partial characterization of a glycoprotein from pea (*Pisum sativum*) with receptor activity for rhicadhesin, an attachment protein of *Rhizobiaceae*. *Plant Mol Biol* 24: 171-183.

Tao, Y., Rao, P.K. Bhattacharjee, S. & Gelvin, S.B. (2004) Expression of plant protein phosphatase 2C interferes with nuclear import of the *Agrobacterium* T-complex protein VirD2. *Proc Natl Acad Sci USA* 101: 5164–5169.

Thomashow M.F., Karlinsey J.E., Marks J.R., & Hurlbert R.E. (1987) Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects polysaccharide composition and plant cell attachment. *J Bacteriol* 169: 3209-3216.

Tinland, B., Koukolikova-Nicola, Z., Hall, M.N., & Hohn, B. (1992) The T-DNA-linked VirD2 protein contains two distinct functional nuclear localization signals. *Proc Natl Acad Sci USA* 89: 7442-7446.

Tinland, B., Schoumacher, F., Gloeckler, V., Bravo-Angel, A.M., & Hohn, B. (1995) The *Agrobacterium tumefaciens* virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. *EMBO J* 14: 3585-3595.

Tomlinson, A.D. & Fuqua, C. (2009) Mechanisms and regulation of polar surface attachment in *Agrobacterium tumefaciens*. *Curr Opin Microbiol.* 12: 708–714.

Turk, S.C.H.J., Van Lange, R.P., Regensburg-Tuink, T.J.G., & Hooykaas, P.J.J. (1994) Localization of the VirA domain involved in acetosyringone-mediated *vir* gene induction in *Agrobacterium tumefaciens*. *Plant Mol Biol* 25: 899-907.

Tzfira, T., & Citovsky, V. (2000) From host recognition to T-DNA integration: function of bacterial and plant genes in the *Agrobacterium*-plant cell interaction. *Mol Plant Pathol* 1: 201-212.

Tzfira, T., & Citovsky, V. (2002) Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. *TRENDS in Cell Biology* 12: 121-129.

Tzfira, T., & Citovsky, V. (2006) *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. *Curr Opin Biotechnol* 17: 147-154.

Tzfira, T., Li, J., Lacroix, B. & Citovsky, V. (2004) *Agrobacterium* T-DNA integration: molecules and models. *Trends Genet* 20: 375–83.

Tzfira, T., Vaidya, M., & Citovsky, V. (2001) VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. *EMBO J* 20: 3596-3607.

Tzfira, T., Vaidya, M. & Citovsky, V. (2002) Increasing plant susceptibility to *Agrobacterium* infection by overexpression of the *Arabidopsis* nuclear protein VIP1. *Proc Natl Acad Sci USA* 99: 10435–440

Van Attikum, H., Bundock, P. & Hooykaas, P.J.J. (2001) Non-homologous end-joining proteins are required for *Agrobacterium* T-DNA integration. *EMBO J* 20: 6550–58.

Van Larebeke, N., Enbler, G., Holsters, M., Van Den Elsacker, S., Zaenen, I., Schilperoort, R.A., & Schell. J. (1974) Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. *Nature* 252: 169-170.

Vanlentine, L. (2003) *Agrobacterium tumefaciens* and the plant: the David and Goliath of modern genetics. *Plant Physiol* 133: 948-955.

Veluthambi, K., Ream, W., & Gelvin, S.B. (1988) Virulence genes, borders, and overdrive generate single-stranded T-DNA molecules from the A6 Ti plasmid of *Agrobacterium tumefaciens*. *J Bacteriol* 170: 1523-1532.

Vergunst, A.C., Lier, M.C.M., Dulk-Ras, A.D., & Hooykaas P.J.J. (2003) Recognition of the *Agrobacterium tumefaciens* VirE2 translocation signal by the VirB/D4 transport system does not require VirE1. *Plant Physiol* 133: 978-988.

Vergunst, A.C., Lier, M.C.M., Dulk-Ras, A.D., Stuve, T.A.G., Ouwehand, A., & Hooykaas P.J.J. (2005) Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of *Agrobacterium*. *Proc Natl Acad Sci USA* 102: 832-837.

Vergunst, A.C., Schrammeijer, B., Dulk-Ras, A., Vlaam, C.M.T., Regensburg-Tuink, T.J.G., & Hooykaas, P.J.J. (2000) VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. *Science* 290: 979-982.

Vogel, A.M., & Das, A. (1992) Mutational analysis of *Agrobacterium tumefaciens* VirD2: tyrosine 29 is essential for endonuclease activity. *J Bacteriol* 174: 303-308.

Vogel, A.M., & Das, A. (1994) Mutational analysis of *Agrobacterium tumefaciens* pTiA6 *virD1*: identification of functionally important residues. *Mol Microbiol* 12: 811-817.

Vogel, A.M., Yoon, J., & Das, A. (1995) Mutational analysis of a conserved motif of *Agrobacterium tumefaciens* VirD2. *Nucleic Acids Res* 23: 4087-4091.

Wagner, V.T., & Matthysse, A.G. (1992) Involvement of a vitronectin-like protein in attachment of *Agrobacterium tumefaciens* to carrot suspension culture cells. *J Bacteriol* 174: 5999-6003.

Wang, K., Herrera-Estrella, A., & Van Montagu, M. (1990) Overexpression of *virD1* and *virD2* genes in *Agrobacterium tumefaciens* enhances T-complex formation and plant transformation. *J Bacteriol* 172: 4432-4440.

Wang, Y., Gao, R. & Lynn, D.G. (2002) Ratcheting up *vir* gene expression in *Agrobacterium tumefaciens*: coiled-coil in histidine kinase signal transduction. *Chembiochem* 3: 311–317.

White, P.R., & Braun, A.C. (1941) Crown gall production by bacteria-free tumor tissues. *Science* 94: 239-241.

Winans, S.P. (1992) Two-way chemical signaling in *Agrobacterium*-plant interactions. *Microbiol Rev* 56: 12-31.

Yusibov, V.M., Steck, T.R., Gupta, V., & Gelvin, S.B. (1994) Association of single-stranded transferred DNA from *Agrobacterium tumefaciens* with tobacco cells. *Proc Natl Acad Sci USA* 91: 2994-2998.

Zaenen, I., Van Larebeke, N., Teuchy, H., Van Montagu, M., & Schell, J. (1974) Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. *J Mol Biol* 86: 109-127.

Zambryski, P., Joos, P.H., Genetello, C., Leemans, J., Van Montagu, M., & Schell, J. (1983) Ti plasmid vector for the intriduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J* 2: 2143-2150.

Zhu, J., Oger, P.M., Schrammeijer, B., Hooykaas, P.J.J., Farrand, S.K., & Winans, S.C. (2000) The bases of crown gall tumorigenesis. *J Bacteriol* 182: 3885-3895.

Zhu, Y., Nam, J., Humara, J.M., Mysore, K.S., Lee, L.Y., Cao, H., Valentine, L., Li, J., Kaiser, A.D., & Kopecky, A.L., et al. (2003) Identification of *Arabidopsis rat* mutants. *Plant Physiol* 132: 494-505.

Ziemienowicz, A. (2001) Odyssey of *Agrobacterium* T-DNA. *Acta Biochimica Polonica* 48: 623-635.

Ziemienowicz, A., Gorlich, D., Lanka, E., Hohn, B., & Rossi, L. (1999) Import of DNA into mammalian nuclei by proteins originating from a plant pathogenic bacterium. *Proc Natl Acad Sci USA* 96: 3729-3733.

Ziemienowicz, A., Merkle, T., Schoumacher, F., Hohn, B., & Rossi, L. (2001) Import of *Agrobacterium* T-DNA into plant nuclei: two distinct functions of VirD2 and VirE2 proteins. *Plant Cell* 13: 369-383.

Zorreguieta, A., & Ugalde, R.A. (1986) Formation in *Rhizobium* and *Agrobacterium* spp. of a 235-kilodalton protein intermediate in β-D-(1-2)glucan synthesis. *J Bacteriol* 167: 947-951.

Zupan, J., & Zambryski, P. (1997) The *Agrobacterium* DNA transfer complex. *Critical Reviews in Plant Sciences* 16: 279-295.

2

Structure-Function Analysis of Transformation Events

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1. Introduction

At the beginning of the twenty-first century, critical reforms were outlined in biology. This was due to the fact that the increase of information garnered from the sequences of informational molecules (DNA, RNA and proteins) and their perception by the scientific community were incompatible with the narrow horizons of the dogmas and paradigms that shaped the ideology of biological knowledge at the end of the last century. The clear linear representations that were invoked during the initial period of the development of molecular biology were at first encountered with numerous exceptions to these "clear" rules and then lost in the avalanche of new representations that were incompatible with the linear schemes. As a result, new directions of study and even meta-directions (e.g., epigenetics, epigenomics and biosemiotics - for reviews, see Allis et al., 2007; Ferguson-Smith et al., 2009; Hoffmeyer, 2008) have arisen, and functionally oriented divisions of biological science have formed that are named as various "–omics" (including the extravagant "biblioma"- Abi-Haidar et al., 2007), the RNA machine (Amaral et al., 2008), and the molecular mechanisms of cell cycle regulation and individual development. In turn, these disciplines have demanded new theoretical implementations involving novel approaches from the theory of networks and systems (Barabasi et al., 2000; West & Brown, 2005; Zaretzky & Letelier, 2002).

The last two decades have been particularly rich in discoveries concerning the mechanisms of the expression of biological information, such as new ways of alternative splicing (Rodríguez-Trelles et al., 2006), a variety of functions for non-coding transcripts and the role of short RNAs as forward and reverse regulators (Mattick et al., 2009). Presently, we have a situation in biology in which it would be nearly impossible to publish a book with the title "DNA Makes RNA Makes Protein" (Pentris et al., 1983). However, another book, with a title equally as concise and perfectly reflecting the new biological paradigm, has not yet been written. In other words, a revision of the old concepts has not been completed with a new and clear way of structuring the data from the quickly growing "body" of biological science. Theoretical biology is also at a critical state, which is characterized by attempts to formulate or reformulate the basic concepts and axioms of the discipline. Signs of such attempts may be the revival of interest in the definition of life, the revision of "sets" and even "types", signs that distinguish the living from the non-living and increased attention to the genotype-phenotype relationship.

This specific creative climate is generated in the interaction zone of theoretical biology and directions relating to the theory of artificial intelligence[1], and it is packed with the new fundamental knowledge that artificial intelligence has absorbed from the different fields of mathematics, physics, and logic (see reviews in Bersini, 2009; Cárdenas et al., 2010; Longo, 2010). It is in this climate that a systems approach to the analysis of biological phenomena is the most productive, yet difficult, for biologists. In journals, the contents of which always gravitate toward theoretical biology, publications on autopoesis, autonomy and incompleteness, and the determinism and unpredictability of biological systems have become commonplace. Notable among these reports is a group of publications that explore the phenomenon of causal closure in living systems as one of the key events on the way to biological complexity (Cárdenas et al., 2010; Kauffmann et al., 2008; Longo, 2010).

Practical achievements of new biological technologies are also impressive. Transgenic plants with useful properties have spread across the globe, displacing more than half of the varieties generated by conventional breeding (Godfray et al., 2010). Information about "cloned" mammals and "replacement components" of mammalian organisms has become standard (e.g., see Ficz et al., 2009; Morgan, et al., 2005). Nevertheless, the theoretical basis for even the most impressive achievements in biotechnology remains underdeveloped, and many promises remain unfulfilled or have been replaced with surrogate solutions.

There is a similar situation with regard to experiments on the genetic transformation of biological objects, where many publications use the terminology and views of the last century. Although many of these terms and concepts are still relevant, it seems appropriate to review the most common events of transformation from a position of a new system of knowledge. We believe that such an analysis will be able to correctly classify several important areas of transformation, to discuss the reasons for failures in some cases and to outline ways to achieve the assigned goals. Therefore, we do not advocate a quick alteration of the terminology and concepts or the creation of a new theoretical foundation of biological science. Personally, we cannot proceed without the concepts of gene, species and many other supposedly "outdated" terms. The task of this publication is to gain a deeper understanding of examples of genetic engineering, those acquired in biotechnology and often having analogs in the wild, with help of a structure-function analysis and to apply new knowledge for the planning of experiments and the prediction of their results. Likely, even this limited goal can scarcely be solved in one study. However, we hope that even the smallest success in this direction will soon be in demand.

We associate certain expectations with the operational presentation of a biological object, where, in the most general mode, the program elements (see below for content on the term "program element" and other elements of the triad) are mapped into the observables. Application of an operational definition of a biological object can help to distinguish (rather conditionally) the mappings associated with the transformation of structures (actual objects) from those associated with the transformation of functions (relations). Such a distinction plays an important role in the interpretation of the transformation events because the result of transformation can be involved primarily in the structural flow or, conversely, in the functional flow of the individual development of the transformed object.

[1] Henceforth, terms and metaphors will be used, which may create the impression that the authors present an organism similar to a computer. In fact, it is not, and we do not know exactly to what extent the notion about the organism can be reduced to the notion about the machine.

In this work, we logically elaborate on the basic ideas of the entity-set representation of biological objects, which has been performed earlier in the framework of plant morphogenesis (Zhuravlev & Omelko, 2008), and show how they can be exploited to describe the individual development of transformed organisms. The chapter is organized as follows. In the first two sections, we determine which transformation events will be included in our analysis and what the relationship is with the individual development of the object. The next two sections present a description of the operational triad as a means of creating a biological object and as a target for transformation. The fifth and largest section provides an analysis of the changes of individual development, which are induced by transformation in the transgenic organisms. Some examples from experiments with producers of secondary metabolites and from experiments on the re-programming of the somatic cells of plants and animals are scrutinized. In the sixth section, the results of the performed analyses are summarized to allow some predictions and suggestions. Last, the conclusion section draws attention to the complex and convoluted character of the mappings between programmed and phenotypic characters that allows for deterministic and probabilistic manifestations.

2. What is a transformation event?

The problem specified in the title of this section is more complex than it seems. This is well illustrated by the example of attempts to define life itself (Luisi, 1998; Ruiz-Mirazo et al., 2004; Zhuravlev & Avetisov, 2006), and defining transformation is equally as difficult. Of course, we can agree to interpret transformation as a particular biotechnological method, consisting of the construction of recombinant DNA and the subsequent introduction of the resulting structure into a living system, but this interpretation does not coincide with all of the similar phenomena in the wild. This idea relates to questions of whether we consider transformation as an exclusively human invention (i.e., as one of the techniques in the arsenal of our human exploration of reality) or an invention of nature, achieved long before the human mind and related to the arsenal of 'becoming' in the living world? The depth of our understanding of transformation events will depend greatly on what point of view is preferred.

The phenomenon of transformation was discovered in the late 1920s, when F. Griffith established that pneumococcal cells could convert from a harmless form to a disease-causing type. This transformation was heritable, and its "transforming principle" was identified as DNA. Since then, and until very recently, transformation events have been associated with DNA. The Encyclopedia Britannica describes transformation in biology as[2]: 'one of several processes by which genetic material in the form of 'naked' deoxyribonucleic acid (DNA) is transferred between microbial cells. Its discovery and elucidation constitutes one of the significant cornerstones of molecular genetics. The term also refers to the change in an animal cell invaded by a tumour-inducing virus.'

In microbiology, transduction events are accepted as distinguishable and considered separately as a process very similar to transformation where genetic recombination in bacteria results from the incorporation of a fragment of bacterial DNA into the genome of a bacteriophage. Then, during infection, this fragment (together with bacteriophage DNA) is

[2] http://www.britannica.com/EBchecked/topic/602613/transformation

carried into another host cell; this process will be covered in more detail in Section 5.1. The field of transformation is closely connected with genetic engineering, which assumes the development of approaches to manipulate the genetic material of a cell to produce new characteristics in an organism. Genes from plants, microbes, and animals can be recombined (recombinant DNA) and introduced into the living cells of any of these organisms. Organisms that have had genes from other species inserted into their genome (the full complement of an organism's genes) are called transgenic.[3]

The abovementioned examples are positioned inside the scope of transformation, but they illustrate a trend toward a greater differentiation in the scope of transformation. For example, there is a tendency in the understanding of transgenic organisms to exclusively those obtained by so-called gene cloning. However, this differentiation gives rise to restrictions. In the specific case of transgenes, primary meaning is associated with the mechanism of the creation of the transgene, and the definition sets aside the result of that action: whether or not a transformed phenotype was created. We emphasize here that the first transformations were found at the phenotypic level, and therefore, the restriction of the notion of transformation strongly constricts the number of examples of transformation and narrows our outlook on this issue. Based on such a narrow view, it is impossible to determine what constitutes a "transformation event". Assume that we have transformed cells using recombinant DNA and have shown the presence of the inserted fragment in the DNA of recipient cell. Can we consider the event of transformation accomplished? Of course, the appearance of the expected sign inclines us to a positive answer, but what can we say when the recombinant DNA is integrated successfully and the expected observable sign does not appear? Some specific cases of transgene silencing will be discussed below, but here, we note only that the transformation event can be viewed as the action itself: the transformation and its result, the manifestation of a new trait. This is a common situation where a function is identified with the result of its action, and this situation tells us that we are dealing with events that can be modeled as a function or as a map. This circumstance will be used in the next section.

It should be emphasized that to understand the mechanism of the expression of observable traits in the cell and to assess the results of an experimental intervention in the operation of this mechanism, we must take the broadest approach possible. To do this, we must replace the concept of the gene with the concept of a hereditary trait, thus referring to the events in transformation as whether the creation (or loss) of the trait is unusual (or usual) in the antecedent cycles of the development of the individual. A direct consequence of such a change will be that the terms of our consideration will include all of the operations that result in the transformation of an organism, whatever their origin may be. This situation will be complicated by the fact that all types of mutations, lateral transfer and hybridization may fall within such operations. We must, for example, classify in this way the phenomenon of the spread of unrelated traits in plants with pollen (in both experimental and natural conditions, equally). Strictly speaking, according to our viewpoint, all of these events *must* be considered in their relation to transformation in the wide sense. For example, viral diseases must be considered here because they induce heritable changes of phenotypic traits. This effect is especially interesting with regard to functional shifts in the host, and it is appropriate place to remember that RNA-directed DNA methylation was discovered in viroid-infected tobacco plants (Wassenegger et al., 1994).

[3] http://www.britannica.com/EBchecked/topic/463327/plant-disease

However, it is clear that such a broad analysis cannot be the subject of merely a single chapter. Therefore, for our analysis, we have chosen only a few examples of the range of events that relate to the production of transgenic organisms, corresponding to the set $\{P_r, P_d\}$, where P_r and P_d denote the sets of recipient and donor characters, respectively. We will include constant reminders that we have consciously limited the scope of our research and that the events analyzed, in fact, can be assessed using scales of different dimensions. Of course, this is a fairly broad idea, but it is this breadth that allows us to make the necessary generalizations.

3. Schematic representation of the development of multicellular organisms

In describing the process of transformation, schemes and diagrams are a common means of representation, but only the structure of the recombinant DNA is usually represented on the schemes. The second participant (i.e., the object of transformation) is usually absent, apparently due to the complexity of its presentation with the same level of detail as for the recombinant DNA. However, to analyze transformation events in this manner, refinement is not required. We need a level of generalization (modeling) of individual development, which will illustrate the relationships of the host and transforming principle.

All of the possible alternatives for the development of an individual can be reduced to a single module, which is the transition (of cells, tissues, organisms, systems) from the less differentiated state to a more differentiated one. The transition itself can occur as a division, multiplication or death. Consequently, this versatile module can be represented as an expression (1):

$$\{(G,Ph)_s \rightarrow (G,Ph)_f\}, \quad (1)$$

where G indicates genotype, Ph indicates phenotype, and s and f indicate the initial (start) and final states, respectively.

A schematic representation of the plant organism *in vitro* has been published previously (Zhuravlev & Omelko, 2008). Additionally, a rather similar schema, based on the conventional binate idea of genotype-phenotype interactions and also demonstrating EVO:DEVO relationships, has been recently published (Andrade, 2010). Here, we present a modified scheme, which provides the relationship between genotype and phenotype as relationships between sets of inherited and observed properties (Fig. 1).

The scheme in Fig. 1 illustrates the relationship between genotype and phenotype in the rather classical representation, namely from the viewpoint that the genotype, G, is a pre-image of the phenotype, Ph. However, the first attempt to differentially trace the relationships of these two moieties already meets with some difficulties. We cannot trace their transmutations as two parallel lines, where each previous state of G or Ph has been converted into the next state. Instead, every division induces changes in G between two states that correspond to the active and condensed state of chromatin. Only the active state of chromatin opens the possibility for G to be mapped into Ph. This type of mapping is dead-end in some sense.

An absence of direct connections between the states of phenotype is of importance in this schema because, for the external observer, the ontogeny of the object appears as a succession of observables. However, we cannot figure the continuous mapping, such as $Ph_1 \rightarrow Ph_2 \rightarrow Ph_3 \ldots$, as the appearance of every new state of Ph is a result of a rather long route of mappings, each starting from new state of G. The diagram (2) below is not commutative.

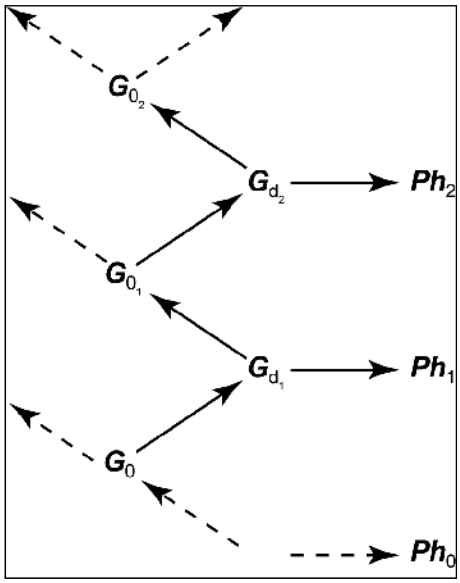

Fig. 1. Scheme of genotype-phenotype interactions during the succession of events in one particular cell line. G_0 corresponds to the compact chromatin state in the course of cell division, and G_d corresponds to the active chromatin state when the expression of the genes responsible for the phenotypic traits is permitted; Ph_i - the phenotypic character.

$$\begin{array}{l} G_1 \rightarrow Ph_1 \\ \searrow \quad \nearrow \\ G_2 \rightarrow Ph_2 \end{array} \tag{2}$$

As we will demonstrate, this idea reflects and explains some of the difficulties of dedifferentiation in experiments on nuclear transfer. We can suppose that this also was a reason why G. Longo and P.-E. Tendero asserted that the existence of empirical correlations between genotypic and phenotypic modifications does not demonstrate the existence of a direct causal relationship between them (Longo, 2009; Longo & Tendero, 2007).

If we use the elaborated diagram (2) and scheme of the development of an individual cell line (Fig. 1) to determine the position of a foreign gene in the transformed genome, we will see that the only place for transgene incorporation is in the set of the oscillating states of G. However, this set is inhomogeneous, and the dynamics of expressions in the transition from G_0 to G_d is not equal to that in the opposite direction because the composition of chromatin, its architecture and the ways of its decoding change in this way. Therefore, in some definite cases, the fate of a transgene will be dependent on its position and the direction of chromatin modeling.

4. Operational triad and individual development

For further analysis of transformation, we require a definition of a biological object. We hypothesized that the definition of a biological object is a task comparable with that for the definition of life itself. Insurmountable obstacles for the latter definition have been reviewed in many publications (Luisi, 1998; Ruiz-Mirazo et al., 2004; Zhuravlev & Avetisov, 2006).

Therefore, we will attempt to define a biological object operationally, in a rather narrow sense, which reflects the individual development of the object. We believe that it will be sufficient to restrict our analytical purpose to the scope of ontogeny. Moreover, we believe that the operational representation of a biological object can be reduced to the analysis of the relationships between the hereditary characteristics of an object and their manifestations; in other words, between the genotype and phenotype. However, the notions of genotype and phenotype have both been subjected to a recent radical revision (Costa, 2008; Fox Keller & Harel, 2007; Gerstein et al., 2007; Snyder & Gerstein, 2003).

With the new molecular knowledge that was partially reviewed in the Introduction section and is partially presented below, it seems problematic to associate the content of an inherited character with a single DNA fragment. As a reflection of the problem, the publications in which the conventional concept of the gene was proposed should be replaced by a "more functional" concept, such as the genon (Scherrer & Jost, 2007) and the deme-bene concept (Fox Keller & Harel, 2007). Within these ideas, the notion of the irreducibility of the content of heritable characters of an organism to a single molecule of DNA has been developed, causing researchers to suggest hereditary mechanisms "beyond the genes" (Amaral & Mattick, 2008; Fox Keller & Harel, 2007), whereas others have defined the genome as an RNA machine (Amaral et al., 2008).

In this context, it seems reasonable to interpret the informational content of DNA and other molecules possessing informational content as a databank or "polytypic library" that causes or begets (in an operational sense) the observable characteristics of the biological object. With this interpretation, hereditary characteristics are not directly associated with certain nucleotide sequences but with a set of characters with respect to which the observable characteristics, such as phenotypic traits, are understood as an operational image of this set. In other words, hereditary characteristics are understood as (remote or direct) operational pre-images of the observable characteristics. However, the cause-and-effect relationships between the pre-images and the observables, even in the case of "typical" transcription/translation, are not (unambiguously) determined. The observable characteristics of the same objects, such as observables of insects during metamorphosis, can vary greatly at different stages of development, while their DNA or other informational structures (pre-images) remains the same. To make the relationship between the pre-image and the image clearer, we must introduce the operational component, which can be attributed neither to pre-image characters nor to the observable characteristics. One can find one of the first rationales of the need for such a similarity of the operating system in Hoffmeyer's early publication, where he stated 'that the conversion of a one-dimensional sequence of symbols, e.g. "DNA inscription" … in a three-dimensional organism' has to be deciphered (Hoffmeyer, 1996, p. 20).

To meet these requirements, we introduce the notion of a function in a broad sense, *F*. Thus, the biological object can be operationally represented by a short universal list:

$$O=(P,F,Ph) \tag{3}$$

The symbol *G*, used above in schemes (2) and (3), is replaced with *P* in expression (3). This may lead to confusion, meaning that we "extract" DNA from the complex image of chromatin to obtain a pure program. Indeed, for our task, we must separate the DNA moiety from the other content of chromatin. However, we interpret the program character more broadly. The programs, *P*, are understood as characteristics of an informational nature

(e.g., instructions or directives) that present the informational content inherent in biological objects. In particular, the programs constitute the operational pre-images of the observables. The most famous, but not the only possible carriers of instructive information, are fragments of DNA that encode proteins. The functions, *F*, are the operations per se that implement interrelationships between the programs, as well as the relationships between the programs and the observables. From the latter, it becomes clear that the part of chromatin released after "DNA extraction" can be considered as a property of *F*.

The observables are the structural and/or functional characteristics of a biological object that can be established by measurements of the object in its interaction with the environment. Phenotypic characteristics of an organism, *Ph*, as observables, are well known, but are not the only possible examples of such characteristics. Therefore, any current state of a biological object is referred to as the particular combination of *P*-, *F*-, and *Ph*-characters (a triad), i.e., the object itself and its current states can be represented by the composition of the following general notations:

$$F: P \rightarrow Ph \tag{4}$$

Such organization allows the object to be represented as both a self-making entity and an element of a self-making entity of higher rank. Similarly, the cell in its operational representation can be considered as an individual entity and as an element of an entity of higher rank (e.g., a tissue or organ). In the biological world, before the appearance of mankind, a biological object was solely executor of any its representation. An object creates itself, displays itself in the surroundings as a successive representation of states of creation (becoming) and interacts with its surroundings in nature.

The triad is understood by us as an operational unit that helps us to represent the individual development as a succession of directed mappings of operational units working in sequential and parallel modes, with cis- and trans-interactions between the units, their compositions, and nests. This can be implemented in the example of a promoter DNA fragment. This fragment can be considered as an argument for several different functions. Particularly, in the course of DNA replication, it is a part of the DNA strand in a chromosome; it is a small part of the bigger argument. However, the promoter can be considered as an entirely independent argument when it is modified by a methyltransferase or other nuclear enzyme. Finally, when specific regulators of transcription, such as protein repressor complexes (Bantignies & Cavalli, 2006), bind to the chromatin, the promoter fragment of DNA can again be considered as part of the larger argument (of collective body of all repressed promoters).

Such manifold representation is characteristic of many informational molecules and their fragments in the cell. A more detailed analysis in this vein requires the assistance of relevant mathematical languages, as was validated in Mossio et al. (2009). To obtain a general image of the diversity of representations characteristic to elements of the triad, we confine ourselves to the following schematic representation (Fig. 2).

The scheme in Fig. 2 is based on the distributed representation of the existence of two functionally different types of cells (germ and somatic) in a multi-cellular organism. Divisions of cells in the germ line are divisions where non-differentiated cells are obtained as a result of the division of pre-existing (meristem or stem) non-differentiated cells. Landscapes of methylation and other labels distributions are very similar in generations of both germ cell lines and meristematic cells. The totipotency of these kinds of cells has been demonstrated in experiments with both plants and animals (Batygina, 2009; Nagy et al.,

1993; Takebe, 1968). Actually, every division in germ cells can be regarded as the identity map or the identity function.

Conversely, the mappings that lead to the creation of observables form a different class of maps, which is performed by different functions. To distinguish between these functions, we introduce the symbols φ and f (see Fig. 2). Both classes of functions can be divided into more-detailed subclasses in accordance with their role in the development of the individual. Thus, the function of the substitution of histones with polyamines in developing sperm can be considered as a subclass of the function φ-class. In turn, functions f_1, f_2, f_3... can be considered as subclasses of functions in different tissues and organs.

It must be emphasized that functional activities corresponding to symbols φ and f are often inconsistent with each other. It can be a consequence of the fact that chromatin expression activity is inconsistent with some stages of the cell cycle (Jacobs, 1995). In contrast to scheme 1 and diagram 2, the relationship between genotype and phenotype is not visible in Fig. 2. This relationship is symbolized by the differential activity of genes in cells in different states (note the different color of the dots in the nuclei). The attempt to represent the relationship in more detail leads to a catastrophic growth in the complexity of the representation, as can be seen in an article representing the genetic landscape of a cell (Costanzo et al., 2010).

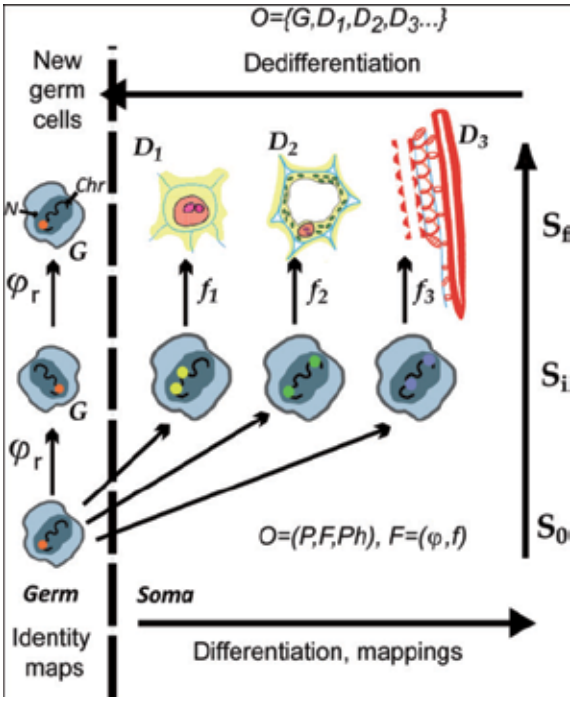

Fig. 2. Scheme illustrating the interrelationships between the elements of the operational triad in two lines of cells in a developing organism. The set of P elements is roughly the same for both germ and somatic cells. However, two different types of functions are associated with these two main directions of cell division in the embryo. Thus, the symbols φ and f are assigned the divisions of the germ and somatic cells, respectively. S_0, S_i and S_f symbolize the initial, intermodal and final states of the object, respectively. The dots of different colors in the nuclei of the cells denote the differential activity of the chromatin.

However, the relationships represented in Fig. 2 are sufficient to argue that these relationships cannot be interpreted as a one-to-one mapping in the majority of cases. Moreover, most of the maps that precede the final (producing the observable) map cannot be expressed in terms of states. The latter entails a specific architecture of the biological object where the set of programs and data are "wrapped" in several shells of specifically arranged mappings. Only the outer shell of observables participates in the contact with surroundings, though many inner layers include the measurable structures that can be identified as observable.

4.1 How many units in dividing cell are portable?

In the beginning of this section, we will emphasize the complex design of chromatin. This complexity is specific to biology because this feature is not an invariant measure, but it develops in the course of the individual development of the object. Moreover, this complex body builds itself of its own accord. During this building, the different structures of chromatin may be interpreted as functions and, thereafter, can be applied to other structures of chromatin as to the arguments. Thus, the repressive complex PRC1 acts as a function for methylated promoter though both, the complex and promoter, are constituents of chromatin. Due to this activity, an inseparable complex of DNA and other chromatin structures carrying general function, *F*, are created.

The idea of the inseparability of DNA and other chromatin structures is not yet generally acknowledged. Researchers and philosophers, seeking to understand the role of DNA in a living organism, have considered the portability feature of DNA as an indication that it belongs to the world of software. For example, G. Longo (2009) writes '…Ending with portability of software: even on different, but suitable environment, a fortiori over identical environments, programs may be repeated at will. And it works'. We contend that this argument is not universal. Any enzyme will work in a suitable environment. The idea of inseparability consists of the affirmation that no single, inseparable part of an object can be extracted from the object (or system) without the complete destruction of the object. In other words, the physical extraction of DNA from the object will destroy this object as an individual.

Of course, naked DNA transferred to another suitable environment (hardware) can realize itself through the interaction with this new environment, but the probability that this realization will result in the creation of an initial object is negligible. Indeed, such is only the case with rather complex entities. We can take numerous examples of the transmission of infectious agents by the naked DNA of viruses and bacteriophages. However, these objects are devoid of individuality hereupon one cannot decide whether the initial object was reproduced or not.

In all cases, when the matter is the division or multiplication of cells, where we have to use the term chromatin, the individuality of the object is connected with the DNA-protein complex. At the current level of knowledge, we cannot exclude the fact that this complex is even more complicated and involves an RNA component (Amaral & Mattick, 2008; Yao, 2008). Of course, these two components (protein and RNA) possess a portable nature.

For example, what is observed in experiments on vegetative plant hybridization? We know that grafting to cold-resistant stock increases the cold resistance of the graft. The graft of a tall plant becomes stunted even if there is a small insertion of the dwarf plant between the root and stem. It is unlikely that we would have reason to believe that these changes in phenotype are associated with the transportability of DNA. The transfer of these properties

can be assumed to involve two other components of chromatin. There is experimental evidence that certain transcription factors (Kim et al., 2002) and short RNAs may be regarded as mobile signaling molecules (Dinger et al., 2008).
The examples of vegetative hybridization discussed here indicate that very distinct and specific phenotypic traits can be created by means of these signaling molecules. However, if this is so, we can no longer assert that all of the specific information is concentrated in the DNA. Thus, we would need to accept the idea that the transportability of the other components of chromatin is relevant for the phenotypic manifestations of living organisms.
We hope that due to this short analysis of chromatin dynamics, we succeeded in the elaboration of the chromatin image as a supramolecular and complex (in chemical sense) body. This body, however, is an indispensible part of the cell and has multifold connections with the rest part of the cell (and organism). Nevertheless, chromatin, as a specific formation of living cell, possesses its own intrinsic topology and dynamics. DNA and some proteins are the regular constituents of chromatin whereas many others signal molecules can incorporate into the system, reactivate and remodel corresponding sites and leave the system. The aggregation of signal molecules with the meaning-bearing fragments of constituent parts can radically convert the functional meaning of these fragments as transmutation functions in arguments. As a result, composition and orientation of operational units in the chromatin body will change too. The individual development of a biological object is, to a large extent, offered by the dynamics of the functional part of chromatin body.

5. Operational triads as targets for transformation

From the operational definition of a biological object established in the previous section, it follows that the individual development can be understood as successions of continuous mappings that build the structure of the object as a complex web, with branchings and the interactions between the operational units. However, these branchings and interactions entangle the web. Even the task of finding one particular fragment of DNA that encodes a necessary protein, among 35,000 such fragments, which together make up scarcely more than 1.5% of the total DNA, cannot be solved by a simple search. This creates a problem that is characteristic of computing science, called data typing, which has some analogs in biology (e.g., blood typing and DNA fingerprinting). In large-scale typing in cell, transposons apparently take part (von Sternberg & Shapiro, 2005). However, in this specific case, DNA typing is a function-oriented process where the typing is performed from the viewpoint (and on behalf) of becoming an operating system. The typed data, P, can be properly treated with the corresponding functions, F, and the results of this processing are handled by other functions. Only after numerous iterations, recursions and destruction of intermediate states are a set of observables, Ph, corresponding to the current state of the object, created.
Only this moment of the creation of a set of observables is critical mapping, in the sense of the creation of Hoffmeyer's three-dimensional organism. However, this critical mapping is preceded by a large and complex task for the proper organization of data and the creation of the necessary operating system. This work remains directly uncommitted in the final mapping, but its role in producing the final observable factor is determinative.
Due to this, any intervention in the structure and organization of the database or in the composition of functions can modify the result of transformation.

5.1 Recombinant DNA carrying one to several genes of known function

The reports described below originated with a study of a phenomenon that Joshua Lederberg and his student Norton D. Zinder termed transduction. In 1952, they revealed that certain bacteriophages were capable of carrying a bacterial gene from one strain of Salmonella to another. Although the capacity of the bacteriophage genome is low and because most bacteriophages can only infect restricted variants of bacterial species, a number of microorganisms that produce important proteins were nonetheless obtained. Worthy of mention are those proteins, hormones, interleukins and other enzymes whose genes are not complex structures and that presented no issues with folding or solubility in the microbe or in the laboratory. These transformations, though producing metabolites unusual for the microbes, ought to be classified as the simplest transformation events because the route from recombinant DNA fragment to observable product is relatively short and because the microbial set of DNA-regulating mechanisms is simple due to their unicellular nature.

Similar results have been obtained in the transformation of plant cells growing in a bioreactor as separated cells or small cell aggregates. However, these cells, their DNA, nucleus and the cell as a whole are equipped with more complex gene expression control mechanisms than bacteria. Furthermore, some intercellular effects can even be observed in thick cell suspensions.

Very often, plant cell transformation is directed at increasing the yield of secondary metabolites that are a characteristic of the plant species. In this case, some evolutionarily developed mechanisms may help the cell to avoid the over-expression of individual genes, including the transgene. A useful way to evade this controlling mechanism is to use viral promoters, which often are unresponsive to host control. This approach protects the transgene from the *cis*-control of the host and from individual *trans*-control, whereas the expression of the transgene can be blocked during the more general form of chromatin remodeling. However, the remodeling of chromatin by such a general transforming principle (e.g., a plant oncogene of a Ti-plasmid) can result in the over-expression of certain sets of host genes (Bulgakov et al., 2009; Zhuravlev et al., 1990). The slow *cis*-control of host DNA (DNA reparation) can destroy the transgene because mutations seem to occur more often in the transgene that in the host DNA (Kiselev et al., 2009).

Several products can be obtained from plant cells through transformation (Godfray et al., 2010). Thus, a certain resonance has been caused by the cultivation of transgenic crops that produce proteins of the human immune system, modified fragments of infectious agents, and other important products of a proteinaceous nature. Important industrial progress has been made in the field of plant protection against pests by the transformation of agricultural crops with the bacillotoxin gene, initially detected in *Bacillus thuringensis.*

The progress of this research was largely determined by the ability of plants to regenerate an entire plant from transformed cells, i.e., it depended on the implementation of the totipotency of plant somatic cells. In cases where the production of somaclones from transformed cells is a complex task, embryogenic culture, embryo or meristem cells are bombarded with microparticles loaded with the corresponding recombinant DNA. Less commonly, the transformation is accomplished by introducing the vector constructs in the course of the "normal" sexual process. This last method is one of few that are suitable for the transformation of multicellular animal species when examples of their somatic embryogenesis are unknown or rare.

The main problem in research of this type is not only the transformation itself but also the regeneration of the transgenic organism from a transformed cell. Until recently, such regeneration from somatic cells was known only in plant systems. Although numerous transformed plants have been obtained by means of somatic embryogenesis, many details of the induction mechanism remain unclear.

We are more interested in the fate of the gene in the context of its relationship with the process of the expression of genetic information of the host cell. A transgene integrated in DNA, as would be assimilated by the database, is indistinguishable from "its own data" in most cases, which (as we shall see below) we cannot say about the transfer of a nucleus. However, in mixed populations, the transformed cells and organisms may differ from untransformed ones by such factors as growth rate and sensitivity to abiotic factors (i.e., being subject to selection). The best results can be obtained when the selection scheme includes the production of cellular clones, such as in the following scheme:

a cell suspension enriched with super-producers → single-cell suspension → the seeding on solid media of a highly diluted suspension → the selection of colonies originating from a single cell → receipt and comparison of clonal lines.

This means that the cell population must originate from a single cell. However, this choice is fraught with the unlikelihood that a uniform population will be obtained with the same resistance characteristics for all of the cells that may result in the decline of the population stability.

5.2 Transfer of the nucleus (nuclear transplantation)

As it is understood in biotechnology and classical genetics (*i.e.* offspring production of one and the same cell), cloning does not belong to the events of transformation. The term cloning, which is often used in the procedure for the reprogramming of a nucleus of the somatic cells of large animals, is used inappropriately, whereas the terms regeneration and hybridization are more adequate in most cases. However, some attempts toward the regeneration of adult animals from somatic cells, including a procedure of transformation with recombinant molecules or nucleus transfer, in the broadest sense, can also be viewed as a transformation. The first phase of experiments with somatic cells is represented by attempts to reprogram somatic cells as a result of a merger with an undifferentiated cell (often an oocyte), enucleated or with an inactivated nucleus (Jaenisch & Gurdon, 2007).

In plant biotechnology, such manipulations are called somatic or parasexual hybridization. Well before the end of the twentieth century, the problem of dedifferentiation (reprogramming) was shown to be less acute in experiments with the cells of higher plants, so the studies were primarily conducted as examples of inter-specific hybridization (Gleba & Sytnik, 1984). However, these experiments have only had a limited theoretical yield. As far as we know, the hybrid Brassica napus was constructed in this way.

In the biotechnology of vertebrates, The Encyclopedia Britannica (2011) cites experiments performed by the British molecular biologist J.B. Gurdon as one of the landmark studies in this direction. J.B. Gurdon transplanted a mature nucleus from an intestinal cell of a tadpole into an enucleated egg of a frog, which subsequently developed into a normal, adult frog. 'Gurdon thus demonstrated that a highly differentiated intestinal cell nucleus, with only intestinal cell genes functioning, could undifferentiate in the environment of the enucleated egg cell and could reactivate those genes necessary to create an entire frog. The frog that was produced was a "clone" in the sense that the entire genome of the donor tadpole was

present in all the cells of the newly formed frog'[4]. For a more detailed history and the recent state of the field, see Jaenisch & Gurdon (2007).

Approximately 20 years later, reports began appearing about the successful development of transplanted mammalian nuclei. In these experiments, the acceptor cell usually originated from germ line cells; more often, it was an oocyte. After fusion, successfully induced embryos were transferred into a surrogate female to promote the full-term development of the new animal. Just as with frogs, the obtained organisms are incorrectly called clones (see, for example, Eggan et al. 2001, 2004).

From the view of knowledge obtained in experiments on plant cells, all of the experiments to obtain "clones" of large animals should be classified as a special case of intra-specific somatic hybridization, where hybridization with undifferentiated enucleated cells was used for the induction of totipotency of a differentiated donor nucleus. All of the examples where the nucleus was taken from one animal and an undifferentiated enucleated cell from another animal are essentially examples of the receipt of a chimeric genotype because cytoplasmic hereditary factors belong to the acceptor cell. For this reason, it is logical to expect that the hybrid offspring would not be an exact copy of the donor; just this has been observed in practice.

The most significant differences of the phenotypic "somatic" descendant from the nucleus-donor phenotype can be assumed to be associated with cytoplasmic hereditary factors, chief of which are the mitochondria. In endothermal animals, mitochondria are inherited through the maternal line. Therefore, when the donor of a somatic nucleus is a male, genome mixing is inevitable because the oocyte can only be obtained through the female line.

However, this is only a *design* part of the problem, whereas a *functional* part also exists. It is important to remember the following details in the technique of cell hybridization (Egli et al., 2007): for the successful induction of embryogenic development of the hybrid cell, the phase of the cell-cycle in which the enucleation of acceptor cell was performed is important. Notably, the state of the nuclear membrane and the level of compactness of chromatin are decisive. The authors believe that the 'removal of the pronuclei during enucleation would deplete these factors and prevent development. In contrast, removal of the condensed chromosomes from a metaphase II, meiotic egg would not do so' (Egli et al., 2007, p.683).

It suggests that some components of the cell-acceptor nucleus, which passed into the cytoplasm in this stage (see subsection 4.1) and, therefore, had the opportunity to interact with the transplanted nucleus, were needed for reprogramming the donor chromatin. Among such important factors, methyltransferase Dnmt1 is suspected. This enzyme is present in the nucleus in the course of one cell cycle only and then is removed from it (Surani & Reik, 2007). This, or some other, transferase can be crucial for the establishment of the specific architecture of donor chromatin as making it available for further control during embryogenesis. In one viewpoint, the post-nuclear transfer development of the hybrid cell can be understood as an interaction between the transplanted nucleus with a soluble part of the acceptor chromatin (i.e., *as an interaction of the nucleus with the nucleus*, which, of course, contradicts the intention of the experiment). It is also important to remember that many successful experiments with nuclear transfer in frogs were performed by the UV inactivation of the recipient nucleus, and such inactivation does not exclude the possibility that some low molecular weight chromatin structure of the recipient remained intact and played some role in reprogramming the donor nucleus.

[4] http://www.britannica.com/EBchecked/topic/262934/heredity

The number of transcription factors required for the induction of embryonic development in mammals has recently been reduced to three (Ficz et al., 2009). However, we have no reason to believe that these transcription factors (and only these transcription factors) were released into the acceptor cytoplasm. Taking into account the possibility that their first "products" can be enzymes of DNA demethylation (Bhutani et al., 2010; Popp et al., 2010), we cannot exclude the possibility that these enzymes are themselves passed into the cytoplasm.
For the hybridization of animal somatic cells, it has been assumed that the donor DNA, as the database, remains intact. Therefore, the main problem is to create a new operating unit, which would start operating from scratch. However, operating from scratch is impossible because the chromatin of the transplanted nucleus is a complex combination of different structures (see above sections), and even the DNA in transplanted chromatin is quite different from that in a zygote. Using the language of artificial intelligence, the problem includes the following: i) a database state that is inappropriate to the initial one, and ii) the remnants of the previous operational structure that support the state available at the moment of nuclear transfer. There is reason to believe that the operating structure is presented mainly by the short-lived regulatory elements. In addition, continuous division, being the basis of development and differentiation, may lead to the rapid dilution of relatively stable regulatory elements. In this respect, experiments on secondary embryogenesis in some plants, rarely giving somaclonal variants, are very informative. For instance, for ginseng (*Panax ginseng* C.A. Meyer), it is relatively easy to obtain vegetative shoots *in vitro*, but they root poorly, presumably because the required level of dedifferentiation in somatic cells was not reached. We can suppose that these effects were associated with DNA demethylation, as demonstrated in other experiments (Bhutani et al., 2010; Laurent et al., 2010; Popp et al., 2010). However, if the ginseng embryoids deficient in root formation were transplanted to a new medium, then after a short period of callus growth, the secondary embryos would develop into normal plants with roots. The molecular mechanism of root initiation was recently revealed to be connected with a movable agent (Schlereth et al., 2010). Thus, additional divisions can result in a decrease in the level of methylation of DNA or in the dilution of some hypothetic factor(s) blocking root initiation. In *Arabidopsis* embryogenesis, an extra-embryonic cell (of the suspensor) is specified to become the founder cell of the primary root meristem, the hypophysis, in response to signals from adjacent embryonic cells (Schlereth et al., 2010). In the course of somatic embryogenesis, no suspensor structure can usually be specified in the callus cells around the embryo (Batygina, 2009). Consequently, two competent cells have to occur in contact to give rise to the somatic embryo and the extra-embryonic root meristem. Of course, this contact is easier when a movable signal exists. Similar events may have taken place in the experiments with vertebrates, where the improvement of embryo formation was obtained due to a procedure known as ”serial cloning”.

5.3 Transformation instead of transplantation

It is known and was reviewed above that the induction of embryo development is a critical phase in experiments on nuclear transfer (Bhutani et al., 2010; Ficz et al., 2009). In this regard, the induction of embryogenesis in plants with phytohormones, antitubulin factors and abiotic factors seems to be more understandable (Zhuravlev & Omelko, 2008). Nevertheless, the problem of induction of embryogenesis remains a major issue for biotechnology in both animal and plant cells. It is particularly urgent in cases that require the generation of a transformed organism in which all of the cells are modified. Currently,

this can only be achieved through the induction of embryogenesis in a separate transformed cell. If the transformant is not a zygote, the difficulties of its induction to embryogenic development may become an insurmountable obstacle to obtaining a transgenic organism. Even in the biotechnology of plant cells, we are faced with fundamental difficulties. For example, the possibility of somatic embryogenesis in such important cultivated plants as soybeans (for a review, see Dos Santos et al., 2006) is severely limited. However, many obstacles impede the application of somatic embryogenesis in the biotechnology of economically important animals. Above, we discussed the problems associated with this so-called cloning. Here, we focus on receiving the reprogrammed fibroblasts through their transformation. As we show below, this process can also be interpreted as applying an induction function, but it is a significantly more economical and accurate attempt than in nuclear transplantation.

The direction of this research came in 2006, after K. Takahashi and S. Yamanaka demonstrated that transformation with vectors carrying genes encoding the Oct4, Sox2, c-Myc and Klf4 transcription factors induced the transformation of mouse fibroblasts into pluripotent stem cells (induced pluripotent stem [iPS] cells). These factors play an important role in the early stages of embryogenesis. Thus, the Oct3/4 and Sox2 genes encode regulatory proteins required for the maintenance of the properties of stem cells (induced in zygote and embryonic tissues). The c-Myc and Klf4 factors maintain the self-renewal of stem cells, and Klf4 also increases the level of Oct4. After the experiments by Takahashi and Yamanaka (2006), similar experiments were performed (Okita et al., 2007; Wering et al., 2007), which successfully demonstrated live-born chimeras using an injection of mixed iPS cells into blastocysts. It became clear that there was a new prospect for cloning, namely the replacement of the nuclear transfer procedure for the induction of somatic embryogenesis from minimally differentiated cells of the connective tissue of animals (i.e., fibroblasts). It should be noted that the wound tissue of plants, callus, is even less differentiated, and the use of this tissue in plant biotechnology has yielded progress in the attainment of somaclonal and gametoclonal variants. Therefore, we expect that this model (induced fibroblasts) will lead to comparable success, though the calli of plants and the connective tissue of animals should not be equated.

Nevertheless, the few achievements obtained with this model confirmed its promise. There is, however, one major difficulty. Transformation, causing an induction, can be considered as the creation of a certain intermediate operating unit, whose fate is not indifferent to the further development of the transformed cells. If it continues to function, it can become an obstacle to further development. We are faced with a similar problem when using the transformation of callus cells with the *rol*C gene to induce embryogenesis in ginseng. The transformed cells are successfully induced and form an embryo-like structure. However, without reaching the torpedo stage, the structures revert to embryogenesis, now secondary, and the cycle is repeated over many passages (Gorpenchenko et al., 2006). We understand the results of this experiment as follows. Under the influence of *rol*C, cells are exposed to dedifferentiation and take the form and properties of embryonic cells. The inductive effect is, however, permanent. With the development of the embryo, and in the course of differentiation, these cells are again faced with factors produced by the recombinant DNA causing the induction. As a result, they revert to an undifferentiated state and, thus, give rise to a new cycle of embryogenesis. In terms of programming, one can say that the operational structure causing the induction is extremely stable, and its function is performed whenever the cells reached a certain degree of differentiation.

Although the molecular mechanism of the induction of embryogenesis with the transformation by *rol* genes or transcription factors genes is still not clear, the fundamental aspect of the matter is plain enough. In addition, the significance of these results for planning experiments on morphogenesis is also obvious: when transformation is used for induction, the transformation must be temporary. How can we halt or dilute the inducing signal?

The number of induction (parental) factors in a "normal" zygote can be assumed to be limited and reduced with each division (perhaps by half). A rapid dilution eliminates the possibility of secondary induction. However, what about a situation where (at this stage of knowledge) transformation seems the only possibility for the implementation of an induction? We see the solution in the creation of short-lived vectors unable to integrate into the host DNA (Zhuravlev & Omelko, 2008). The creation of such structures in plants (e.g., the use of attenuated tobacco mosaic virus, containing the mRNA from transcription factors genes) can be used as a method in the induction of embryogenesis in crop plants, whose somaclonal variants are difficult to obtain.

A successful implementation has been achieved by K. Kaji et al. (2009) in their attempt to obtain the virus-free induction of pluripotency in mouse and human fibroblasts and a subsequent excision of reprogramming factors. A drug-inducible lentiviral reprogramming strategy has also been designed to achieve the tight control of transgene expression in iPS cells (Boland et al., 2009). In this work, the four original reprogramming factors (Oct4, Sox2, Klf4 and c-Myc) were placed under the control of the tetO promoter, which is activated by the reverse tetracycline transactivator (rtTA) protein in the presence of the tetracycline analogue doxycycline (dox). This construct makes possible the control of the level of expression of reprogramming factors.

However, the task of managing the development of an individual is not always reduced to the necessity of the rapid dilution or direct removal of initiation factors; occasionally, the opposite is necessary. The low content of such factors in the zygote has apparently caused the failure of attempts to split the embryos of cattle. If this is predominantly due to the lack of transcription factors it is possible that their direct injection into blastomeres will be decisive in the embryo splitting technique for rapid breeding of cattle.

6. The problems of transformation and some routes to success

This section concerns the question of the unpredictability of the results of transformation and, in more general terms, the uncertainty and unpredictability of the individual development of any organism. This question in the aspect of embryogenesis in plants *in vitro* has been partially discussed in a previous publication (Zhuravlev & Omelko, 2008).

Those working in biotechnology are often faced with a problem when a transformation does not produce the desired result and the target product accumulates only in small amounts. These phenomena are associated with the uncertainty of the location of transgene insertion, variations in the number of integrated copies and the active defense of the cell against the expression of foreign DNA fragments. The first two facts are usually clear and established by experiments. With regard to the protective measures, there are less data, and these data are more difficult to interpret. Indeed, the number of mutations in the DNA inserts from plasmids is significantly above the average mutation rate in the non-transformed DNA of ginseng (Kiselev et al., 2009).

Perhaps these considerations may have a more general nature, and the very unpredictable results of transformation may be associated with the uncertainty of the developmental paths

of the transformed organism. Another basis for the unpredictability of the results of morphogenesis *in vitro* can often be the indeterminate number of induced cells. The point here is that when the induction covers not just one but a number of neighboring cells, each characterized by different states of chromatin activity, the overall result is difficult to predict. Simultaneous operation of many sources of signal molecules complicates the model and deprives its predictive power.

Another important condition that leads to unpredictability in plant morphogenesis is the low level of specificity of the signal that induces the start of the morphogenetic transformations. Such signals in plants may be various biotic and abiotic factors, such as stress, phytohormones, a shift of the ionic environment, or an electrical impulse. To induce morphogenesis in animal cells *in vitro*, most of these factors have proven to be ineffective, indicating the greater specificity of the primary signal in animal cells. The structural features of plant cells, especially the cytoskeleton, predispose the cell to the perception of external signals of an abiotic nature (see review in Rowat et al., 2008). This structural feature, among others, likely makes the induction of morphogenesis in plants easier but less selective in comparison with the systems of an animal nature. The induction of somatic embryogenesis in animals, with the many known limitations, requires more specific endogenous inducers. In addition to transcription factors, which have already been mentioned, transcribed and non-transcribed RNAs of the mother organism may be such highly specific inducers, which are deposited in the egg and direct the early stages of development. Maternal RNA degrades during embryogenesis and is replaced by zygotic RNA as the transcription of zygotic genes is triggered. Before this, the zygotic genome in animals is transcriptionally inactive (Amaral & Mattick, 2008; Schier, 2007; Yao, 2008).

Taking these facts into consideration, it is logical to expect that the unpredictability of results of transformation is associated with the indirect mode of mapping the genotype on the phenotype, which was apparently a historical necessity because the organisms were placed in front of an intractable task to increase and diversify the phenotype without significantly increasing the genotype. In artificial intellect programs, this problem occurs very rapidly. One possible solution may be to use an indirect genetic encoding that takes the form of a developmental process (Nowacki et al., 2008; Roggen et al., 2007).

In this chapter, we developed a dual idea representing the organism as the following: i) an integral, indivisible entity, and ii) a complex construction in which three functionally entangled bodies can be abstracted, namely, the *P*, *F* and *Ph* sets. The task to describe the relationships of these sets resembles the Poincare's three-body problem, which was recently analyzed by G. Longo (2009) in its relation to biological objects.

7. Conclusion

The current shift of paradigm in the science of complex systems has affected our view of biological object, its development and its transformation. Depending on the construct used, transformation can relate to different intracellular processes, which are characterized by non-linearity and self-referencing, self-construction and self-modification. The problem now is how long distance is between the introduced DNA fragment and the desirable phenotypic character and how many side effects will be induced on this route.

This route is short only in the simplest unicellular organisms. In more complex organisms and especially in those organisms that manifest individuality, the route includes numerous iterations and alternatives, which are incompatible with the idea of predictability of results

of transformation. In situation where the transformation aims for induction (of embryogenesis) only, the problem of eliminating the transforming agent after the execution of its signal function necessarily arises. However, recent publications have given us confidence that these problems can be solved in the biotechnology of both mammals and seed plants.

Transformation, with the aim to produce a specific product, has been more successful when the site of incorporation of the transforming agent is closer to the final map in observables. In this case, however, there are some additional uncertainties. First, the introduction of one or two genes is not always sufficient to produce the final product. Second, the over-accumulation of the product may be harmful to the host (the conflict of phenotypes). Third, the conditions of the host may be inconsistent with the demands for correct labeling and folding of target product (usually of protein nature) and, therefore, inactivate it.

In addition to these features, there is another uncertainty of a more common nature, which is peculiar to the individual development of biological systems in general. It lies in the fact that there is not a strict determinism in the ways in which and in what order the information of a DNA fragment to its phenotypic embodiment will be realized. Nevertheless, there are apparently still some obscure channels that allow a biological object to successfully realize its individual development by the creation of the phenotype with properties similar to those expected.

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9. References

Abi-Haidar, A., Kaur, J., Maguitman, A., Radivojac, P., Retchsteiner, A., Verspoor, K., Wang, Z. & Rocha, L.M. (2007). Uncovering Protein-Protein Interactions in the Bibliome. *Proceedings of the Second BioCreative Challenge Evaluation Workshop*, pp. 247-255, ISBN 84-933255-6-2, Madrid, Spain, April 23-25, 2007.

Adami, C. (2002). What is Complexity? *BioEssays*, Vol.24, N12, (December 2002), pp. 1085–1094, ISSN 1521-1878.

Allis, C.D., Jenuwein, T. & Reinberg, D. (Eds.). (2007). *Epigenetics,* CSHL Press, ISBN 0879697245, New York, U.S.A.

Amaral, P., Dinger, M., Mercer, T. & Mattick, J. (2008). The Eukaryotic Genome as an RNA Machine. *Science*, Vol.319, N.5871, (28 March 2008), pp. 1787-1789, ISSN 0036-8075.

Amaral, P. & Mattick, J. (2008). Noncoding RNA in Development. *Mammalian Genome*, Vol.19, N.7-8, (August 2008), pp. 454-492, ISSN 0938-8990.

Andrade, E. (2005). The Interrelations between Genotype/Phenotype/Environment: A Semiotic Contribution to the Evo:Devo Debate. *S.E.E.D. Journal (Semiotics, Evolution, Energy, and Development),* Vol.5, N.2, (December 2005), pp. 27-65.

Bantignies, F. & Cavalli, G. (2006). Cellular Memory and Dynamic Regulation of Polycomb Group Proteins. *Current Opinion in Cell Biology,* Vol.18, N3, (June 2006), pp. 275-283, ISSN 0955-0674.

Barabasi, A. L., Albert, R. & Jeong, H. (2000). Scale-Free Characteristics of Random Networks: the Topology of the World-Wide Web. *Physica A: Statistical Mechanics and its Applications*, Vol.281, N1-4, (15 June 2000), pp. 69-77, ISSN 0378-4371.

Batygina, T.B. (Ed.) (2009). *Embryology of Flowering Plants: Terminology and Concepts. Reproductive Systems*. Science Publisher Inc., ISBN 978-1-57808-265-0, U.S.A.

Bersini, H. (2009). How artificial life relates to theoretical biology, In: *Origins of Life: Self-Organization and/or Biological Evolution?* M. Gérin and M.-C. Maurel (Eds.), 61–78, EDP Sciences, ISBN: 978-2-7598-0476-4, Paris, France.

Bhutani, N., Brady, J., Damian, M., Sacco, A., Corbel, S. & Blau, H. (2010). Reprogramming Towards Pluripotency Requires AID-dependent DNA Demethylation. *Nature,* Vol.463 N7284, (25 February 2010), pp. 1042-1047, ISSN 0028-0836.

Boland, M., Hazen, J., Nazor, K., Rodriguez, A., Gifford, W., Martin, G., Kupriyanov, S. & Baldwin, K. (2009). Adult Mice Generated from Induced Pluripotent Stem Cells. *Nature*, Vol.461, N7260, (3 September 2009), pp. 91-94, ISSN 0028-0836.

Bulgakov, V.P., Inyushkina, Y.V., Gorpenchenko, T.Y., Koren, O.G., Shkryl, Y.N. & Zhuravlev, Y.N. (2009). Emerging Roles of Agrobacterial Plant-Transforming Oncogens in Plant Defence Reaction. *AIP Conference Proceedings*, Vol.1089, (12 January 2009), pp. 94-103, ISBN 978-0-7354-0622-3.

Cárdenas, M.L., Letelier, J.C., Gutierrez, C., Cornish-Bowden, A. & Soto-Andrade, J. (2010) Closure to Efficient Causation, Computability and Artificial Life, *Journal of Theoretical Biology*, Vol.263, N1, (March 2010), pp. 79-92, ISSN 0022-5193.

Costanzo, M., Baryshnikova, A., Bellay, J., Kim, Y., Spear, E.D., Sevier, C.S., Ding, H., Koh, J.L.Y., Toufighi, K., Mostafavi, S., Prinz, J., St.Onge, R.P., VanderSluis, B., Makhnevych, T., Vizeacoumar, F.J., Alizadeh, S., Bahr, S., Brost, R.L., Chen, Y., Cokol, M., Deshpande, R., Li, Z., Lin, Z.Y., Liang, W., Marback, M., Paw, J., San Luis, B.J., Shuteriqi, E., Tong, A.H.Y., van Dyk, N., Wallace, I.M., Whitney, J.A., Weirauch, M. T., Zhong, G., Zhu, H., Houry, W.A., Brudno, M., Ragibizadeh, S., Papp, B., C. Pal, C., Roth, F.P., Giaever, G., Nislow, C., Troyanskaya, O.G., Bussey, H., Bader, G.D., Gingras, A.C., Morris, Q.D., Kim, P.M., Kaiser, C.A., Myers, C.L., Andrews, B.J. & Boone, C. (2010). The Genetic Landscape of a Cell. *Science*, Vol.327, N5964, (22 January 2010), pp. 425-431, ISSN 0036-8075.

Costa, F.F. (2008) Non-coding RNAs, epigenetics and complexity, *Gene*, Vol.410, N1, (29 February 2008), pp. 9–17, ISSN 0378-1119.

de Vries, S.C. (1998) Making Embryos in Plants. *Trends in Plant Sciences,* Vol.3, N12, (1 December 1998), pp. 451-452, ISSN 1360-1385.

Dinger, M.E., Mercer, T.R. & Mattick, J.S. (2008). RNAs as Extracellular Signaling Molecules. *Journal of Molecular Endocrinology,* Vol.40, N4, (April 2008), pp. 151-159, ISSN 0952-5041.

Dos Santos, K.G.B., de Araujo, M.J.E., Moco, M.C.C. & Bodanese-Zanettini. M.H. (2006). Somatic Embryogenesis from Immature Cotyledons of Soybean (Glycine max (L.) Merr.): Ontogeny of Somatic Embryos. *Brazilian Archivs of Biology and Technology,* V. 49. N1, (January 2006), pp. 49–55, ISSN 1516-8913.

Eggan, K., Akutsu, H., Loring, J., Jackson-Grusby, L., Klemm, M., Rideout 3rd, W.M., Yanagimachi, R. & Jaenisch, R. (2001). Hybrid Vigor, Fetal Overgrowth, and Viability of Mice Derived by Nuclear Cloning and Tetraploid Embryo Complementation. *Proceedings of the National Academy of Sciences U.S.A.*, Vol.98, N11, (May 2001), pp. 6209-6214, ISSN 0027-8424.

Eggan, K., Baldwin, K., Tackett, M., Osborne, J., Gogos, J., Chess, A., Axel, R. & Jaenisch, R. (2004) Mice Cloned from Olfactory Sensory Neurons. *Nature,* Vol.428, N6978, (4 March 2004), pp. 44 -49, ISSN 0028-0836.

Egli, D., Rosains, J., Birkhoff, G. & Eggan, K. (2007). Developmental Reprogramming After Chromosome Transfer into Mitotic Mouse Zygotes. *Nature*, Vol.447, N7338, (7 June 2007), pp. 679-686, ISSN 0028-0836.

Ferguson-Smith, A.C., Greally, J.M., Martienssen, R.A. (Eds.). (2009). *Epigenomics,* Springer, ISBN: 978-1-4020-9186-5.

Ficz, G., Farthing, C.R. & Reik, W. (2009). The Epigenomic Landscape of Reprogramming in Mammals. In: *Epigenomics,* A.C. Ferguson-Smith, J.M. Greally, R.A. Martienssen (Eds.), 259-282, Springer, ISBN: 978-1-4020-9186-5.

Fox Keller, E. & Harel, D, (2007). Beyond the Gene. *PLoS ONE*, Vol.2, N11, (28 November 2007), e1231, eISSN-1932-6203.

Gerstein, M.B., Bruce, C., Rozowsky, J.S., Zheng, D., Du, J., Korbel, J.O., Emanuelsson, O., Zhang, Z.D., Weissman, S. & Snyder, M. (2007). What is a Gene, Post-ENCODE? History and Updated Definition. *Genome Research*, Vol.17, N6, (June 2007), pp. 669-681, ISSN 1088-9051.

Gleba, Y.Y., Sytnik, K.M. (1984). *Protoplast fusion: Genetic engineering in higher plants*. Springer-Verlag, ISBN 3540132848, Berlin-Heidelberg-New York-Tokyo.

Godfray, H.C., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M. & Toulmin, C. (2010). Food Security: The Challenge of Feeding 9 Billion People. *Science*, Vol.327, N5967, (12 February 2010), pp. 812-818, ISSN 0036-8075.

Gorpenchenko, T.Y., Kiselev, K.V., Bulgakov, V.P. Tchernoded, G.K., Bragina, E.A., Khodakovskaya, M.V., Koren, O.G., Batygina, T.B. & Zhuravlev, Yu.N. (2006). The *Agrobacterium rhizogenes* rolC-Gene-Induced Somatic Embryogenesis and Shoot Organogenesis in *Panax ginseng* Transformed Calluses. *Planta*, Vol.223, N 3, (February 2006), pp. 457–467, ISSN 0032-0935.

Hoffmeyer, J. (1996). *Signs of Meaning in the Universe*. Indiana University Press, ISBN 0-253-33233-8, Bloomington, U.S.A.

Hoffmeyer, J. (2008). *Biosemiotics: An Examination into the Signs of Life and the Life of Signs*. University of Scranton Press, ISBN: 9781589661691, Scranton PA, U.S.A.

Jacobs, T. (1995). Cell Cycle Control. *Annual Review of Plant Physiology and Plant Molecular Biology*, Vol.46, N, (June 1995), pp. 317–339, ISSN 1040-2519.

Jaenisch, R. & Gurdon, J. (2007). Nuclear Transplantation and and the Reprogramming of the Genome. In: *Epigenetics,* Allis, C.D., Jenuwein, T. & Reinberg, D. (Eds.). CSHL Press, ISBN 0879697245, New York, U.S.A.

Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P. & Woltjen, K. (2009). Virus-free Induction of Pluripotency and Subsequent Excision of Reprogramming Factors. *Nature*, Vol.458, N7239, (9 April 2009), pp. 771-775, ISSN 0028-0836.

Kauffman, S., Logan, R., Este, R., Goebel, R., Hobill, D. & Shmulevich, I. (2008) Propagating organization: an enquiry. *Biology and Philosophy*, Vol.23, N1, (January 2008), pp. 27-45, ISSN 0169-3867.

Kim, J.Y., Yuan, Z., Cilia, M., Khalfan-Jagani, Z. & Jackson, D. (2002). Intercellular Trafficking of A KNOTTED1 Green Fluorescent Protein Fusion in the Leaf and Shoot Meristem of *Arabidopsis*. *Proceedings of the National Academy of Sciences U.S.A.*, Vol.99, N6, (19 March 2002), pp. 4103–4108, ISSN 0027-8424.

Kiselev, K.V., Turlenko, A.V., Tchernoded, G.K. & Zhuravlev, Yu.N. (2009). Nucleotide Substitutions in *rolC* and *nptII* Gene Sequences During Long-term Cultivation of *Panax ginseng* Cell Cultures. *Plant Cell Reports*, Vol.28, N8, (August 2009), pp. 1273-1278, ISSN 0721-7714.

Laurent, L., Wong, E., Li, G., Huynh, T., Tsirigos, A., Ong, C., Low, H., Sung, K., Rigoutsos, I., Loring, J. & Wei, C. (2010). Dynamic Changes in the Human Methylome During Differentiation. *Genome Research*, Vol.20, N3, (March 2010), pp. 320–331, ISSN 1088-9051.

Longo, G. (2010). Incomputability in Physics and Biology. Extended and revised version of an Invited Lecture, 6th Conference on Computability in Europe, CiE 2010, Ponta Delgada, Azores, Portugal, June 30 – July 4, 2010.

Longo, G. (2009). Randomness and Determination, from Physics and Computing towards Biology. *Lecture Notes in Computer Science*, Vol. 5404, pp. 49-61, ISSN 0302-9743.

Longo, G. & Tendero, P.-E. (2007). The Differential Method and the Causal Incompleteness of Programming Theory in Molecular Biology. In *Foundation of Science*, Vol.12, N4, (December 2007), pp. 337-366, ISSN 1233-1821.

Luisi, P. (1998). About Various Definitions of Life. *Origins of Life and Evolution of Biospheres*, Vol.28, N4-6, (October 1998), pp. 613–622, ISSN 0169-6149.

Mattick, J., Amaral, P., Dinger, M., Mercer, T. & Mehler, M. (2009). RNA Regulation of Epigenetic Processes. *BioEssays*, Vol.31, N1, (January 2009), pp. 51–59, ISSN 1521-1878.

Morgan, H.D., Santos, F., Green, K., Dean, W. & Reik, W. (2005). Epigenetic Reprogramming in Mammals. *Human Molecular Genetics,* Vol.14, Siuppl.1, (15 April 2005), R47–R58, ISSN 0964-6906

Mossio, M., Longo, G. & Stewart, J. (2009). An Expression of Closure to Efficient Causation in Terms of Lambda-calculus. *Journal of Theoretical Biology*, Vol.257, N3, (7 April 2009), pp. 489-498, ISSN 0022-5193.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. & Roder, J.C. (1993). Derivation of Completely Cell Culture-derived Mice from Early-passage Embryonic Stem Cells. *Proceedings of the National Academy of Sciences U.S.A.*, Vol.90, N 18, (15 September 1993), pp. 8424–8428, ISSN 0027-8424.

Okita, K., Ichisaka, T. & Yamanaka, S. (2007). Generation of Germline-competent Induced Pluripotent Stem Cells. *Nature*, Vol.448, N7151, (19 July 2007), pp. 313–317, ISSN 0028-0836.

Pentris, S., Hunt, T. & Tooze, J. (Eds.) (1983). *DNA Makes RNA Makes Protein*. Elsevier, ISBN 0444804919, U.S.A.

Popp, C., Dean, W., Feng, S., Cokus, S.J., Andrews, S., Pellegrini, M., Jacobsen, S.E. & Reik, W. (2010). Genome-wide Erasure of DNA Methylation in Mouse Primordial Germ

Cells Is Affected by AID Deficiency. *Nature*, Vol.463, N 7284, (22 January 2010), pp. 1101-1105, ISSN 0028-0836.

Rodríguez-Trelles, F., Tarrío, R. & Ayala, F.J. (2006). Origins and Evolution of Spliceosomal Introns. *Annual Review of Genetics*, Vol.40, pp. 47-76, ISSN 0066-4197.

Roggen, D., Federici, D. & Floreano, D. (2007). Evolutionary Morphogenesis for Multi-cellular Systems. *Genetic Programming and Evolvable Machines*, Vol.8, N1, (March 2007), pp. 61–96, ISSN 1389-2576.

Rowat, A., Lammerding, J., Herrmann, H. & Aebi, U. (2008). Towards an Integrated Understanding of the Structure and Mechanics of the Cell Nucleus. *BioEssays,* Vol.30, N3, (March 2008), pp. 226–236, ISSN 1521-1878.

Ruiz-Mirazo, K., Peretó, J. & Moreno, A. (2004). A Universal Definition of Life: Autonomy and Open-Ended Evolution. *Origins of Life and Evolution of Biospheres,* Vol.34, N3, (June 2004), pp. 323-346, ISSN 0169-6149.

Scherrer, K. & Jost, J. (2007). Gene and Genon Concept: Coding Versus Regulation. *Theory in Biosciences,* Vol.126, N2-3, (October 2007), pp. 65-113, ISSN 1431-7613.

Schlereth, A., Möller, B., Liu, W., Kientz, M., Flipse, J., Rademacher, E.H., Schmid, M., Jürgens, G. & Weijers, D. (2010). MONOPTEROS Controls Embryonic Root Initiation by Regulating a Mobile Transcription Factor. *Nature*, Vol.464, N 7290, (8 April 2010), pp. 913-917, ISSN 0028-0836.

Schier, A.F. (2007). The Maternal-Zygotic Transition: Death and Birth of RNAs. *Science*, Vol.316, N 5823, (20 April 2007), pp. 406-407, ISSN 0036-8075.

Snyder, M. & Gerstein, M. (2003). Defining Genes in the Genomics Era. *Science*, Vol.300, N5617, (11 April 2003), pp. 258–260, ISSN 0036-8075.

von Sternberg, R. & Shapiro, J.A. (2005). How Repeated Retroelements Format Genome Function. *Cytogenetic and Genome Research*, Vol.110, N1-4, (), pp. 108-116, ISBN 978-3-8055-7964-3.

Takahashi, K. & Yamanaka, S. (2006). Induction of Pluripotent Stem Cells From Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell*, Vol.126, N4, (25 August 2006), pp. 663-676, ISSN 0092-8674.

Takebe, I., Otsuki, Y. & Aoki, S. (1968). Isolation of Tobacco Mesophyll Cells in Intact and Active State. *Plant Cell Physiology*, Vol.9, N1, pp. 115–124, ISSN 0032-0781.

Wassenegger, M., Heimes, S., Riedel, L. & Sänger, H.L. (1994) RNA-Directed de novo Methylation of Genome Sequences in Plants. *Cell*, Vol.76, N3, (11 February 1994), pp. 567-576, ISSN 0092-8674.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E. & Jaenisch, R. (2007). In vitro Reprogramming of Fibroblasts into a Pluripotent ES-cell-like State. *Nature*, Vol.448, N7151, (July 2007), pp. 318–324, ISSN 0028-0836.

West, G.B. & Brown, J.H. (2005). The Origin of Allometric Scaling Laws in Biology from Genomes to Ecosystems: towards a Quantitative Unifying Theory of Biological Structure and Organization. *Journal of Experimental Biology,* Vol.208, N9, (1 May 2005), pp. 1575-1592, ISSN 0022-0949.

Yao, M.C. (2008). RNA Rules. *Nature*, Vol.451, N7175, (10 January 2008), pp. 131-132, ISSN 0028-0836.

Zaretzky, A.N. & Letelier, J.C. (2002). Metabolic Networks from (M,R) Systems and Autopoiesis Perspective. *Journal of Biological Systems,* Vol.10, N3, (September 2002), pp. 265-280, ISSN 0218-3390.

Zhuravlev, Yu.N. & Avetisov, V.A. (2006). The Definition of Life in the Context of its Origin. Biogeosciences, Vol.3, N3, (10 July 2006), pp. 281-291, ISSN 1726-4170.

Zhuravlev, Yu.N., Bulgakov, V.P., Moroz, L.A., Uvarova, N.I., Makhan'kov, V.V., Malinovskaya, G.V., Artyukov, A.A. & Elyakov, G.B. (1990). Accumulation of Panaxosides in a Culture of Cells of *Panax ginseng* C. A. Mey Transformed with the Aid of *Agrobacterium rhizogenes*. Doklady Akademii Nauk SSSR (Bot.Sci). New York: Springer Consultants Bureau. Vol.311, pp. 22-23. NAL Call No.: 511-P444AE

Zhuravlev, Yu.N. & Omelko, A.M. (2008). Plant Morphogenesis in vitro. *Russian Journal of Plant Physiology*, Vol.55, N5, (September 2008), pp. 579-596, ISSN 1021-4437.

Part 2

Plant Transformation: Improving Quality of Fruits, Crops and Trees - Molecular Farming

3

Genetic Transformation in Tomato: Novel Tools to Improve Fruit Quality and Pharmaceutical Production

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1. Introduction

Tomato is one of the most important vegetable crop worldwide with a total production of around 141 million tons on a cultivated area of around 5 million hectares (FAOSTAT, 2009, http://faostato.fao.org). Among the most representative countries, Italy contributes with more than 6 million tons to the world production, on a cultivated area of around 117.000 hectares, both in open fields and greenhouses (FAOSTAT, 2009). This crop represents also one of the major products of the food industry worldwide and Italy ranks first for processing tomato production among Countries of the Mediterranean Region (World Processing Tomato Council, 2009, www.wptc.to). Indeed, the high variability of tomato fruits, ranging from the cherry type to the big round or elongated berry, supplies both fresh market and processing products, such as paste, juice, sauce, powder or whole. In the last years, tomato consumption has further increased since it was demonstrated that tomato fruit could protect against diseases, such as cancer and cardiovascular disorders, due to its antioxidant properties (Rein et al., 2006). Tomato fruits are particularly rich of nutritional compounds such as lycopene and alfa-carotene, vitamin C, flavonoids and hydroxycinnamic acid derivatives whose intake would account for health benefits.

The cultivated tomato (*Solanum lycopersicum*) belongs to the *Solanaceae* family that includes more than 3.000 species, among which 12 represent tomato wild relatives. These species exhibit a wide variety of adaptation to diverse habitats, plant morphology, fruit size and colour, the latter varying from green to white, yellow, pink, red, brown, depending mainly on the metabolites fruit content. The wild related tomato species represent a potential reservoir of useful genes that have been greatly used in breeding programs (Bai & Lindhout, 2007; Gur & Zamir, 2004). Indeed, this vegetable is one of the most investigated crop both at genetic and genomic level not only because of its economic importance but also because it is one of the best characterized plant systems. It has diploid genetics (24 somatic chromosomes), a small genome size (950 Mb per haploid nucleus), is self-pollinated, has a short generation time, is easily reproduced by seed and vegetative propagation and is cross-compatible with many wild species. All these characteristics make it amenable to genetic analysis.

The huge amount of researches focused on tomato allowed the development of new tools and platforms for genetics and genomics analyses (Barone et al., 2008). Since tomato is considered the model species among the *Solanaceae*, these novel techniques have been also exploited for other economically important crops, such as potato, pepper, and eggplant. Moreover, due to the high sinteny existing among *Solanaceae* species, tomato was chosen as reference genome to be completely sequenced by the International Tomato Genome Sequencing Consortium at the end of the year 2003 (Mueller et al., 2005). Molecular comparative mapping studies revealed a high level of conserved gene content and order within this family (Wu & Tanksley, 2010), as well as within other families (i.e grasses, crucifers, legumes). Indeed, a high level of microsinteny amongst the genomes of tomato, potato, pepper and eggplant was observed. Therefore, determination of the tomato genome sequence could allow extending information among species, thus creating a common map-based framework of knowledge. This could allow inferring the sequence organization of other *Solanaceae* crops as basis for understanding how plants diversify and adapt to new and adverse environments.

The recent release of the tomato genome sequence (Mueller et al., 2009), together with the powerful genetic and genomic resources available today for this species, allowed plant biotechnologists to implement novel methods to obtain new genotypes that could answer to new consumer, producer and processor requirements. These resources, in fact, could help the transfer of useful genes among species and/or improved genotypes through assisted breeding programs as well as through genetic transformation technologies.

In the present review, after providing some information on tomato genetic and genomic resources, we will give an overview of genetic transformation techniques and biotechnology applications investigated in this species. Several recent review reported new studies on tomato genetic transformation as a tool for the improvement of resistance to pests and pathogens (Balaji & Smart, 2011; Khan et al., 2011; Panthee & Chen, 2010; Wu et al., 2011; Zhang et al., 2010). Therefore, after a short description of main transformation techniques to which tomato is well adapted, herein we will focus on the use of genetic transformation for fruit quality engineering and pharmaceutical production.

2. Genetic and genomic resources

Among cultivated species, tomato is one of the richest in genetic and genomic resources (Table 1 and Table 2), including information now available from the complete genome sequencing that was released in the last year in a preliminary version. All these tools, used together or separately, are having a great impact on tomato breeding and genetics (Barone et al., 2009; Foolad, 2007).

This cultivated species could count on a number of wild and related species, on a wide collection of naturally or induced mutants and on many well-characterized genetic stocks, such as cultivars and landraces, cytogenetic stocks and pre-bred lines. Today this germplasm is publicly available (Table 1). In the miscellaneous group, the Backcross Recombinant Inbreds and Introgression Lines are particularly useful for the identification of genes and/or QTLs, since they constitute "immortal" population to be used for quantitative analyses (Grandillo et al., 2008). In addition, they also represent exotic libraries that allow to better exploit biodiversity exhibited by wild species. Indeed, the IL population is composed by many lines, each carrying a single homozygous genomic region from the wild species, altogether covering the whole wild genome (Eshed & Zamir, 1995; Fridman et al., 2004).

GENETIC RESOURCE	NOTES	WEBSITE
Wild species	More than 1.100 accessions	TGRC (http://tgrc.ucdavis.edu), NPGS (www.ars-usda.gov)
Monogenic mutants	More than 600 mutants	TGRC
Miscellaneous stock	More than 1.500 accessions	TGRC, NPGS
Introgression lines (IL)	from *S. pennellii*, *S. habrochaites*, *S. lycopersicoides*	TGRC
Backcross Recombinant Inbreds (RIL)	*S. lycopersicum* x *S. pimpinellifolium*	TGRC
Induced-mutant stocks	More than 3.400 induced-mutants from *cv* M82	SGN (http://zamir.sgn.cornell.edu/mutants)
	Around 1000 induced-mutants from Micro-Tom	TOMATOMA (http://tomatoma.nbrp.jp)
	More than 5.000 mutants from *cv* Red Setter	LycoTill (www.agrobios.it/tilling/index.html)

Table 1. Tomato genetic resources publicly accessible via web

Currently, IL populations that derive from various wild species are available, even though others are being generated (Barone et al., 2009). The first population (from *S. pennellii*) has been so far widely used to localize QTLs (Lippman et al., 2007) and to clone them (Frary et al., 2000; Fridman et al., 2000).

In addition to a collection of natural mutants available at TGRC (Tomato Genetic Resource Centre), wide collections of induced mutants were generated in different genetic backgrounds, by chemical or physical mutagenesis (Emmanuel & Levy, 2002; Menda et al., 2004; Watanabe et al., 2007). These mutants were widely phenotyped for many traits and contributed to better understand some developmental processes, such as growth habit, flowering and fruit ripening (Giovannoni, 2007; Pineda et al., 2010; Saito et al., 2011). In addition, induced mutagenesis has often been implemented with gene-specific detection of single-nucleotide mutations to generate TILLING platforms. So far, TILLING was developed for the cv. M82 (Piron et al., 2010), Red Setter (Minoia et al., 2010), Tpaadasu (Gady et al., 2009) and Micro-Tom (Saito et al., 2011) and its use has allowed the pinpointing of mutations in genes of interest.

The variability displayed by the different sources of germplasm available for tomato could be explored to search for new genes or favourable alleles to be transferred by conventional breeding and/or genetic transformation in selected genotypes to obtain new varieties.

In recent years, genetic resources combined with tomato specific genomic tools (Barone et al., 2009) allowed to successfully achieve various objectives, including the development of new varieties resistant to biotic and abiotic stresses and with improved fruit quality traits and yield. Most of these resources are also publicly available for the scientific community and are accessible *via* web (Table 2).

GENOMIC RESOURCE	NOTES	WEBSITE
Molecular markers	Thousands markers (i.e RFLP, AFLP, SSR, COS, CAPS, SNP)	SGN (http://solgenomics.net)
Molecular maps	10 genetic maps involving crosses among different species and varieties	SGN
Physical map	from *S. lycopersicum*	SGN
Complete genome sequence	released version SL2.40 January 2011	SGN
EST collections	Around 300.000 from various tissues and developmental stages	SOLESTdb (http://biosrv.cab.unina/solestdb) Tomato Gene Index (http://compbio.dfci.harvard.edu/tgi), plantGDB (http://www.plantgdb.org), MiBASE (http://www.kazusa.or.jp/jsol/microtom)
transcriptomic array	TOM1 (approx. 8000 unigenes)	Tomato Functional Genomics database (http://ted.bti.cornell.edu)
	TOM2 (approx. 11.000 independent genes)	TFGD
	Affimetrix (approx. 10.000 genes)	(http://www.affymetrix.com)
	Combimatrix TomatoArray1.0 (more than 20.000 probes)	Functional Genomic Center (http://ddlab.sci.univr.it)
Metabolomic platforms	Metabolites from *S. pennellii* and *S. habrochaites* ILs , metabolomics of tomato fruit from 96 cultivars	TFGD, MoToDB (http://appliedbioinformatics.wur.nl)
TILLING platforms	From *cv*. Red Setter, M82	LycoTill, UTill (http://urgv.evry.inra.fr/UTILLdb), (http://tilling.ucdavis.edu/index.php/TomatoTilling)
SNP array	SolCAP approx. 8000 SNPs from 6 genotypes	SolCAP (http://solcap.msu.edu)
Bioinformatic platforms	Data mining and integration, genome annotation	SGN, TFGD

Table 2. Tomato genomic resources publicly accessible via web

Since the beginning of 1990s, the contribution of molecular markers and maps to tomato breeding and gene identification has been widely documented (Foolad, 2007; Frary et al., 2005; Gupta et al., 2009), and more than 15.000 different markers are collected in the SGN database, where markers can be searched by name, chromosome position and mapping population. Moreover, cytological and cytogenetic maps are also available, as well as a detailed physical map, which was the foundation for the tomato genome sequencing project (Mueller et al., 2005). Contemporarily, gene expression analyses performed on different tissues and developmental stages, as well as on genotypes that differ in their answer to environmental *stimuli*, have dramatically raised the number of ESTs available at various websites. Consequently, several microarray platforms have being designed and are being used for transcriptional profiling, thus contributing to the identification of novel genes (Baxter et al., 2005b; Di Matteo et al., 2010). In addition bioinformatics resources aiming at integrating the forthcoming tomato genome sequence, wide collections of ESTs and data from transcriptomic, proteomic and metabolomic platforms available for tomato will enhance the design and management of genetic transformation approaches, such as those pointing at fruit quality engineering and production of pharmaceutical proteins.

3. Techniques for tomato genetic transformation

Since the 1980s several *Agrobacterium*-mediated transformation protocols have been developed in tomato, using cotyledons or leaves (Pino et al., 2010; Sharma et al.; 2009; Van Eck et al., 2006). Transformation efficiencies obtained in various cultivars range from 10 to 41%. Many factors were believed to be crucial for tomato transformation using *Agrobacterium tumefaciens*, including the application of nurse cells or acetosyringone to the culture or pre-culture media, the type of explants, the *Agrobacterium* strain used and its concentration, co-cultivation period and the concentration of thiamine, 6-benzylamino purine (BAP), zeatin and indole acetic acid (IAA). Also, new transformation procedures have been developed for tomato varieties with low *in vitro* regeneration capacity (Fuentes et al., 2008) and alternative transformation methods, such as floral dip, have been tested (Yasmeen et al., 2009). In addition, novel resources for temporal and tissue-specific manipulation of gene expression in tomato plants are now available for the scientific community. In this regard, it is noteworthy the work from Fernandez et al. (2009) and Estornell et al. (2009) that created new *Solanaceae* genetic toolkit for targeted gene expression and silencing in tomato fruits.

Recently, as the information provided by the tomato genome sequencing become available, the demand for efficient functional genomics tools are increasing. Functional genomics studies of the tomato plant require the use of high-throughput methods for functional analysis of many genes including simple and easily reproducible plant transformation systems. The miniature tomato cultivar MicroTom is a rapid-cycling cherry tomato variety that differs from standard tomato cultivars primarily by two recessive genes that confer the dwarf genotype (Dan et al., 2006). MicroTom shares some traits with the model plant *Arabidopsis thaliana* such as the small size, short life cycle (70-90 days from sowing to fruit-ripening) and small genome (950 Mb) and it is therefore considered a model cultivar for tomato genetics and functional genomics. Several studies investigated the production of improved protocols for *Agrobacterium*-mediated MicroTom transformation obtaining a transformation efficiencies ranging from 20 to 56% (Dan et al., 2006; Qiu et al., 2007; Sun et al., 2006). Recently, Pino et al. (2010) developed an efficient and inexpensive method for

MicroTom transformation using a new tomato genotype harbouring the allele *Rg1* that greatly improves tomato *in vitro* regeneration.

Another breakthrough in the field of tomato genetic transformation was the development of a system for stable genetic transformation of tomato plastids (Ruf et al., 2001). In comparison with conventional nuclear transformation, the integration of transgenes in the plastid genome presents several advantages: 1) high expression levels of recombinant proteins attainable owing to the high ploidy level of the plastid genome (up to 10,000 plastid genomes per cell); 2) efficient transgene integration since integration into the plastid genome relies on homologous recombination between the targeting regions of the transformation vector and the wild-type plastid DNA; 3) absence of epigenetic effects (gene silencing); 4) increased biosafety due to the biological containment of transgenes and recombinant products owing to maternal inheritance of plastid and plastid transgenes and absence of dispersal in the environment through the pollen; 5) possibility to express multiple transgenes from prokaryotic-like operons, thus simplifying engineering metabolic pathways (Bock & Warzecha; Cardi et al., 2010; Ruf et al., 2001; Wurbs et al., 2007).

The availability of a technology for transgene expression from the tomato plastid genome opened up new possibilities for metabolic engineering and the use of plants as bioreactors for the production of pharmaceuticals (Ruf et al., 2001, Wurbs et al., 2007). The group of Ralph Bock investigated the possibility to elevate the pro-vitamin A content of tomatoes using the chloroplast transformation technology (Apel & Bock, 2009; Wurbs et al., 2007). Apel & Bock (2009) introduced the lycopene β-cyclase genes from the eubacterium *Erwinia herbicola* and the plant daffodil (*Narcissus pseudonarcissus*) into the tomato plastid genome in order to enhance carotenoid biosynthesis inducing lycopene-to-provitamin A conversion. The expression of the enzyme from the higher plant daffodil in fruits of transplastomic tomato plants triggered efficient conversion of lycopene to β-carotene and resulted in a >50% increase in total carotenoid accumulation. Zhou et al. (2008) studied the feasibility of producing human immunodeficiency virus (HIV) antigen in transplastomic plant and demonstrated that the HIV antigens p24 and Nef in the plastid could be expressed in plastid of tomato plants.

Today, the technology of stable plant transformation is successful in tomato; however, the lack of an efficient, simple and reliable protocol and the length of time required to produce transgenic lines complicate the analysis of gene function. In alternative, transient assays could provide a rapid tool for the functional analysis of transgenes and have been often used as an alternative to the analysis of stably transformed lines (Wroblewsky et al., 2005). A powerful tool for fast reverse genetics is the virus-induced gene silencing (VIGS) technology (Orzaez & Granell, 2009). Using this method, recombinant virus vectors carrying host-derived sequences are used to infect the plant; systemic spreading of this recombinant virus causes specific degradation of the endogenous gene transcripts by PTGS (post-transcriptional gene silencing) (Dinesh-Kumar et al., 2003; Liu et al., 2002). In 2002, Liu and colleagues demonstrated that a tobacco rattle virus (TRV)-based VIGS vector could be used in tomato to silence genes efficiently. To shorten the time and simplify the functional analysis in fruits, Orzaez et al. (2006) developed a methodology that allowed transient expression of transgenes directly in fruit tissues. However, the identification and quantification of non-visual phenotypes could be hampered by the irregular distribution of fruit VIGS. In a recent paper Orzaez et al. (2009) developed an anthocyanin-guided VIGS in order to overcome the limitations of this technique such as its irregular distribution and efficiency. To develop a visually traceable system the authors developed a method

comprising: 1) a tomato line expressing *Rosea1* and *Delila* transcription factors under the control of the E8 promoter that showed a purple-fruited phenotype and 2) a modified TRV VIGS vector incorporating partial *Rosea1* and *Delila* sequences agro-injected in the transformed lines and that was able to restore the red-fruited phenotype.

4. Biotechnology applications

4.1 Fruit quality engineering

Tomato fruit quality includes several aspects that may be grouped into two categories: organoleptic properties and nutritious contents. Organoleptic quality involves color and texture of the fruit, but also taste and aroma, whereas nutritional quality refers to the content of metabolites contributing to the intake of nutritious such as sugars, carotenoids, flavonoids, ascorbic acid and folate.

Most of the quality traits show a continuous variation, are attributed to the joint action of many genes and are strongly induced by environmental conditions. Beside their complex inheritance, fruit quality traits have often been engineered in tomato through approaches of reverse genetics, such as genetic transformation and mutagenesis, pointing at controlling the expression of single major genes involved in the regulation of a desirable phenotype. In addition, genetic transformation has often been successful in enhancing fruit quality-related traits in tomato investigating simultaneously the role of candidate genes in specific biological processes in the fruit.

In general, there are three main goals of engineering strategies in plants (Verpoorte et al., 2000): the enhancement of a desired trait, the decrease in the expression of a specific unwanted trait, and the development of a novel trait (i.e. a molecule that is produced in nature but not usually in the host plant, or a completely novel compound). Strategies aimed at inducing changes in the expression of a trait changing the synthesis of a specific metabolite are referred to as metabolic engineering. Approaches for achieving the redirection of metabolic fluxes include the engineering of single steps in a pathway to increase or decrease metabolic flux to target compounds, to block competitive pathways or to introduce short cuts that divert metabolic flux in a particular way. However, this strategy has only limited value because the effects of modulating single enzymatic steps are often absorbed by the system in an attempt to restore homeostasis. Recently, strategies aimed at targeting multiple steps in the same pathway are gaining increasing interest because they help to control metabolic flux in a more predictable manner. This might involve up-regulating several consecutive enzymes in a pathway; up-regulating enzymes in one pathway while suppressing those in another competing pathway; or using regulatory genes such as transcription factors (TF) to establish multipoint control over one or more pathways in the cell. Since technical hurdles limits the number of genes that can be transferred to plants and pyramiding of transgenes by crossing transformants for single targets is a highly time-consuming approach, researchers developed new transformation methods to introduce multiple transgenes into plants and express them in a coordinated manner (Navqvi et al., 2009). In addition, controlling the expression of a single TF or a combination of TFs provides attractive tools for overcoming flux bottlenecks involving multiple enzymatic steps, or for deploying pathway genes in specific organs, cell types or even plants where they normally do not express.

A schematic description of successful metabolic engineering for enhancement of fruit quality in tomato is provided in Table 3 and Table 4. Genetic transformation targeting a single TF has been used to successfully engineer tomato for inducing development of parthenocarpic and

seedless fruit. Parthenocarpy enables fruit set and growth to be independent from pollination, fertilization and seed development circumventing the environmental constraints on fruit production and ensuring yield stability. Seedless fruits enhance consumer appeal and could also be a valuable trait for industrial tomatoes because parthenocarpy increases the content of soluble solids, improves yield and flavour of paste and reduces processing costs. Reported applications involved the overexpression of an auxin response factor 8 (*ARF8*) from *Arabidopsis* (Goetz et al., 2007) and downregulation of *Aux/IAA9* transcription factor (Wang et al., 2005) to promote fruit parthenocarpic development.

Modifications of fruit softening and of the overall firmness have been achieved mostly by engineering genes controlling single enzymatic steps in cell wall-associated pathways. In particular, polygalacturonase (Kramer et al., 1992; Langley et al. 1994; Smith et al., 1990), pectin methylesterase (Tieman & Handa, 1994), expansin (Brummell et al., 1999) and β-galactosidase (Smith et al., 2002) genes showed effectiveness in controlling fruit firmness and softening in transgenic tomato plants. A dosage series of the gene *fw2.2,* a negative regulator of cell division (Frary et al., 2000) was generated in tomato by genetic transformation allowing to modulate fruit weight in tomato without affecting cell size in pericarp and placenta tissues (Liu et al., 2003).

Two examples of successful metabolic engineering modifying tomato fruit flavour relayed on heterologous single-gene expression to introduce in tomato untypical traits. In the first example, a biologically active thaumatin, a sweet-tasting, flavour-enhancing protein from the African plant *Thaumatococcus daniellii* Benth was expressed in transgenic tomatoes that produced sweeter fruits with a specific aftertaste (Bartoszewski et al., 2003). In the second example, the lemon basil geraniol synthase (*GES*) gene was overexpressed under the control of the strong fruit-ripening-specific tomato polygalacturonase promoter (*PG*). *GES* encodes the enzyme responsible for the production of geraniol from GDP and its expression caused the plastidial terpenoid biosynthetic flux to divert, leading to a reduced lycopene accumulation and to dramatic changes in the aroma and overall flavour of the transgenic fruits (Davidovich-Rikanati et al., 2007).

In another study, the overexpression of either *LeAADC1A* or *LeAADC2*, encoding for phenylalanine decarboxylases that are involved in the synthesis of 2-phenylethanol from phenylalanine, resulted in fruits with up to 10-fold increased emissions of the products of the pathway, including 2-phenylacetaldehyde, 2-phenylethanol, and 1-nitro-2-phenylethane. On the other hand, antisense reduction of *LeAADC2* significantly reduced emissions of these volatiles (Tieman et al., 2006).

In addition to organoleptic fruit quality, nutritional attributes of tomato fruit have recently received increasing attention by molecular biologists. For instance, the fruit soluble solid content was engineered by using an RNAi approach to generate transgenic plants that were exclusively altered in the expression of a specific isoform of the cell wall invertase *LIN5* (Baxter et al., 2005a; Fridman et al., 2000, 2004; Schauer et al., 2006; Zanor et al., 2009).

Several attempts have been made also to engineer higher carotenoid contents in tomato fruit and a number of tomato lines have been generated with enhanced levels of lycopene, β-carotene and xanthophylls (mainly zeaxanthin and lutein) and low levels of non-endogenous carotenoids such as ketocarotenoids (Fraser et al., 2009). One of the most interesting achievements is the HighCaro (HC) tomato plant (D'Ambrosio et al. 2004), a transgenic line carrying the tomato lycopene β-cyclase (*tLcy-b*) cDNA. Carotenoid biosynthetic pathway is a highly regulated, interconnected, compartmentalized, membrane bound pathway that can be successfully engineered to enhance carotenoids in crop plants

circumventing homeostasis. Carotenoids are biosynthetically related to gibberellins via geranyl-geranyl pyrophosphate and isopentenyl pyrophosphate and this often caused in transgenic plants unpredictable phenotypes. For example, in transgenic lines overexpressing the endogenous gene *Psy-1*, besides an effect on gibberellins formation, the levels of other isoprenoid derived phytohormones were altered in vegetative tissues as well as chlorophyll and tocopherol contents in fruit (Fray et al., 1995). In order to minimize these detrimental effects, engineering approaches to enhance carotenoids in tomato have recently focused on the use of tissue-specific promoters. The use of tomato ripening enhanced promoters allowed a controlled expression at this stage facilitating co-ordination with endogenous carotenoid formation and reducing competition with other branches of the isoprenoid pathway. On the other hand, transcriptional up-regulation of a gene does not always correlate to increased protein or enzyme activity and forward-feed regulation mechanisms could operate within the pathway to maintain homeostasis. For example, in tomato lines expressing a bacterial derived phytoene synthase (*CrtB*) the subsequent desaturation step in the pathway was reduced (Fraser et al., 2002). Moreover, transgenic lines expressing a bacterial desaturase had a reduced phytoene synthase transcription and enzyme activity. In addition, tomato lines overexpressing deoxy-D-xylulose 5-phosphate synthase (*Dxs*) showed elevated phytoene formation in ripe fruit, however desaturation limited progression through the pathway (Enfissi et al., 2005). Finally in tomato lines overexpressing *Psy-1* a lycopene cyclase (*CYC-B*) is induced resulting in increased enzyme activity generating β-carotene as an unintended end-product (Fraser et al., 2007). By contrast, feedback inhibition could also limit accumulation of end-products as was the case of tomato lines expressing the *CrtI* enzyme where the elevated β-carotene levels reduced phytoene synthase (Romer et al., 2000). In contrast with results obtained in rice and potato, multiple step engineering strategies in the carotenoid and isoprenoid precursor pathways in tomato were only partially successful (Diretto et al., 2007). Finally, the simultaneous expression of an Arabidopsis *LCY-B* gene and a pepper *CHY-B* gene resulted in the production of xanthophylls, while the expression of *CrtW* and *CrtZ* from *Paracoccus* spp. leaded to the formation of low fruit levels of ketocarotenoids (Dharmapuri et al., 2002; Ralley et al., 2004). Innovative strategies for carotenoid engineering in tomato fruit consist in alteration of cryptochromes and components of the light signal transduction pathway. These approaches have the advantages of elevating the carotenoid content of the fruit and also other important health related phytochemicals such as phenylpropanoids and flavonoids (Davuluri et al., 2005).

Due to their presumed health benefits, there is growing interest in the development of food crops with tailor-made levels and composition of flavonoids. The repertoire of case studies aimed at increasing the levels of flavonoids in tomato fruit also offers the wider range of examples of successful engineering strategies ever realized. Herein we will list some of the results recently obtained.

The first strategy is related to engineering single structural genes controlling key steps in the pathway, such as a chalcone isomerase (CHI) (Muir et al., 2001) and a chalcone synthase (CHS) (Colliver et al., 2002). More encouraging results were obtained targeting multiple constitutive genes within the flavonoid pathway. For instance, the concomitant ectopic expression of *Petunia CHS, CHI, F3H* (flavanone hydroxylase) and *FLS* (flavonol synthase) in tomato fruit led to increased levels of flavonols in both peel (primarily quercetin glycosides) and flesh (primarily kaempferol glycosides). In another case, the concomitant expression of both *CHS* and *FLS* had a synergistic effect resulting in a significant accumulation of both

naringenin- and kaempferol-glycosides in tomato flesh (Colliver et al., 2002). Secondly, in order to increase the range of flavonoids produced in tomato fruit, a different strategy was taken that consisted in introducing branches to the pathway leading to the synthesis of atypical flavonoids. The overexpression of a grape stylbene synthase (STS) resulted in the accumulation of resveratrol aglycon and its glucoside in tomato fruit peel, while the level of naringenine chalcone was negatively affected because of a competition effect with the main pathway (Schijlen et al., 2006). Similarly, the concomitant overexpression of a petunia CHS and an alfalfa chalcone reductase (CHR) allowed deoxychalcones to accumulate in the tomato peel. When a gerbera *FNS-II* gene and a *Petunia CHI* gene were simultaneous overexpressed in tomato, flavones (mainly as luteolin aglycon) accumulated in their peel (Schijlen et al., 2006). The third strategy involved engineering of transcription factors to enhance a wider range of flavonoid compounds. Besides the increased level of flavonoids induced in tomato fruit by silencing DET1, the expression in tomato of the transcription regulator AtMYB12 activated flavonol biosynthesis as well as the caffeoylquinic acid biosynthetic pathway (Adato et al., 2009). Also, a 60-fold increase in kaempferol glycosides has been achieved in tomato flesh tissue by simultaneous ectopic expression of the two maize transcription factors Lc and C1 (Bovy et al., 2002). Most surprisingly, the expression of the Delila (*Del*) and Rosea1 (*Ros1*) genes, two transcription factors from the snapdragon *Antirrhinum majus*, in the fruit of transgenic tomatoes induced the accumulation of high levels of anthocyanins in tomato (Butelli et al., 2008) through the activation of a broad range of flavonoid biosynthetic pathway related genes.

In contrast with flavonoid metabolism, so far a reduced number of efforts have been placed into genetic transformation-mediated metabolic engineering of tomato fruit for enhanced ascorbic acid levels. Only few of them succeeded in effectively affect ascorbic acid content and only for a limited number of structural genes within the ascorbic acid pathway. In a fruit systems biological approach, transgenic tomato lines silenced for a mitochondrial ascorbic acid synthesizing enzyme L-galactono-1,4-lactone dehydrogenase performed an increased fruit ascorbic acid level (Garcia et al., 2009) whereas the silencing of an GDP-D-mannose-3',5'-epimerase resulted in a reduced fruit ascorbic acid accumulation (Gilbert et al., 2009). On the other hand, overexpression of GDP-D-mannose-3',5'-epimerase genes resulted in enhances ascorbic acid accumulation in tomato fruit (Zhang et al., 2011).

Similarly to ascorbic acid, the opportunity of engineering folate accumulation in tomato fruit has been mostly overlooked and only a few attempts gave rise to successful outcomes. In order to increase pteridines, which act as folate precursors and are synthesized from *p*-aminobenzoate, a GTP cyclohydrolase I was overexpressed and a 2-fold increase in folate level in tomato fruit was gained (de la Garza et al., 2004). A higher folate accumulation (up to 25-fold increase) was achieved in tomato fruit by combining in the same plant the overexpression of an aminodeoxychorismate synthase, the *p*-aminobenzoate-forming enzyme, and the GTP cyclohydrolase I (de la Garza et al., 2007).

Comprehensively, within genetic engineering strategies for crop improvements, the most striking advances so far have involved plants engineered to produce missing nutrients or increase the level of nutrients that are already synthesized. An important trend is to move away from plants engineered to produce single nutritional compounds towards those simultaneously engineered to produce multiple nutrients, a development made possible by the increasing use of multigene engineering or regulative genetic element with pleiotropic effects.

Trait	Engineering strategy	Inserted target	Fruit phenotype	Reference
Partheno-carpy	single TF	*Arf8* *IAA9*	induced parthenocarpy	Goetz et al., 2007; Wang et al., 2005
Firmness	single biosynthetic key gene	*PG*	reduced softening	Langley et al. 1994
		PME	reduced shelf-life	Tieman and Handa, 1994
		EXP1A	reduced firmness	Brummell et al., 1999
		β-galactosidase	increased firmness	Smith et al., 2002
Size	dosage series of a single gene	*fw2.2*	increased size	Frary et al., 2000
Flavour	heterologous single gene	thaumatin	enhanced flavour	Bartoszewski et al., 2003
Flavour and aroma	heterologous single gene for diverting biosynthetic flux	*GES*	changes in flavor & aroma	Davidovich-Rikanati et al., 2007
	single biosynthetic key gene	*LeAADC1A*, *LeAADC2*	Increased/ decreased 2-phenylacetaldehyd, 2-phenylethanol, and 1-nitro-2-phenylethane	Tieman et al., 2006

Table 3. Examples of successful fruit engineering for organoleptic quality trait in tomato. Abbreviations: TF, transcription factor; SG, silencing; SI, serial increase of gene dosage

Trait	Engineering strategy	Inserted target	Fruit phenotype	Reference
Soluble solids content	single biosynthetic key gene	*Lin5*	reduced sugars accumulation	Zanor et al., 2009
Carotenoid content	single biosynthetic key gene	*Dxs*	increased phytoene & carotenoids	Enfissi et al., 2005
		CrtB, *CrtI*, *CrtY*	increased carotenoids	Fraser et al., 2002, 2007; Wurbs et al., 2007
		PSY-1	increased carotenoids	Römer et al., 2000
		CYC-B, *LCY-B*	increased lycopene & β-carotene	Rosati et al., 2000 D'Ambrosio et al., 2004; Ronen et al., 2000
	genes targeting biosynthetic steps	*LCY-B*, *CHY-B*	β-cryptoxanthin & zeaxanthin	Dharmapuri et al., 2002
	single regulative gene	*CRY-2*	increased carotenoid	Giliberto et al., 2005
		DET-1, *COP1LIKE*, *CUL4*	increased carotenoid and flavonoid	Liu et al., 2004; Wang et al., 2008; Davuluri et al., 2005
		FIBRILLIN	increased carotenoids and volatiles	Smikin et al., 2007
	single biosynthetic key gene	*spermidine synthase*	increased lycopene	**Neily et al., 2011**
Flavonoid content	single biosynthetic key gene	*CHI*	increased fruit peel flavonol	Muir et al., 2001
	genes targeting biosynthetic steps	*CHS*, *CHI*, *F3H*, *FLS*	increased flavonols	Colliver et al., 2002
	heterologous gene/genes for diverting flux	*STS*, *CHS*, *CHR*, *FNS-II*, *CHI*	accumulation of resveratrol, deoxychalcones, & flavones	Schijlen et al., 2006
	single TF	*MYB12*	accumulation of flavonols	Adato et al., 2009
	Multiple TFs	*Del*, *Ros1*	high levels of anthocyanins	Butelli et al., 2008

Trait	Engineering strategy	Inserted target	Fruit phenotype	Reference
Ascorbic acid content	single biosynthetic key gene	*GalLDH*, *GME*	increased/ decreased fruit ascorbic acid	Garcia et al., 2009; Gilbert et al., 2011; Zhang et al., 2011
	genes targeting consecutive biosynthetic steps	*GCHI* and/or *ADCS*	increased fruit folate	Diaz de la Garza et al., 2004; 2007

Table 4. Examples of successful fruit engineering for nutritional quality traits in tomato. Abbreviations: TF, transcription factor; SG, silencing; IPP, isopenthenilpyrophosphate

4.2 Production of pharmaceutical proteins

Genetically modified plants are currently being evaluated as promising alternative for the production of recombinant proteins and antigens. Major advantages of plant-made pharmaceuticals include low cost of production, higher scale-up capacity and lack of risk of contamination with mammalian pathogens. Several antigenic proteins have been produced in plant, examples are plant-made vaccines against smallpox, HIV and HPV (Human Papilloma Virus) (Lenzi et al., 2008; Rigano et al., 2009; Scotti et al., 2009). In addition, transgenic plants can represent a suitable vehicle for oral delivery of pharmaceuticals since the plant cell wall protects the recombinant antigen in the harsh condition of stomach and intestine (Sharma et al., 2008a). The delivery of vaccines to mucosal surface makes immunization practise safe and acceptable and is capable of inducing both humoral and cell-mediated immune responses (Salyaev et al., 2010). The production of plant-made mucosal vaccines eliminates needle-associated risks and downstream processing of traditional vaccines such as purification, sterilization and refrigeration. Recently, in addition to other systems, tomato plants have been used as vehicles for the expression and oral delivery of vaccines since tomato is edible, generates abundant biomass at low cost, has flexible growth conditions and contains the natural adjuvant α-tomatine (Salyaev et al., 2010; Soria-Guerra et al., 2011). In this regards, it is noteworthy the work from Zhang and colleagues (2006) that expressed the recombinant Norwalk virus capsid protein in tomato and potato and demonstrated that, although in mice oral immunization with both dried tomato fruit and potato tuber elicited systemic and mucosal antibody responses, the recombinant vaccine in transgenic tomato fruit, especially in air-dried material, was a more potent oral immunogen than potato. The authors speculated that the robust immunogenicity of tomato-derived vaccines was due to natural bioencapsulation by the plant cell matrix and membrane systems, larger amount of smaller 23 nm Virus-like particles and the presence of the natural adjuvant α-tomatine. In this paragraph, we will describe several examples of pharmaceuticals produced in tomato plants focusing on the most recently reported studies.

Several studies reported the production of transgenic tomato plants for the expression of viral antigens. In 2008, Perea Arango and colleagues reported high-level expression of the entire coding region of the nucleoprotein (N) gene of rabies virus in transgenic tomato plants. When mice were immunized both intraperitoneally (i.p.) and orally with the tomato-made N protein, the antibody titer of mice immunized i.p. was at least four times higher

than that of mice immunized orally. In addition, only mice immunized i.p. were partially protected against a peripheral virus challenge. In the same year, Pan et al. (2008) described the production of genetically modified tomato plants that expressed the structural polyprotein, P1-2A, and protease, 3C, from foot-and-mouth disease virus (FMDV). Guinea pigs vaccinated intramuscularly with foliar extracts from the transgenic material developed a virus-specific antibody response and were protected against a challenge infection. Recently, in order to develop a vaccine against HPV Paz De la Rosa et al. (2009) expressed in tomato plants chimeric particles containing the HPV 16 L1 sequence fused to a string of T-cell epitopes from HPV 16 E6 and E7 proteins. L1 fused to the string of epitopes was able to assemble into chimeric VLPs (Virus-like particles); in addition, intraperitoneal administration in mice of the transgenic material was able to induce both neutralizing antibodies against the viral particle and a cytotoxic T-lymphocytes activity against the epitopes. Up to date, several groups investigated the production of a mucosal vaccine against HIV and HBV (Hepatitis B virus) in genetically modified tomato plants (Lou et al., 2007; Salyaev et al., 2010; Zhou et al., 2008). For instance, Shchelkunov et al. (2006) investigated the production of transgenic plants expressing a synthetic chimeric gene, *TBI-HBS*, encoding the immunogenic ENV and GAG epitopes of HIV-1 and the surface protein antigen (HBsAg) of HBV and investigated the immunogenicity of the transgenic material fed to experimental mice. Peña Ramirez and colleagues (2007) investigated the possibility of expressing the HIV-1 Tat protein in fruits of tomato plants. In mice, oral feeding with the tomato-based vaccine was able to raise mucosal IgAs and induce serum IgGs with neutralizing activity. More recently, Cueno at al. (2010) expressed the HIV-1 protein Tat in tomato plants reaching up to 4 µg of recombinant protein per milligram plant protein. In addition, tomato extracts intradermally inoculated into mice were found to induce both humoral and cellular immune responses.

Bacterial antigens have also been expressed in transgenic tomato plants. Alvarez and colleagues (2006) expressed in transgenic tomato plants the FI-V antigen fusion protein for the production of a vaccine against pneumonic and bubonic plague. The authors tested the immunogenicity of the tomato-made vaccine in mice which were primed subcutaneously with bacterially produced F1-V and boosted orally with freeze-dried, powdered transgenic tomato fruit and demonstrated that the vaccine elicited IgG1 in serum and mucosal IgA in fecal pellets. In 2007, Soria-Guerra and collegues expressed in tomato a plant-optimized synthetic gene encoding the recombinant polypeptide sDTP (diphtheria-pertussis-tetanus), containing six DTP immunoprotective exotoxin epitopes and two adjuvants in order to develop an edible multicomponent DPT vaccine. Recently, the same group examined whether immunization of mice fed with freeze-dried tomato material elicited specific antibody responses. Sera of immunized mice tested for IgG antibody response to pertussis, tetanus and diphtheria toxin showed responses to the foreign antigens; in addition, high response of IgA against tetanus toxin was evident in gut (Soria-Guerra et al., 2011). In addition, several studies investigated the feasibility of production of a safe, inexpensive plant-based mucosal vaccine against cholera. For instance, Jang et al. (2007) expressed the Cholera toxin B subunit (CTB) in transgenic tomato fruits and demonstrated the immunogenicity of the tomato-made vaccine in mice. In alternative, Sharma and colleagues (2008b) produced the toxin co-regulated pilus subunit A (TCPA) of *Vibrio cholerae* and its immunogenic epitopes P4 or P6 fused to cholera toxin B subunit (CTB) in tomato plants. In the same year, the same research group reported the production of genetically modified

tomato plants for the expression of accessory colonization factor subunit A (ACFA) of *Vibrio cholerae* and ACFA fused to CTB (Sharma et al., 2008a).

Another advantage of using transgenic plants for the production of recombinant protein of biopharmaceutical and industrial importance is that plant cells are able to perform complex post-translational modification, including glycosylation (Agarwal et al., 2008). In this regard, the feasibility of expression of glycosylated and biologically active recombinant human α-1-antitrypsin (AAT) protein in transgenic tomato plants was demonstrated. In this study, in order to achieve high-level expression of recombinant protein in transgenic plant cells, the gene encoding human AAT protein was optimized by codon adjustment and elimination of mRNA destabilizing sequences. In addition, the synthetic gene was expressed with different signal sequences, translation initiation context sequence, Alfalfa mosaic virus UTR (untranslated region) at 5′ end and ER (endoplasmic reticulum) retention signal sequence (KDEL) at 3′ end. The modified gene driven by CaMV35S duplicated enhancer promoter resulted in high-level expression (up to 1.55% of TSP) of recombinant protein in transgenic tomato plants. Elias-Lopez et al. (2008) described the production of transgenic tomato plants expressing interleukin-12. BALB/c mice were infected with either *Mycobacterium tuberculosis* H37Rv strain or multi-drug-resistant clinical isolate (MDR) and treated with a daily oral dose of transgenic fruit extracts. Oral administration of the transgenic plant material improved protective immunity and induced higher resistance to mycobacterial infection, when administered the day before infection or during late progressive disease induced by virulent mycobacteria. Other therapeutic proteins produced in transgenic tomato plants include the analgesic-antitumor peptide (AGAP) from the venom of *Buthus martensii* Karsch (Lai et al., 2009) and human beta-amyloid for the production of a vaccine against Alzheimer`s disease (Youm et al., 2008).

Another alternative for the production of recombinant antigens in plant cells is transgene expression from the plastid genome. Chloroplast transformation offers a number of advantages, including the potential to accumulate enormous amounts of recombinant protein, uniform transgene expression rates, no gene silencing and transgene containment. Recently, Zhou et al. (2008) expressed HIV antigens p24 and Nef from tomato`s plastid genome. In tomato, antigen accumulation reached values of approximately 40% of total leaf protein. When the authors determined p24-Nef accumulation in fruits they found that although green tomatoes accumulated the HIV antigens to approximately 2.5% of the TSP, there was no expression in ripe fruits. The authors speculated that this was due to the presence in red-fruited tomatoes of chromoplasts that, compared to chloroplasts, are usually less active in plastid gene expression.

Up to date, several studies demonstrated the feasibility of using tomato plants as vehicles for the production of pharmaceuticals. One drawback of a tomato-made vaccine could be the short shelf-life of fresh fruits. To provide antigen stability during storage, food-processing techniques, such as freeze-drying, could be applied to transgenic tomato fruits expressing recombinant proteins. Freeze-dried plant material could be stored for long time and consumed without cooking; in addition, this technique could allow to standardize and concentrate the plant-made vaccine. Several studies applied this technique to vaccine produced in transgenic tomato and demonstrated that freeze-dried produced stable formulations for oral delivery (Alvarez et al., 2006; Salyaev et al., 2010; Shchelkunov et al., 2006; Soria-Guerra et al., 2011; Zhang et al., 2006).

5. Conclusion

In the present review, we underlined the role of genetic transformation as method to improve fruit quality and pharmaceutical production. In addition, we highlighted the double role of genetic transformation as tool for biotechnology applications and functional analyses of genes of interest (Figure 1). For tomato, these approaches are feasible following strategies of gene/QTL identification based on the use of genetic and genomic resources today available for this species.

Nowadays, European politicians often debate about perceived risks of genetically modified crops, while ignoring potential benefits; therefore, it is highly unlikely that engineered crops will be adopted in the short-to-medium term.

Considering these constraints, mutants could be envisaged as valid alternative to engineer tomato plants for enhanced fruit quality (Figure 1). Mutants could be selected from natural variation or generated using different approaches. In addition, if the mutant exhibits superior alleles, it could be used as improved genotypes or as donor parent in backcrossing breeding schemes to deliver the desirable trait. The isogenic mutant resources available today for tomato are useful for dissecting the mechanisms underlying mutant phenotypes, and such mutagenized populations are also being used to develop targeting induced local lesions in genomes (TILLING) platforms, which represent a high-throughput genetic strategy to screen for point mutations in specific regions of targeted genes, and to validate gene function (McCallum et al., 2000).

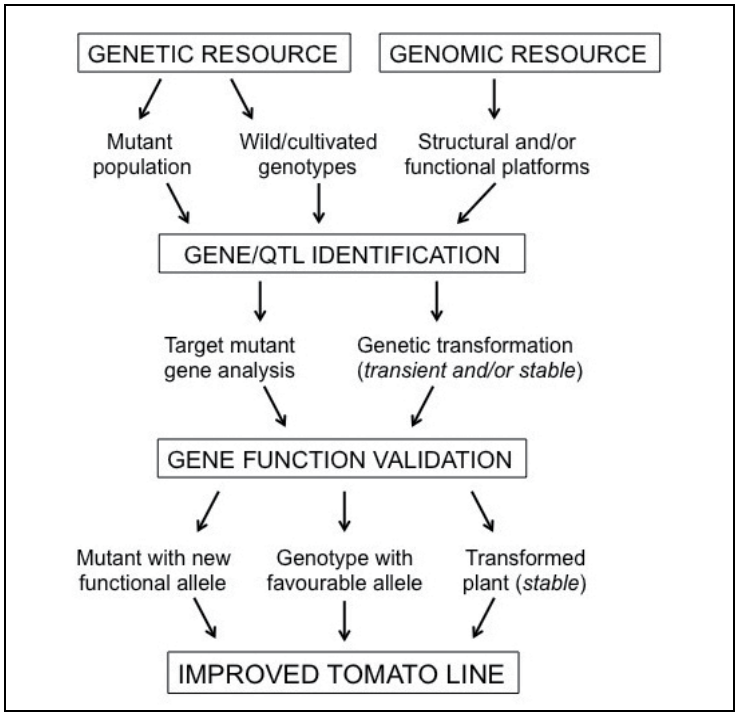

Fig. 1. Flow-Chart of steps from the screening of genetic and genomic resources to improved tomato lines

Another alternative approach to obtain tomato with desirable traits is to discover gene markers that discriminate contrasting alleles in genes or QTLs that control the trait(s) of interest (Figure 1). Following their identification, useful genes or QTLs can be introgressed into desirable genetic backgrounds via Marker Assisted Selection (MAS), where the selection for a trait is based on the genotype rather that the trait itself (Foolad, 2007). The knowledge of the tomato genome sequence dramatically enhances identification of novel molecular markers. Indeed we can envisage that, notwithstanding the implementation of recently developed Next Generation Sequencing technologies, the routine application of markers in tomato breeding will increase (Varshney et al., 2009).

In conclusion, the use of different approaches, such as tomato genetic transformation, exploiting of mutants and identification of allele-specific markers, could not only speed up the process of gene transfer, but it could also allow pyramiding of desirable genes and QTLs from different genetic backgrounds. The rapid integration of new alleles in elite tomato lines will allow new cultivars with desirable traits to enter the market in a shorter time compared to cultivar obtained through traditional breeding.

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Contribution no.Book 007 from the DISSPAPA

7. References

Adato, A.; Mandel, T.; Mintz-Oron, S.; Venger, I.; Levy, D.; Yativ, M.; Dominguez, E.; Wang, Z.; De Vos, R. C.; Jetter, R.; Schreiber, L.; Heredia, A.; Rogachev, I. & Aharoni, A. (2009). Fruit-surface flavonoid accumulation in tomato is controlled by a SlMYB12-regulated transcriptional network. *PLoS Genetics,* Vol.5, No.12, e1000777

Agarwal, S.; Singh, R.; Sanyal, I. & Amla, D.V. (2008) Expression of modified gene encoding functional human α-1-antitrypsin protein in transgenic tomato plants. *Transgenic Research*, Vol.17, pp. 881-896

Alvarez, M.L.; Pinyeard, H.L.; Crisantes, J.D.; Rigano, M.M; Pinkhasov J.; Walsmley, A.M.; Mason, H.S. & Cardineau, G.A. (2006) Plant-made subunit vaccine against pneumonic and bubonic plague is orally immunogenic in mice. *Vaccine,* Vol.24, pp.2477-2490

Apel, W. & Bock, R. (2009) Enhancement of carotenoid biosynthesis in transplastomic tomatoes by induced lycopene-to-provitamin A conversion. *Plant Physiology,* Vol.151, pp.59-66

Bai, Y. & Lindhout, P. (2007). Domestication and Breeding of Tomatoes: What have We Gained and What Can We Gain in the Future? *Annals of Botany*, Vol.100, pp. 1085–1094

Balaji, V. & Smart, C.D. (2011). Over-expression of snakin-2 and extensin-like protein genes restricts pathogen invasiveness and enhances tolerance to Clavibacter michiganensis subsp. michiganensis in transgenic tomato (*Solanum lycopersicum*). *Transgenic Research*, doi:10.1007/s11248-011-9506-x

Barone, A.; Chiusano, M. L.; Ercolano, M.R.; Giuliano, G.; Grandillo, S. & Frusciante, L. (2008). Structural and functional genomics of tomato. *International Journal Plant Genomics*, doi:10.1155/2008/820274

Barone, A.; Di Matteo, A.; Carputo, D. & Frusciante, L. (2009). High-throughput genomics enhances tomato breeding efficiency. *Current Genomics*, Vol.10, No.1, pp. 1-9

Bartoszewski, G.; Niedziela, A.; Szwacka, M. & Niemirowicz-Szczytt, K. (2003). Modification of tomato taste in transgenic plants carrying a thaumatin gene from Thaumatococcus daniellii Benth. Plant Breeding, *Vol.122, No.4,* pp.347–351

Baxter, C. J.; Carrari, F.; Bauke, A.; Overy, S.; Hill, S. A.; Quick, P. W.; Fernie, A. R. & Sweetlove, L. J. (2005a). Fruit carbohydrate metabolism in an introgression line of tomato with increased fruit soluble solids. *Plant Cell Physiology*, Vol.46, pp.425-437

Baxter, C.J.; Sabar, M.; Quick, W.P.& Sweetlove, L.J. (2005b). Comparison of changes in fruit gene expression in tomato introgression lines provides evidence of genome-wide transcriptional changes and reveals links to mapped QTLs and described traits. *Journal of Experimental Botany*, Vol.56, pp. 1591–1604

Bock, R. & Warzecha H. (2010) Solar-powered factories for new vaccines and antibiotics. *Trends in Biotechnology,* Vol. 28, Issue No 5, pp.246-252.

Bovy, A.; de Vos, R.; Kemper, M.; Schijlen, E.; Pertejo, M.A.; Muir, S.; Collins, G.; Robinson, S.; Verhoeyen, M.; Hughes, S.; Santos-Buelga, C. & van Tunen, A. (2002). High flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes Lc and C1. *Plant Cell*, Vol.14, pp. 2509-2526

Brummell, D. A.; Harpster, M. H.; Civello, P. M.; Palys, J. M., Bennett, A. B. & Dunsmuir P. (1999). Modification of Expansin Protein Abundance in Tomato Fruit Alters Softening and Cell Wall Polymer Metabolism during Ripening. *The Plant Cell*, Vol. 11, pp.2203–2216

Butelli, E.; Titta, L.; Giorgio, M.; Mock, H.P.; Matros, A.; Peterek, S.; Schijlen, E.G.; Hall, R.D.; Bovy, A.G.; Luo, J. & Martin, C. (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nature Biotechnology*,Vol.26, pp. 1301-1308

Cardi, T.; Lenzi, P. & Maliga, P. (2010). Chloroplasts as expression platforms for plant-produced vaccines. *Expert Review of Vaccines*, Vol.9, Issue No 8, pp.893-911

Chen, H.; Chang, M.; Chiang, B. & Jeng, S. (2006). Oral immunization of mice using transgenic tomato fruit expressing VP1 protein from enterovirus 71. *Vaccine,* Vol.24, pp.2944-2951

Colliver, S.; Bovy,A.; Collins, G.; Muir, S.; Robinson, S.; de Vos,C.H.R. & Verhoeyen, M.E. (2002). Improving the nutritional content of tomatoes through reprogramming their flavonoid biosynthetic pathway. *Phytochemistry Reviews*, Vol.1, pp. 113-123

Cueno, M.E.; Hibi, Y.; Karamatsu, K.; Yasutomi, Y.; Imai, K.; Laurena, A.C. & Okamoto, T. (2010). Preferential expression and immunogenicity of HIV-1 Tat fusion protein expressed in tomato plant. *Transgenic Research*,Vol.19, pp.889-895

D'Ambrosio, C.; Giorio, G.; Marino, I.; Merendino, A.; Petrozza, A.; Salfi, L.; Stigliani, A. L. & Cellini, F. (2004). Virtually complete conversion of lycopene into β-carotene in fruits of tomato plants transformed with the tomato lycopene β-cyclase (tlcy-b) cDNA. *Plant Science*, Vol.166, pp.207–214

Dan, Y.; Yan, H.; Munyikw, T.; Dong, J.; Zhang, Y. & Armstrong, C.L. (2006) MicroTom - a high-throughput model transformation system for functional genomics. *Plant Cell Reports,* Vol.25, pp.432 – 441

Davidovich-Rikanati, R.; Sitrit, Y.; Tadmor, Y.; Iijima, Y.; Bilenko, N.; Bar, E.; Carmona, B.; Fallik, E.; Dudai, N.; Simon, J. E.; Pichersky, E. & Lewinsohn E. (2007). Enrichment

of tomato flavor by diversion of the early plastidial terpenoid pathway. *Nature Biotechnology*, Vol.25, pp.899-901

Davuluri, G. R.; van Tuinen, A.; Fraser, P. D.; Manfredonia, A.; Newman, R.; Burgess, D.; Brummell, D. A.; King, S. R.; Palys, J.; Uhlig, J.; Bramley, P. M.; Pennings, H. M. & Bowler, C. (2005). Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nature Biotechnology*, Vol.23, pp.890-895

de la Garza, R. I. D.; Gregory, J.F. & Hanson, A.D. (2007). Folate biofortification of tomato fruit. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.104 No.10, pp. 4218-4222

de la Garza, R. I. D.; Quinlivan, R.; Klaus, E.P.; Basset, S.M.; Gregory, G.J.; & Hanson, A.D. (2004). Folate biofortification in tomatoes by engineering the pteridine branch of folate synthesis. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.101, pp 13720-13725

Dharmapuri, S.; Rosati, C.; Pallara, P.; Aquilani, R.; Bouvier, F.; Camara, B. & Giuliano, G. (2002). Metabolic engineering of xanthophyll content in tomato fruits. *FEBS Letters*, Vol.519, pp.30-34

Di Matteo, A.; Sacco, A.; Anacleria, M.; Pezzotti, M.; Delledonne, M.; Ferrarini, A.; Frusciante, L. & Barone, A. (2010). The ascorbic acid content of tomato fruits is associated with the expression of genes involved in pectin degradation. *BMC Plant Biology*, Vol.10, pp. 163-173

Dinesh-Kumar, S.P.; Anandalakshmi, R.; Marathe, R.; Schiff, M. & Liu, Y. (2003). Virus-Induced gene silencing. *Methods in Molecular Biology*, Vol.236, pp.287-94

Diretto, G.; Al-Babili, S.; Tavazza, R.; Papacchioli, V.; Beyer, P. & Giuliano, G. (2007). Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. *PLoS ONE*, Vol.2, e350

Elías-López, A.L.; Marquina, B.; Gutiérrez-Ortega, A.; Aguilar, D.; Gomez-Lim, M. & Hernández-Pando, R. (2008). Transgenic tomato expressing interleukin-12 has a therapeutic effect in a murine model of progressive pulmonary tuberculosis. *Clinical and Experimental Immunology*, Vol.154, pp.123-133

Emmanuel, E. & Levy, A.A. (2002). Tomato mutants as tools for functional genomics. *Current Opinion in Plant Biology*, Vol.5, pp. 112-117

Enfissi, E. M. A.; Fraser, P. D.; Lois, L. M.; Boronat, A.; Schuch, W. & Bramley; P. M. (2005). Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids. *Plant Biotechnology Journal*, Vol.3, pp.17–27

Eshed, Y. & Zamir, D. (1995). An introgression line population of Lycopersicon pennellii in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. *Genetics*, Vol.141, pp. 1147-1162

Estornell, L.H.; Orzaez, D.; Lopez-Pena, L.; Pineda, B.; Anton, M.T.; Moreno, V. & Granell, A. (2009) A multisite gateway-based toolkit for targeted gene expression and hairpin RNA silencing in tomato fruits. *Plant Biotechnology Journal*, Vol.7, pp. 298-309

Fernandez, A.I.; Viron, N.; Alhagdow, M.; Karimi, M.; Jones, M.; Amsellem, Z.; Sicard, A.; Czerednik, A.; Angenent, G.; Grierson, D.; May, S.; Seymour, G.; Eshed, Y.;

Lemaire-Chamkey, M.; Rothan, C. & Hilson P. (2009). Flexible tools for gene expression and silencing in tomato. *Plant Physiology*, Vol. 151, pp. 1729-1740

Foolad, M.R. (2007). Genome Mapping and Molecular Breeding of Tomato. *International Journal of Plant Genomics*, doi:10.1155/2007/64358

Frary, A.; Nesbitt, T.C.; Grandillo, S.; van der Knaap, E.; Cong, B.; Liu, J.; Meller, J.; Elber, R.; Alpert, K.B. & Tanksley, S.D. (2000). fw2.2: A Quantitative Trait Locus Key to the Evolution of Tomato Fruit Size. *Science*, Vol.289, No.5476, pp. 85-88

Frary, A.; Xu, Y.; Liu, J.; et al. (2005). Development of a set of PCR-based anchor markers encompassing the tomato genome and evaluation of their usefulness for genetics and breeding experiments. *Theoretical and Applied Genetics*, Vol.111, No.2, pp. 291-312

Fraser, P. D.; Enfissi, E. M. & Bramley, P. M. (2009). Genetic engineering of carotenoid formation in tomato fruit and the potential application of systems and synthetic biology approaches. Archives of Biochemistry and Biophysics, Vol.483, pp.196-204

Fraser, P. D.; Enfissi, E. M.; Halket, J. M.; Truesdale, M. R.; Yu, D.; Gerrish, C. & Bramley, P. M. (2007). Manipulation of phytoene levels in tomato fruit: effects on isoprenoids, plastids, and intermediary metabolism. *Plant Cell*, Vol.19, pp.3194-3211

Fraser, P.D., Roemer, S., Shipton, C.A., Mills, P.B., Kiano, J.W., Misawa, N., Drake, R.G., Schuch, W. & Bramley, P. M. (2002). Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.99, pp.1092–1097

Fray, R. G.; Wallace, A.; Fraser, P. D.; Valero, D.; Hedden, P.; Bramley, P. M. & Grierson, D. (1995). Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. *Plant Journal*, Vol.8, pp.693–701

Fridman, E.; Carrari, F.; Liu, Y. S.; Fernie, A. R. & Zamir, D. (2004). Zooming in on a quantitative trait for tomato yield using interspecific introgressions. *Science*, Vol.305, pp.1786-1789

Fridman, E.; Pleban, T. & Zamir, D. (2000). A recombination hotspot delimits a wild-species quantitative trait locus for tomato sugar content to 484 bp within an invertase gene. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.97, pp.4718-4723

Fuentes, A. D.; Ramos, P. L.; Sanchez, Y.; Callard, D.; Ferreira, A.; Tiel, K.; Cobas, K.; Rodriguez, R.; Borroto, C.; Doreste, V. & Pujol, M. (2008). A transformation procedure for recalcitrant tomato by addressing transgenic plant-recovery limiting factors. Biotechnology Journal, Vol.3, pp. 1088-1093

Gady, A. L. F.; Hermans, F. W. K.; Van de Wal M. H. B. J.; van Loo, E. N.; Visser, R. G. F. & Bachem, C. W. B. (2009). Implementation of two high through-put tech- niques in a novel application: detecting point mutations in large EMS mutated plant populations. *Plant Methods*, Vol.5, pp. 1-14

Garcia, V.; Stevens, R.; Gil, L.; Gilbert, L.; Gest, N.; Petit, J.; Faurobert, M.; Maucourt, M.; Deborde, C.; Moing, A.; Poessel, J.L.; Jacob, D.; Bouchet, J.P.; Giraudel, J.L.; Gouble, B.; Page, D.; Alhagdow, M.; Massot, C.; Gautier, H.; Lemaire-Chamley, M.; Rolin, D.; Usadel, B.; Lahaye, M.; Causse, M.; Baldet, P. & Rothan, C. (2009). An integrative genomics approach for deciphering the complex interactions between

ascorbate metabolism and fruit growth and composition in tomato. *Comptes Rendus Biologies*, Vol.332, pp. 1007-1021

Gilbert, L.; Alhagdow, M.; Nunes-Nesi, A.; Quemener, B.; Guillon, F.; Bouchet, B.; Faurobert, M.; Gouble, B.; Page, D.; Garcia, V.; Petit, J.; Stevens, R.; Causse, M.; Fernie, A.R.; Lahaye, M.; Rothan, C. & Baldet, P. (2009). GDP-D-mannose 3,5-epimerase (GME) plays a key role at the intersection of ascorbate and non-cellulosic cell-wall biosynthesis in tomato. *The Plant Journal*, Vol.60, pp. 499-508

Giliberto, L.; Perrotta, G.; Pallara, P.; Weller, J. L.; Fraser, P. D.; Bramley, P. M.; Fiore, A., Tavazza, M. & Giuliano, G. (2005). Manipulation of the Blue Light Photoreceptor Cryptochrome 2 in Tomato Affects Vegetative Development, Flowering Time, and Fruit Antioxidant Content. *Plant Physiology*, Vol.137, pp.199–208

Giovannoni, J.J. (2007). Fruit ripening mutants yield insights into ripening control. *Current Opinion in Plant Biology*, Vol.10, No.3, pp. 283-289

Goetz, M.; Hooper, L. C.; Johnson, S. D.; Rodrigues, J.C.; Vivian-Smith, A. & Koltunow, A. M. (2007). Expression of aberrant forms of AUXIN RESPONSE FACTOR8 stimulates parthenocarpy in Arabidopsis and tomato. *Plant Physiology*, Vol.145, pp.351-366

Grandillo, S.; Tanksley, S.D. & Zamir, D. (2008). Exploitation of natural biodiversity through genomics, *In Genomics Assisted Crop Improvement*: *Vol I Genomics Approaches and Platforms* (R.K. Varshney & R. Tuberosa Ed.) Springer, The Netherlands: pp 121-150

Gupta, V.; Mathur, S.; Solanke, A.U.; Sharma, M.K.; Kumar, R.; Vyas, S.; Khurana, P.; Khurana, J.P.; Tyagi, A.K. & Sharma, A.K. (2009). Genome analysis and genetic enhancement of tomato. *Critical Reviews in Biotechnology*, Vol.29, No.2, pp 152-181

Gur, A. & Zamir D. (2004). Unused natural variation can lift yield barriers in plant breeding. *PLoS Biol*ogy,Vol.2, No.10, e245

Jang, X.; He, Z.; Peng, Z.; Qi, Y.; Chen, Q. & Yu, S. (2007). Cholera toxin B protein in transgenic tomato fruit induces systemic immune response in mice. *Transgenic Research,* Vol.16, pp.169- 175

Khan, R. S.; Nakamura, I. & Mii, M. (2011). Development of disease-resistant marker-free tomato by R/RS site-specific recombination. *Plant Cell Reports*, doi:10.1007/s00299-011-1011-4

Kramer, M.; Sanders, R.; Bolkan, H.; Waters, C., Sheeny, R. E. & Hiatt W. R. (1992). Postharvest evaluation of transgenic tomatoes with reduced levels of polygalacturonase: processing, firmness and disease resistance. *Postharvest Biology and Technology*, Vol.1, No.3, pp.241-255

Lai, L.; Huang, T.; Wang, Y.; Liu, Y.; Zhang, J. & Song, Y. (2009). The expression of analgesic-antitumor peptide (AGAP) from Chinese *Buthus martensii* Karsch in transgenic tobacco and tomato. *Molecular Biology Reports,* Vol.36, pp.1033-1039

Langley, K. R., Martin, A., Stenning, R.; Murray, A. J.; Hobson, G. E.; Schuch, W. W. & Bird, C. R. (1994). Mechanical and optical assessment of the ripening of tomato fruit with reduced polygalacturonase activity. Journal of the Science of Food and Agriculture, *Vol.66, No.4,* pp.547–554

Lenzi, P.; Scotti, N.; Alagna, F.; Tornesello, M.L.; Pompa, A.; Vitale, A.; De Stradis, A.; Monti, L.; Grillo, S.; Buonaguro, F.M.; Maliga, P. & Cardi T. (2008). Translational fusion of chloroplast-expressed human papillomavirus type 16 L1 capsid protein enhances

antigen accumulation in transplastomic tobacco. *Transgenic Research*, Vol.17, Issue No 6, pp.1091-102

Lippman, Z.B.; Semel, Y. & Zamir, D. (2007). An integrated view of quantitative trait variation using tomato interspecific introgression lines. *Current Opinion in Genetics & Development*, Vol.17, pp. 545-552

Liu, J.; Cong, B. & Tanksley, S. D. (2003). Generation and analysis of an artificial gene dosage series in tomato to study the mechanisms by which the cloned quantitative trait locus fw2.2 controls fruit size. *Plant Physiology*, Vol.132, pp.292-299

Liu, Y.; Roof, S.; Ye, Z.; Barry, C.; van Tuinen, A.; Vrebalov, J.; Bowler, C. & Giovannoni, J. (2004). Manipulation of light signal transduction as a means of modifying fruit nutritional quality in tomato. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.101, pp.9897-9902

Liu, Y.; Schiff, M. & Dinesh-Kumar, S. P. (2002). Virus-Induced gene silencing in tomato. *The Plant Journal*, Vol.31, Issue No 6, pp. 777-786

Lou, X.; Yao, Q.; Zhang, Z.; Peng, R.; Xiong, A. & Wang, H. (2007). Expression of the human hepatitis B virus large surface antigen gene in transgenic tomato plants. *Clinical and Vaccine Immunology,* Vol.14, Issue No.4, pp. 464-469

McCallum, C. M.; Comai L.; Greene, E. A. & Henikoff, S. (2000). Targeting induced local lesions in genomes (TILLING) for plant functional genomics. *Plant Physiology*, Vol.123, pp.439-442.

Menda, N.; Semel, Y.; Peled, D.; Eshed, Y. & Zamir, D. (2004). In silico screening of a saturated mutation library of tomato. *Plant Journal*, Vol.38, No.5, pp. 861-872

Minoia, S.; Petrozza, A.; D'Onofrio, O.; Piron, F.; Mosca, G.; Sozio, G.; Cellini, F.; Bendahmane, A. & Carriero, F. (2010). A new mutant genetic resource for tomato crop improvement by TILLING technology. *BMC Research Notes*, Vol.3, pp. 69

Mueller, L.A.; Lankhorst, R.K.; Tanksley, S.D. et al. (2009). A Snapshot of the Emerging Tomato Genome Sequence. *The Plant Genome*, Vol.2, No.1, pp. 78-92

Mueller, L.A.; Tanksley, S.D.; Giovannoni, J.J. et al. (2005). The Tomato Sequencing Project, the first cornerstone of the International Solanaceae Project (SOL). *Comparative and Functional Genomics*, Vol.6, No.3, pp. 153-158

Muir, S. R.; Collins, G. J.; Robinson, S.; Hughes, S. G.; Bovy, A. G.; de Vos, C. H.; van Tunen, A. J. & Verhoeyen, M. E. (2001). Overexpression of petunia chalcone isomerase in tomato results in fruit containing dramatically increased levels of flavonols. *Nature Biotechnology*, Vol.19, pp.470–474

Naqvi, S.; Farré, G.; Sanahuja, G.; Capell, T.; Zhu C. & Christou, P. (2009). When more is better: multigene engineering in plants. *Trends in Plant Science*, Vol. 15, No. 1, pp. 48-56

Neily, M. H.; Matsukura, C.; Maucourt, M.; Bernillon, S.; Deborde, C.; Moing, A., Yin, Y.-G.; Saito, T.; Mori, K., Asamizu, E.; Rolin, D.; Moriguchi, T. & Ezura, H. (2011). Enhanced polyamine accumulation alters carotenoid metabolism at the transcriptional level in tomato fruit over-expressing spermidine synthase. *Journal of Plant Physiology*, Vol.168, No.3, pp.242-252

Orzaez, D. & Granell, A. (2009). Reverse genetics and transient gene expression in fleshy fruits. *Plant Signaling and Behaviour*, Vol.4, Issue No 9, pp.864- 86

Orzaez, D.; Medina, A.; Torre, S.; Fernandez-Moreno, J.P.; Rambla, J.L., Fernandez-del-Carmen, A.; Butelli, E.; Martin, C. & Granell, A. (2009). A visual-reporter system for

virus-induced gene silencing in tomato fruit based on anthocyanin accumulation. *Plant Physiology*, Vol.150, pp. 1122.1134

Orzaez, D.; Mirabel, S.; Wieland, W.H & Granell, A. (2006). Agroinjection of tomato fruits. A tool for rapid functional analysis of transgenes directly in fruit. *Plant Physiology*, Vol.140, pp.3-11

Pan, L.; Zhang, Y.; Wang, Y.; Wang, B.; Wang, W.; Fang, Y.; Jiang, S.; Lv, J.; Wang, W.; Sun, Y. & Xie, Q. (2008). Foliar extracts from transgenic tomato plants expressing the structural polyprotein, P1-2A, and protease, 3C, from foot-and-mouth disease virus elicit a protective response in guinea pigs. *Veterinary Immunology and Immunopathology,* Vol.121, pp.83-90

Panthee, D.R. & Chen, F. (2010). Genomics of Fungal Disease Resistance in Tomato. *Current Genomics*, Vol.31, No.1, pp. 30, ISSN: 1389-2029

Paz De la Rosa, G.; Monroy-Garcia, A.; de Lourdes Mora-Garcia, M.; Reynaga Peña, C.G.; Hernández-Montes, J.; Weiss-Steider, B. & Gómez Lim, A.M. (2009). An HPV 16 L1-based chimeric human papilloma virus-like particles containing a string of epitopes produced in plants is able to elicit humoral and cytotoxic T-cell activity in mice. *Virology Journal,* Vol.6, pp.2

Peña Ramírez, Y.J.; Tasciotti, E.; Gutierrez-Ortega, A.; Donayre Torres, A.J.; Olivera Flores, M.T.; Giacca, M. & Gómez Lim, M.A. (2007). Fruit-specific expression of the human immunodeficiency virus type 1 tat gene in tomato plants and its immunogenic potential in mice. *Clinical and Vaccine Immunology,* Vol.14, pp.685-692

Perea Arango, I.; Loza Rubio, E.; Rojas Anaya, E.; Olivera Flores, T.; Gonzalez de la Vara, L. & Gomez Lim, M.A. (2008). Expression of the rabies virus nucleoprotein in plants at high-levels and evaluation of immune responses in mice. *Plant Cell Reports,* Vol.27, pp.677-685

Pineda, B.; Gimenez-Caminero, E.; Garcia-Sogo, B.; Anton, M.T.; Atares, A.; Capel, J. et al. (2010). Genetic and physiological charac- terization of the arlequin insertional mutant reveals a key regulator of reproductive development in tomato. *Plant Cell Physiology*, Vol.51, pp. 435–447

Pino, L.E.; Lombardi-Crestana, S.; Azevedo, M.S.; Scotton, D.C.; Borgo, L.; Quecini, V.; Figueira, A. & Peres, L.E.P. (2010). The *Rg1* allele as a valuable tool for genetic transformation of the tomato "Micro-Tom" model system. *Plant Methods,* Vol.6, pp.23

Piron, F.; Nicolai, M.; Minoia, S.; Piednoir, E.; Moretti, A.; Salgues, A.; Zamir, D.; Caranta, C. & Bendahmane, A. (2010). An induced mutation in tomato eIF4E leads to immunity to two potyviruses. *PLoS One*, Vol.5, No.6, pp. e11313

Qiu, D.; Diretto, G.; Tavarza, R.& Giuliano, G. (2007). Improved protocol for Agrobacterium mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene *CsZCD*. *Scientia Horticulturae,* Vol.112, pp.172-175

Ralley, L., Enfissi, E.M.; Misawa, N.; Schuch, W.; Bramley, P. M. & Fraser, P. D. (2004). Metabolic engineering of ketocarotenoid formation in higher plants. *Plant Journal*, Vol.39, pp.477-486

Rein, D.; Schijlen, E.; Kooistra, T.; Herbers, K.; Verschuren, L.; Hall, R.; Sonnewald, U.; Bovy, A. & Kleemann, R. (2006). Transgenic flavonoid tomato intake reduces C- reactive protein in human C-reactive protein transgenic mice more than wild-type tomato. *Jouirnal of Nutrition*, Vol.136, pp. 2331-2337

Rigano, M.M.; Manna, C.; Giulini, A.; Pedrazzini, E.; Capobianchi, M.; Castilletti, C.; Di Caro, A.; Ippolito, G.; Beggio, P.; De Giuli Morghen, C.; Monti, L.; Vitale, A. & Cardi, T (2009).Transgenic chloroplasts are efficient sites for high-yield production of the vaccinia virus envelope protein A27L in plant cells. *Plant Biotechnology Journal,* Vol.7, Issue No 6, pp. 577–591

Römer, S.; Fraser, P. D.; Kiano, J. W.; Shipton, C. A.; Mills, P. B.; Drake, R.; Schuch, W. & Bramley, P. M. (2000). Elevation of the provitamin A content of transgenic tomato plants. *Nature Biotechnology*, Vol.18, pp.666–669

Ronen, G.; Carmel-Goren, L.; Zamir, D. & Hirschberg, J. (2000). An alternative pathway to β-carotene formation in plant chromoplasts discovered by map-based cloning of beta and old-gold color mutations in tomato. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.97, pp.11102–11107

Rosati, C.; Aquilani, R.; Dharmapuri, S.; Pallara, P.; Marusic, C.; Tavazza, R.; Bouvier, F.; Camara, B. & Giuliano, G. (2000). Metabolic engineering of β-carotene and lycopene content in tomato fruit. *Plant Journal*, Vol.24, pp.413–420

Ruf, S.; Hermann, M.; Berger, I.J.; Carrer, H. & Bock, R. (2001). Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nature Biotechnology,* Vol.19, pp.870- 875

Saito, T.; Ariizumi, T.; Okabe, Y.; Asamizu, E.; Hiwasa-Tanase, K. Fukuda, N.; Mizoguchi, T.; Yamazaki, Y.; Aoki, K. & Ezura, H. (2011). TOMATOMA: A Novel Tomato Mutant Database Distributing Micro-Tom Mutant Collections. *Plant and Cell Physiology*, Vol.52, No.2, pp. 283-296

Salyaev, R.K.; Rigano, M.M. & Rekoslavskaya, N.I. (2010) Development of plant-based mucosal vaccines against widespread infectious diseases. *Expert Reviews of Vaccines*, Vol.9, Issue No 8, pp.937-46

Schauer, N.; Semel, Y.; Roessner, U.; Gur, A.; Balbo, I.; Carrari, F.; Pleban, T.; Perez-Melis, A.; Bruedigam, C.; Kopka, J.; Willmitzer, L.; Zamir, D. & Fernie, A. R. (2006). Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. *Nature Biotechnology*, Vol.24, pp.447-454

Schijlen, E.; de Vos C.H.R; Jonker, H.; van den Broeck, H.; Molthoff, J.; van Tunen, A.; Martens, S. & Bovy,A. (2006). Pathway engineering for healthy phytochemicals leading to the production of novel flavonoids in tomato fruit. *Plant Biotechnology Journal*, Vol.4, pp. 433-444

Scotti, N.; Alagna, F.; Ferraiolo, E.; Formisano, G.; Sannino, L.; Buonaguro, L.; De Stradis, A.; Vitale, A.; Monti, L.; Grillo, S.; Buonaguro, F.M. & Cardi, T. (2009). High-level expression of the HIV-1 Pr55gag polyprotein in transgenic tobacco chloroplasts. *Planta*, Vol.229, Issue No 5, pp.1109-22

Sharma, M.K.; Jani, D.; Thungapathra, M.; Gautam, J.K.; Meena, L.S.; Singh, Y.; Ghosh, A.; Tyagi, A.K.; Sharma, A.K. (2008a). Expression of accessory colonization factor subunit A (ACFA) of *Vibrio cholerae* and ACFA fused to cholera toxin B subunit in transgenic tomato (*Solanum lycopersicum*). *Journal of Biotechnology,* Vol.135, pp.22-27

Sharma, M.K.; Singh, N.K.; Jani, D.; Sisodia, R.; Thungapathra, M.; Gautam, J.K.; Meena, L.S.; Singh, Y.; Ghosh, A.; Tyagi, A.K.; Sharma, A.K. (2008b). Expression of toxin co-regulated pilus subunit A (TCPA) of *Vibrio cholerae* and its immunogenic epitopes fused to cholera toxin B subunit in transgenic tomato (*Solanum lycopersicum*). *Plant Cell Reports*, Vol.27, pp.307-318

Sharma, M.K.; Solanke, A.U.; Jani, D.; Singh, Y.; Sharma, A.K. (2009). A simple and efficient *Agrobacterium*-mediated procedure for transformation of tomato. *Journal of Biosciences,* Vol.34, Issue No 3, pp.423-433

Shchelkunov, S.N.; Salyaev, R.K.; Posdnyakov, S.G.; Rekoslavskaya, N.I.; Nesterov, A.E.; Ryzhova, T.S.; Sumtsova, V.M.; Pakova, N.V.; Mishutina, U.O.; Kopytina, T.V. & Hammond, R.W. (2006). Immunogenicity of a novel, bivalent, plant-based oral vaccine against hepatitis B and human immunodeficiency viruses. *Biotechnology Letters*, Vol.28, pp.959-967

Simkin, A. J.; Gaffé, J.; Alcaraz, J. P.; Carde, J. P.; Bramley, P. M.; Fraser, P. D. & Kuntz, M. (2007). Fibrillin influence on plastid ultrastructure and pigment content in tomato fruit. *Phytochemistry*, Vol.68, No.11, pp.1545-1556

Smith, C. J. S.; Watson, C. F.; Bird, C. R.; Ray, J.; Schuch, W. & Grierson, D. (1990). Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. *Molecular and General Genetics*, Vol.224, No.3, pp.477-481

Smith, D. L.; Abbott, J. A. & Gross, K. C. (2002). Down-regulation of tomato beta-galactosidase 4 results in decreased fruit softening. *Plant Physiology,* Vol.129, pp.1755-1762

Soria-Guerra, R.E.; Rosales-Mendoza, S.; Márquez-Mercado, C.; López-Revilla, R.; Castilli-Collazo, R. & Alpuche-Solís, AG. (2007). Transgenic tomatoes express an antigenic polypeptide containing epitopes of the diphtheria, pertussis and tetanus exotoxins, encoded by a synthetic gene. *Plant Cell Reports,* Vol.26, pp.961-968

Soria-Guerra, R.E.; Rosales-Mendoza, S.; Moreno-Fierros, L.; López-Revilla, R. & Alpuche-Solís, A.G. (2011). Oral immunogenicity of tomato derived sDPT polypeptide containing *Corynebacterium diphteriae*, *Bordetella pertussis* and *Clostridium tetani* exotoxin epitopes. *Plant Cell Reports,* Vol.30, pp.417-424

Sun, H.; Uchii, S.; Watanabe, S. & Ezura, H. (2006). A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. *Plant and Cell Physiology,* Vol.47, Issue No 3, pp.426-431

Tieman, D. M.; Handa, A. K. (1994). Reduction in pectin methyltransferase activity modifies tissue integrity and cation levels in ripening tomato (Lycopersicon esculentum Mill.) fruits. Plant Physiology, Vol.106, pp.429-436

Tieman, D. M.; Zeigler, M.; Schmelz, E. A.; Taylor, M. G.; Bliss, P.; M.Kirst & Klee H. J. (2006). Identification of loci affecting flavour volatile emissions in tomato fruits. *Journal of Experimental Botany,* Vol.57, pp.887-896

Van Eck, J.; Kirk, D.D. & Walsmley, A.M. (2006). Tomato (*Lycopersicum esculentum*). *Methods in Molecular Biology,* Vol.343, pp.459-4773

Varshney, R. K.; Nayak, S. N.; May, G. D. & Jackson, S. A. (2009). Next-generation sequencing technologies and their implications for crop genetics and breeding. *Trends in Biotechnology,* Vol.27, No.9, pp.522-530

Verpoorte, R.; van der Heijdenvan, R. & Memelink, J. (2000). Engineering the plant cell factory for secondary metabolite production. *Transgenic Research*, Vol.9, pp. 323-343

Wang, H.; Jones, B.; Li, Z.; Frasse, P.; Delalande, C.; Regad, F.; Chaabouni, S.; Latche, A.; Pech, J. C. & Bouzayen, M. (2005). The tomato Aux/IAA transcription factor IAA9 is involved in fruit development and leaf morphogenesis. Plant Cell, Vol.17, pp.2676-2692

Wang, S.; Liu, J.; Feng, Y.; Niu, X.; Giovannoni, J. & Liu, Y. (2008). Altered plastid levels and potential for improved fruit nutrient content by downregulation of the tomato DDB1-interacting protein CUL4. The Plant Journal, *Vol.55, No.1,* pp.89–103

Watanabe, S.; Mizoguchi, T.; Aoki, K.; Kubo, Y.; Mori, H.; Imanishi, S.; Yamazaki, Y.; Shibata, D.; Ezura, H. (2007). Ethylmethanesulfonate (EMS) mutagenesis of Solanum lycopersicum cv. Micro-Tom for large-scale mutant screens. *Plant Biotechnol*ogy, Vol.24, pp. 33–38

Wroblewski, T.; Tomczak, A. & Michelmore, R. (2005). Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*. *Plant Biotechnology Journal,* Vol. 3, pp.259-273

Wu, F. & Tanksley, S.D. (2010). Chromosomal evolution in the plant family Solanaceae. *BMC Genomics*, Vol.17, No.11, pp. 182

Wu, Y.; He, Y. & Ge, X. (2011). Functional characterization of the recombinant antimicrobial peptide Trx-Ace-AMP1 and its application on the control of tomato early blight disease. *Applied Microbiology and Biotechnology*, doi:10.1007/s00253-011-3166-x

Wurbs, D.; Ruf, S. & Bock, B. (2007). Contained metabolic engineering in tomatoes by expression of carotenoid biosynthesis genes from the plastid genome. *The Plant Journal*, Vol.49, pp.276-288

Yasmeen, A.; Mirza, B.; Inayatullah, S.; Safdar, N.; Jamil, M.; Ali, S. & Choudhry, M.F. (2009). In planta transformation of tomato. *Plant Molecular Biology Reporter*, Vol.27, pp.20–28

Youm, J.W.; Jeon, J.H.; Kim, H.; Kim, Y.H.; Ko, K.; Joung, H. & Kim, H. (2008). Transgenic tomatoes expressing human beta-amyloid for use as a vaccine against Alzheimer`s disease. *Biotechnology Letters*, Vol.30, pp.1839 – 1845

Zanor, M. I.; Osorio, S.; Nunes-Nesi, A.; Carrari, F.; Lohse, M.; Usadel, B.; Kuhn, C.; Bleiss, W.; Giavalisco, P.; Willmitzer, L.; Sulpice, R.; Zhou, Y. H. & Fernie, A. R. (2009). RNA interference of LIN5 in tomato confirms its role in controlling Brix content, uncovers the influence of sugars on the levels of fruit hormones, and demonstrates the importance of sucrose cleavage for normal fruit development and fertility. *Plant Physiology*, Vol.150, pp.1204-1218

Zhang, C.; Liu, J.; Zhang, Y.; Cai, X.; Gong, P., Zhang, J.; Wang, T.; Li, H. & Ye, Z. (2011). Overexpression of SlGMEs leads to ascorbate accumulation with enhanced oxidative stress, cold, and salt tolerance in tomato. *Plant Cell Reports*, Vol.30, pp. 389-398

Zhang, X.; Buehner, N.A.; Hutson, A.M.; Estes, M.L. & Mason, H.S. (2006). Tomato is a highly effective vehicle for expression and oral immunization with Norwalk virus capsid protein. *Plant Biotechnology Journal,* Vol.4, pp.419-432

Zhang, X.; Li, H.; Zhang, J.; Zhang, C.; Gong, P.; Ziaf, K.; Xiao, F. & Ye, Z. (2010). Expression of artificial microRNAs in tomato confers efficient and stable virus resistance in a cell-autonomous manner. *Transgenic Research*, doi:10.1007/s11248-010-9440-3

Zhou, F.; Badillo-Corona, J.A.; Karcher, D.; Gonzalez-Rabade, N.; Piepenburg, K.; Borchers, A.M. Maloney, A.P.; Kavanagh, T.A.; Gray, J.C. & Bock, R. (2008). High-level expression of human immunodeficiency virus antigens from the tobacco and tomato plastid genomes. *Plant Biotechnology J*ournal, Vol.6, pp.897-913.

4

Genetic Transformation Strategies in Fruit Crops

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1. Introduction

Genetic transformation provides the means for adding single horticultural traits in existing cultivars without modify their commercial characteristics. This capability is particularly valuable for perennial plants and fruit tree species, in which conventional breeding is hampered by their long generation time and juvenile periods, complex reproductive biology, high levels of heterozygosity, limited genetic sources and linkage drag of undesirable traits from wild relatives. In addition, gene transfer technologies for fruit tree species take the inherent advantage of vegetative propagation used for their reproduction, which allowed for the application of a high scale production of the desired transgenic line starting from one successful transformed line. Despite this opportunity, final setting of transformation protocols in this type of species, endures major limiting factors preventing the development of new varieties: a) explants recalcitrance to regenerate adventitious transformed shoots and b) a limited regeneration capability, usually extended to just few genotypes (i.e. cultivar dependence).
This chapter illustrates the road between the establishment of transformation methodologies on particular species of *Vitis* spp. and *Prunus* spp. and their use as technical baselines for achievement of transformation procedures in new, eventually more recalcitrant, cultivars or genus members.

1.1 Genetic transformation of fruits in the current research era

Genetic improvement of fruit trees is essential for increasing fruit production. For most of these species, the desired new varieties contemplate the presence of agronomic and horticultural traits related to propagation, yield, appearance, quality, disease and pest control, abiotic stress and shelf-life. Incorporation of these traits into the genetic backgrounds of species by conventional breeding needs overcome some major disadvantages, including long juvenile periods and reduced possibility of introgression of the suitable traits (when available) into commercially relevant cultivars. Although currently the use of new technologies based on high throughput platforms for sequencing and genotyping has deeply contributed to accelerate the association of molecular markers and major genes to these relevant traits, there exists a bottle neck in this strategy when

phenotyping must be carried out. In addition, breeding by controlled crosses is hampered due to factors specifically related to complex characteristics belonging to these species, such as delayed flowering, unsuccessful fruit setting due to abortive embryos, massive fruit drop, and self-incompatibility barriers found in many of them.

Genetic transformation represents inherent advantages for fruit tree improvement, although in fruit trees this area of research is not a routine procedure. The transversal negative perception about the "transgenic technology" is added to an additional degree of difficulty for setting up adequate technical systems in fruit tree species. Eventually, if a proper regenerative system has been established, any DNA construct designed for either a major gene over-expression or gene silencing (i.e. interfering RNA´s *in vivo* generation) can be introduced into a desired genome. Consequently, the feasibility of genetic modification relies on adequate technical systems which allowed for results in a reasonable time frame. Regardless the final objectives of transformed events (a product or fundamental research), highly regenerative systems for explants production and whole plants regeneration are key steps of fruit tree genetic transformation. In addition, the relevance of these procedures is even higher when an era of candidate genes evaluation has begun as a result of the current knowledge about genomes.

2. Use of grapevine systems as a model in fruit species

2.1 Grapevine somatic embryogenesis and genetic transformation of somatic embryos

Since its first report in 1976 (Mullins and Srinivasan, 1976), somatic embryogenesis (SE) in *Vitis vinifera* L. has been described in different cultivars and their hybrids (Martinelli and Gribaudo, 2001, 2009), becoming the most efficient procedure for the generation of *in vitro* cultures prone to genetic transformation (Stamp and Meredith, 1988; Scorza *et al*., 1996; Martinelli *et al*., 2001a; Iocco *et al*., 2001; Torregrosa *et al*., 2002; Hinrichsen *et al*., 2005, Li *et al*., 2008). As described by Ammirato (1983), SE is understood as the initiation of embryos from plant somatic tissues closely resembling their zygotic counterparts. As a fruit species, grapevine SE is not a routine procedure that can be easily and efficiently reproduced among different cultivars (Martinelli *et al*., 2001; Araya *et al*., 2008). Grapevine SE has been successfully reached using as source explants stamens and pistils (Rajasekaran and Mullins, 1983; Martinelli *et al*., 2001a, Araya *et al*., 2008), unfertilized ovules (Mullins and Srinivasan, 1976), ovaries (Martinelli *et al*., 2001), leaves (Martinelli *et al*., 1993), petioles (Martinelli *et al*., 1993), and tendrils (Salunkhe *et al*., 1997). In the conventional approach, grapevine SE is induced to the generation of pro-embryogenic (PE) and embryogenic (E) cell masses by cultivation of these explants in solid X6 medium using TC agar Petri dishes for 30 days (Li *et al*., 2001). X6 corresponds to a modified MS (Murashige and Skoog, 1962) medium lacking glycine and supplemented with KNO_3 and NH_4Cl as the sole nitrogen source, in addition to sucrose, myo-inositol, and activated charcoal. For transformation, cells are pre-conditioned by a seven days treatment in DM (Driver and Kuniyuki, 1984) solid medium and then infected with *Agrobacterium tumefaciens* by immersion of explants in liquid DM medium containing the bacteria. After two days in co-cultivation, an early selection is applied using solid DMcck (DM medium supplemented with carbenicillin, cefotaxime, and kanamycin) medium for 21 days. Transformed cells are, again, induced to generate E cells in solid X6

medium up to appearance of mature structures and fully developed somatic embryos, which are picked up and harvested as putative transgenic lines (Figure 1).
SE process based on the use of Petri dishes is illustrated for both leaves and inflorescences as source explants. This process leads to somatic embryo generation (embryogenic masses) prone for *Agrobacterium* infection. After co-cultivation, a selection step is applied by 21 days. Afterwards, embryo development leading to the regeneration of whole plants, acclimatization and field trial of the obtained individuals are carried out. The field trial shown in Figure 1 corresponds to an assay of genetically modified plants generated to introduce tolerance to the fungus *Botrytis cinerea* carried out since 2004 at La Platina Station in the National Institute of Agriculture, Santiago, Chile.

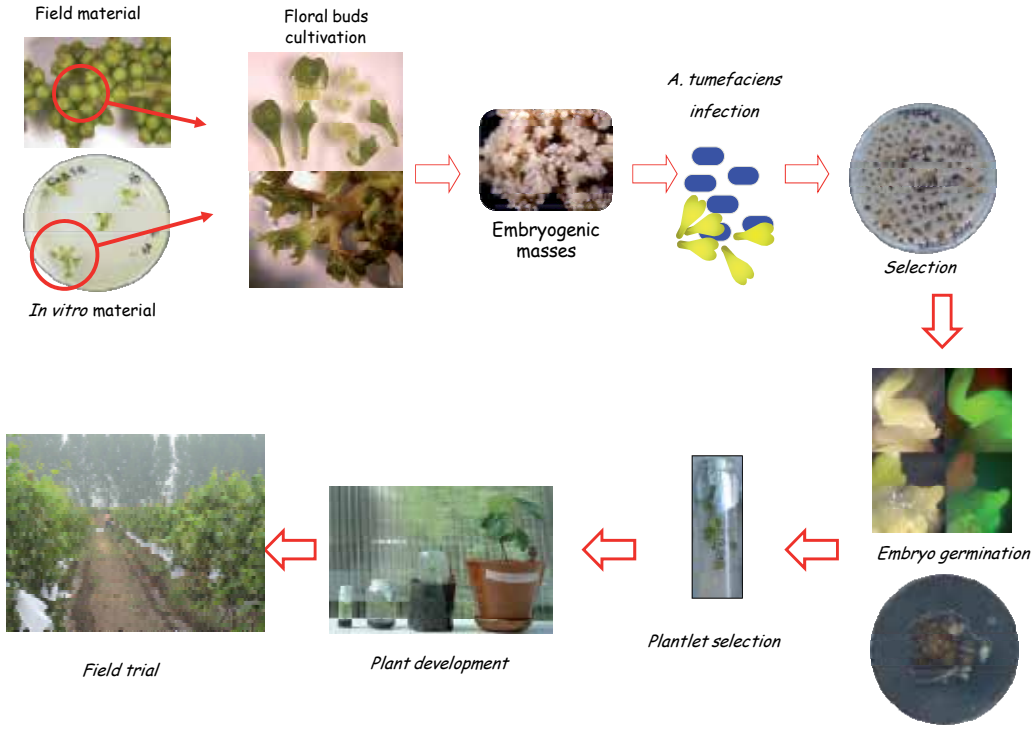

Fig. 1. Conventional work flow involved in somatic embryogenesis (SE) and *Agrobacterium*-mediated genetic transformation of grapevine ´Thompson Seedless´.

2.2 Current requirements in grapevine genetic transformation

The convergence of genome sequencing studies on *V. vinifera* cv. ´Pinot Noir´ (Jaillon *et al.*, 2007; Velasco *et al.*, 2007) and a high through-put transformation pipeline to carry out the evaluation of candidate genes, seem a current major priority. The SE-mediated transformation process of grapevine has not been directed to the massive generation of transformable explants. Improvements to the technology have recently showed up by maintenance of embryogenic cultures using suspension cultures in flasks. These efforts did not report major morphological or anatomical differences in the generated E and PE masses when compared to the above described procedures using solid media (Jayasankar *et al.*,

2003). Recently, Li *et al*. (2008) improved this fundamental methodology by introduction of a flask-based calli processing before starting the second round of sub-culturing in solid X6 medium. This additional step consisted of four consecutive sub-cultures using agitated flasks containing DMcck; i.e. under a permanent selection by increasing the kanamycin working concentrations (in grapevines this is referred as concentrations from 20 to 50 mg/L). A final selective pressure is applied (for instance 100 mg/L) by addition of kanamycin to solid DMcck Petri dishes. The processed calli were reinserted into the second round of culturing in solid X6 medium dishes, until embryo harvesting and development for evaluation.

In general terms, the whole process requires a total time of 24 to 25 weeks and leads to the generation of candidate genetically modified plantlets ready for a primary, PCR-based, screening.

Regardless the strategy (solid media Petri dishes or solid media plus the inclusion of a flasks' step), both routes share a critical and consistent problem referred to the massive generation of adequate amounts of E or PE masses supporting routine transformation experiments.

2.3 Optimizing somatic embryogenesis platforms. Different strategies

It is accepted that the developmental stage of source explants is of great importance in grapevine SE setting (Martinelli and Gribaudo, 2009). Commonly, SE systems in grapes are initiated from stamens and pistils and responses are variety-dependent. The best developmental stages to initiate embryogenic cultures have been deduced for some genotypes using the basis of phenological stages of inflorescences (Dhekney *et al*., 2009); whereas stamens and pistils from some cultivars such as ´Pinot Gris´ must be collected at early developmental stages; other genotypes such as ´Merlot´, ´Sauvignon Blanc´ and ´Freedom´ must be induced using explants at more advanced maturity stages.

In vitro leaves have been also proposed as source explants for SE induction in grapevines. Although lower efficiencies than stamens and pistils have been obtained, the use of unopened leaves (i.e. between 1.5 to 5.0 mm long) placed abaxial side down on Petri dishes supplemented with solid NB2 medium leads to the generation of PE masses that later will regenerate into whole plants in ´Superior Seedless´, ´Thompson Seedless´ and ´Freedom´ genotypes (Dhekney *et al*., 2009). Alternatively, convenient procedures to introduce material from the field have been reached by proper cultivation of grapevine sterile buds in solid C2D4B medium (Araya *et al*., 2008; Gray, 1995) by one to four months and then culturing the processed tissues into NB2 solid medium.

The use of induction media based on modified MS or Nitsch (described by Li *et al*., 2008), established the basis for additional improvements in grapevine SE. This time, authors were focused on the yield of the system. Solid cultures are heterogeneous and diffusion-limited; on the other hand, agitated liquid cultures, involve mainly faster, more uniform, efficient, and controllable mass transfer processes. It is accepted that use of liquid cultures offers numerous technical advantages over solid cultures (Archambault *et al*., 1994). However, the actual evaluation of embryogenic protocols must be carried out on the basis of volumetric productions, true plant organ (i.e. torpedo shape, Jayasankar *et al*., 2003), system homogeneity, and finally, conversion of embryo cells into whole plants (Archambault *et al*., 1994). In ´Thompson Seedless´, application of an induction period by six weeks using Li´s

modifications generated PE masses that are transferred into maintenance liquid media based on B5 major salts (Gamborg *et al.*, 1968) and vitamins from MS. These flask assays have been the basis for the generation of an air-lift bioreactor as recently described Tapia *et al.* (2009). The system was designed to improve biomass production of ´Thompson Seedless´ somatic embryos and, at the same time, enabled a preliminary characterization of cell´s behavior during the grapevine SE time-course. The very first improvement derived from the use of liquid systems was that biomass multiplication rate decreased from 60 to about 40 days due to the use of agitated flasks (Figure 2a). Even better, the use of an air-lift bioreactor improved this rate to seven days (Tapia *et al.*, 2009). In addition, a lower than expected sugar consumption was observed during the SE process, suggesting side roles for this substrate during culturing. Li´s procedures described that flasks culturing leaded to the generation of up to 400 mg of biomass, obtaining PE and E cells; in those experiments, bigger inocula leaded to explants' oxidation. On the contrary, the 2 L vessel's reactor (Figure 2b) regularly admitted 2 g inocula without affecting the process, including explants viability and duplication of this biomass at the seventh day of batching. Genetic transformation procedures of somatic embryos obtained from this system did not show any difference compared to explants produced by the regular solid media-based system (depicted in Figure 1), generating fully regenerated transgenic plants (Tapia *et al.*, 2009).

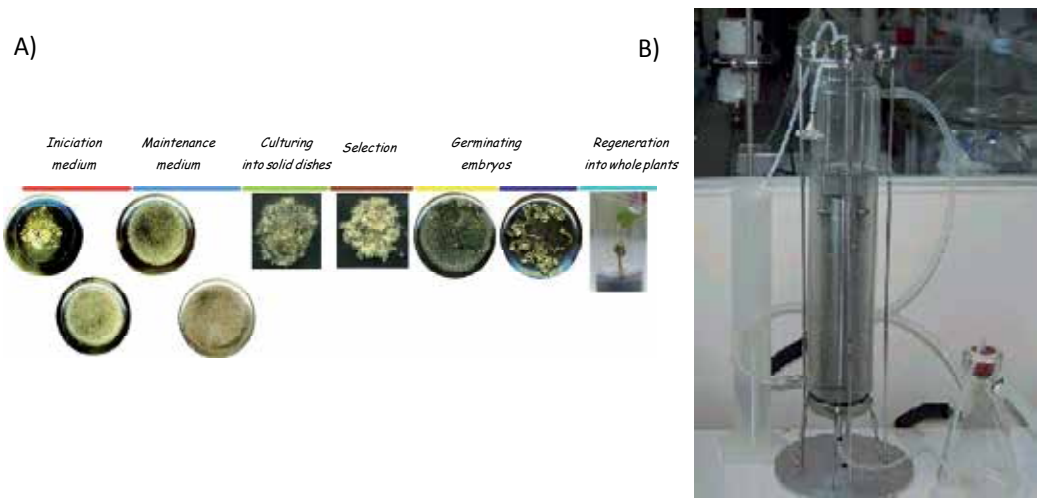

Fig. 2. Improved pipeline for somatic embryogenesis and *Agrobacterium*-mediated genetic transformation of grapevine ´Thompson Seedless´ and ´Princess´ using liquid media (A) and an air-lift bioreactor (B). Main steps are indicated.

2.4 Inducing somatic embryogenesis in recalcitrant germplasms. New strategies

Embryogenic competence can be considered as an exception more than a rule. Several genotypes have shown low or null responses to protocols that have been successfully evaluated in certain varieties. An optimized SE procedure for ´Thompson Seedless´ (Figure 3a) is not as efficient as applied on ´Red Globe´ (Figure 3b). Analyses of factors affecting competence have been recently reported (Dhekney *et al.*, 2009) by exploring changes in the induction phase. The use of MS and Nitsch macro- and micro-elements supplemented with

6-bencyl aminopurine (BAP) at 4.5 and 8.9 µM plus 2,4-dichlorophenoxy acetic acid (2,4-D) at 8.9 and 4.5 µM, respectively, leaded to most of the 18 evaluated cultivars and eight *Vitis* hybrids, to produce SE to some degree in that work. In that line, from the learning process to design and build an air-lift system, the use of liquid media offered important data about the effect of different nutrients applied in closer contact with grapevine PE cells. These findings have leaded to the use of mixed (i.e. having solid and liquid steps) protocols on diverse low-competence backgrounds such as ´Freedom´ or ´Red Globe´.

Fig. 3. Differential results of SE procedures in ´Thompson Seedless´ and ´Red Globe´.
A, An efficient SE system in ´Thompson Seedless´ generating PE and E cells (right picture, whitest cells); B, the same system applied on ´Red Globe´ showing an inefficient result.

The new strategies include the above referred use of *in vitro* leaf explants as a source of starting material for SE. Multiple *in vitro* buds are obtained from *in vitro* leaves using C2D4B (Araya *et al.*, 2008; Gray, 1995) by at least 30 days of cultivation. When these buds are transferred to solid NB2 media supplemented with 2,4-D (4.5 µM) and BAP (1 µM) by additional 120 days, PE masses are generated. These masses are the pivot for a new branch of procedures in which solid and liquid cultures are used. In ´Thompson Seedless´ or ´Princess´, bud production by culturing of leaves in C2D4B media prepare cells that will produce as many somatic embryos as required during the phase of cultivation in NB2 medium; these cultivars describe high yield enough to accomplish considerable number of transformation experiments (Dhekney *et al.*, 2009) (Figure 3a). However, such yield is not observed when the strategy is applied on ´Harmony´, ´Freedom´ or ´Red Globe´ genotypes (Figure 4a). Although very few somatic embryos are prone for transformation purposes from these materials (see whitest cells in the right picture on Figure 3b), the strategy leads to the production of a considerable remaining material, which in our hands was formerly discarded for transformation assays (Figure 4a). After several trials for recovering these masses and re-convert them into SE competent cells, a highly productive cycle was observed by culturing them solid X6 media and the addition of a liquid pulse of MS modified medium supplemented with glutamine (400 mg/L) and kinetin (4.6 µM). Under such treatments, cells are propagated, disintegrated into minimal groups of cells and leaded to

new developmental stages; as a result, the generation of an extremely high amount of somatic embryos after 30 days of treatment is achieved (Figure 4b). Although these procedures have fixed the competence for SE in these genotypes, it remains to be evaluated if such combined treatments could help this process in other grape genotypes. In the meantime, genetic transformation of grapevine rootstocks is now plausible and efforts evaluating genes related to root-linked disorders can be now evaluated.

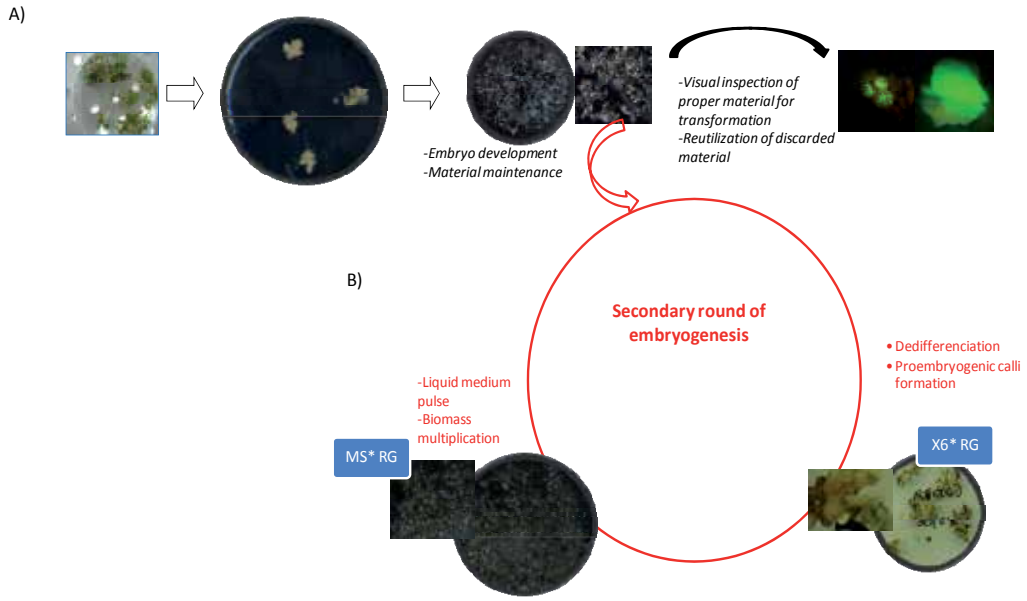

Fig. 4. Improved procedures to induce somatic embryogenesis in ´Red Globe ´. The low efficiency protocol to induce and transform embryos (black arrows, A) leads to the formation of remaining cells that can be incorporated into a new round of SE based on the use of glutamine and kinetin (red circle in the diagram, B) for yield improvement.

3. Genetic transformation of plums in the waiting for a peach transformation system

It has already been almost 20 years since the generation of the most highlighted event in the field of stone fruits genetic transformation: the *Plum Pox Virus* (PPV) *Prunus domestica* resistant line named C5. Obtained by transformation of ´Bluebyrd´ explants, C5 was derived from transformation events using *A. tumefaciens* strain C58/Z707 containing a binary plasmid with the coat protein (CP) PPV gene plus the 3' non-translated region of the viral genome (EMBL Accession No X16415, Teycheney *et al*., 1989). The high degree of resistance observed has been stable in more than 10 years of evaluations in greenhouses and in the field (Hily *et al*., 2004; Malinowski *et al*., 2006; Polák *et al*.; 2008). In C5, the clone has exhibited resistance associated to RNA interference (RNAi), i.e. a high level of transgene transcription in the nucleus associated to a low level of mRNA in the cytoplasm, whereas the genetic analysis has revealed multicopy transgene insertions with repeated sequences in the presence of additional one or more aberrant copies (Scorza *et al*. 2001). RNAi in C5 was

later confirmed by detection of small interfering RNAs (siRNAs) homologous to PPV sequences (Hily *et al*., 2005); siRNAs of 22 and 25-26 nucleotides in length were found in challenged C5 plants, whereas short siRNAs were found exclusively in wild type infected *P. domestica* controls. Results suggested that the high level virus resistance in C5 is connected with the production of this long-sized class of siRNAs (Hily *et al*., 2005). Recently, multicopy arrangements of T-DNA fragments from the transformation plasmid in the C5´s genome have been determined, showing the occurrence of an *in planta* hairpin structure of the introduced CP gene (Kundu *et al*., 2008; Scorza *et al*., 2010) and explaining the possible source for RNAi in C5.

Multiple tissues have been used for plant regeneration in the *Prunus* genus, including leaves (Gentile *et al*., 2002; Yancheva, 2002; Dolgov *et al*., 2005), cotyledons (Mante *et al*., 1989), embryos (Pérez-Clemene *et al*., 2004), and hypocotyls (Mante *et al*., 1991; Gonzalez-Padilla *et al*., 2003). As a system, generation of C5 represents to date the more successful and reproducible procedure for plant regeneration in this genus, obtained by the use of medial hypocotyl segments (Mante *et al*., 1991). To get this procedure, 1-phenyl-3-(1,2,3-thidiazol-5-il) urea (thidiazuron, TDZ) at 7.5 μM and indole-3-butyric acid (IBA) at 0.25 μM were applied to the explants in order to regenerate *Agrobacterium*-mediated transformed hypocotyl segments. The hypocotyls based system was later improved by González-Padilla *et al.* (2003); these authors described a reduction in the total number of regenerated shoots without affecting the transformation rate by application of early selection (80 mg/L of kanamycin and 300 mg/L of Timentin) during shoot regeneration in a medium consisting on half-strength MS salts and vitamins supplemented with 5 mM α-naphthaleneacetic acid (NAA) and 0.01 mM kinetin.

From these works, new improvement was made in the diploid species *Prunus salicina*. The regeneration and genetic transformation of Japanese plum using the approach of hypocotyl segments described in *P. domestica* was described by Urtubia *et al.* in 2008 (Figure 5). Previously, Tian *et al.* (2007) used a constant concentration of TDZ (7.5 μM) that eventually was combined with variable amounts of BAP (2.5 or 12.5 μM) or kinetin (12.5 μM) to induce shoot formation in ´Shiro´ and ´Early Golden´; additional evaluation of ´Redheart´ hypocotyl segments did not generate plantlets by this protocol. In successful genotypes, results demonstrated that the use either of TDZ or of TDZ plus BAP have allowed the establishment of whole plants acclimatized at greenhouse level. For transformation setting up of this species, Urtubia *et al.* (2008) described the use of different TDZ/IBA ratios (6:1 to 10:1) to regenerate *Agrobacterium* transformed ´Angeleno´ and ´Larry Ann´ hypocotyl segments. The shooting on average on 12% of the total cultured explants and the establishment of whole plants expressing the green fluorescent protein in the greenhouse concluded this research and established Japanese plum as a model diploid species in *Prunus* transformation. Despite this, the observed varietal dependence of results and the low number of confirmed positive transgenic lines ratified the difficulty to obtain genetic transformation systems in stone fruit species. Authors also described the effect of using different *Agrobacterium* strains on the co-cultivated *P. salicina* explants; whereas the use of LBA4404 strain leaded to significant oxidation in the treated explants, infections with EHA or GV strains led to the production of whole plants with no major disadvantages throughout the protocol.

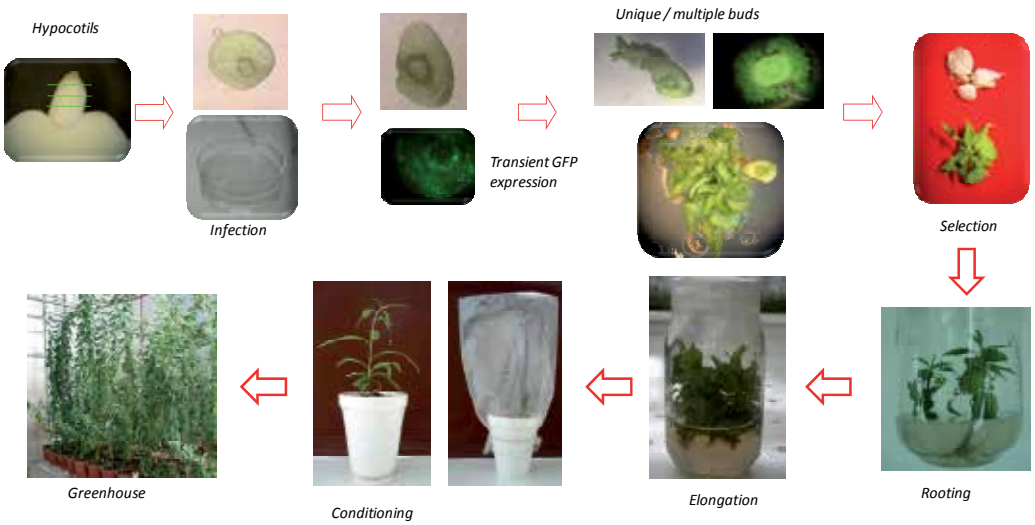

Fig. 5. ´Angeleno´ Japanese plum genetic transformation procedures. The complete pipeline for *Agrobacterium*-mediated genetic transformation of *P. salicina* is showed.

3.1 A new example: RNAi-based PPV resistance in Japanese plums

Holding the research focus on PPV, new strategies against the virus can be evaluated with improved expectations, including new technology development obtained for gene silencing. As mentioned, an additive effect of multicopy T-DNA fragments arranged in C5´s genome and the occurrence of an *in planta* hairpin structure for the introduced CP gene (Kundu *et al*., 2008; Scorza *et al*., 2010) onset an adequate silencing scenario in the plant. Hairpin RNA inducing DNAs have been described as an important activator of RNAi in transgenic plants (Watson *et al*., 2005). Artificial *in vivo* generation of siRNA was soon published by introduction of hairpin RNA inducing plasmids into target cells (Wesley *et al*., 2001); since then, multiple findings have been focused on the development of optimal constructs to generate siRNAs in transgenic plants (Watson *et al*., 2005; Qu *et al*., 2007), and making gene silencing through RNAi one of the most promissory strategies to boost plant immunity nowadays (Pennisi, 2010).

Recently, Dolgov *et al.* (2010) developed and used a coat protein gene-based hairpin inducing plasmid to generate RNAi transgenic *P. domestica* L. 'Startovaya' plants. This group had previously generated a transformation protocol for European plum using adult tissue-derived explants by use of 5-12 days old leaves (Mikhaylov and Dolgov, 2007). This alternate transformation strategy involves slight wounding of explants and the application of a five hours auxin shock in liquid MS medium supplemented with indole-3-acetic acid (28 µM). After co-cultivation procedures, regeneration is induced by culturing in MS modified salts including BAP (22 µM) and IBA (1.96 µM) and the presence of convenient selection antibiotics (hygromycin) plus cefotaxime to keep *A. tumefaciens* AGL0 used in the process under control. Elongation of treated explants is obtained by use of the same growth regulators in lower quantities than used for regeneration (BAP 8.8 µM, IBA 0.5 µM) and the inclusion of a 16 h photoperiod. As preliminary results of transformed individuals challenged by grafting experiments, these authors have indicated the potential development of five candidate lines that challenged by grafting on PPV-infected rootstocks demonstrated successfully disease resistance with no virus accumulation as suggested the use of Western blotting experiments.

Considering that genome annotation for peach is expected in the very near future, the advantage of transforming a diploid species in the *Prunus* genus opened space for candidate gene evaluation in this type of fruit trees, making very attractive the use of Japanese plums as a closer model. The approach of silencing PPV sequences has been under development in *P. salicina*; an arrangement of "hot-spot" target sequences from the coat protein gene and predicted as highly sensitive to gene silencing, have been located in tandem in a construct designed to generate *in vivo* RNA hairpin. The use of this construct for genetic transformation of Japanese plum hypocotyls leaded to the generation of six transgenic lines that putatively could silence the PPV coat protein gene. Acclimatized and rooted transgenic plants harboring this construct were used has a source of scions for grafting experiments on PPV-infected *Prunus insititia* rootstocks that previously had been used for a confined evaluation of C5 scions (Wong *et al.*, 2010). Symptomatology (Figure 6) and ELISA data from the first season of evaluations for these lines have indicated the existence of two transgenic lines showing the recovery and resistant phenotypes, respectively (Figure 6). Massive sequencing data is under development for these lines in order to corroborate small RNAs populations generated in these challenged ´Angeleno´ scions.

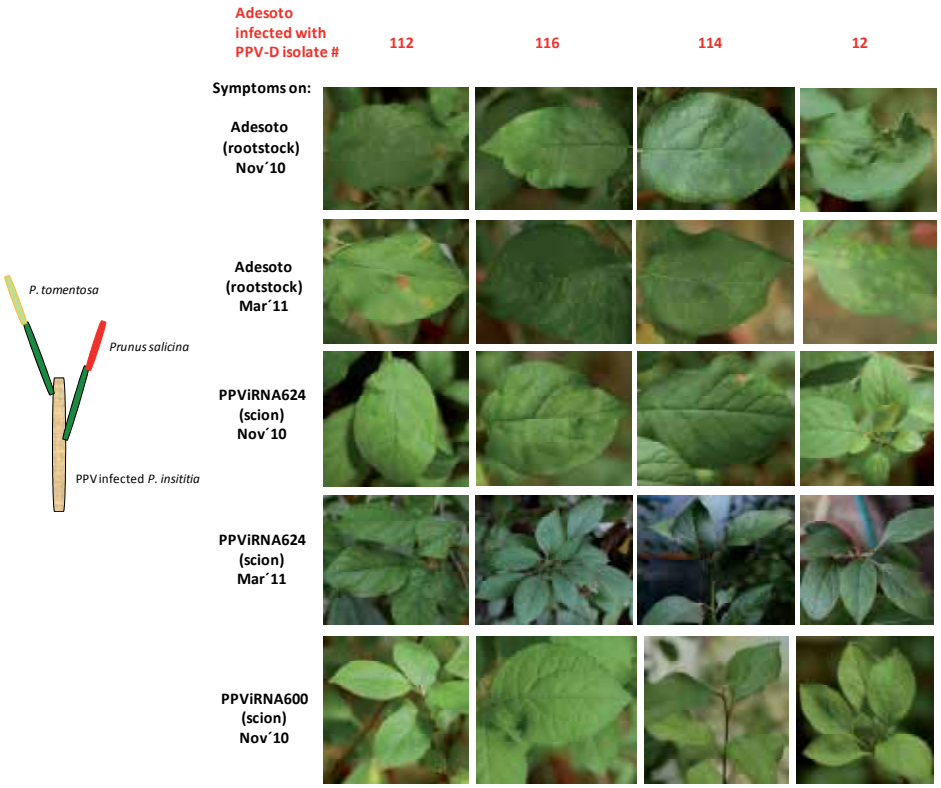

Fig. 6. Genetically modified ´Angeleno´ plants challenged by micro-grafting onto previously infected 'Adesoto' rootstocks. Four different PPV-D Chilean isolates (numbers) have been evaluated.

These assays are carried out in a biosafety greenhouse at La Platina Station of the National Institute of Agriculture, Santiago, Chile.

4. Towards peach genetic transformation

In parallel to the systems developed for plum, the race for a *Prunus persica* genetic transformation procedure has been run with not such a clear achievement. Peach highlights as one of the more recalcitrant species regarding the *in vitro* organogenesis process. First attempts for tissue culture in peach were focused on propagation (Hammerschlag, 1982). From these studies, some factors affecting the establishment of apical buds were identified, such as disinfection procedures for introduced material from the field, plant growth regulators applied and temperature of culturing. Also, as observed in grapevines, genotype-dependent responses were obtained mainly in the rooting and acclimatization steps during *in vitro* establishment. Authors suggested that endogenous hormone variations were responsible for these differential responses obtained at culturing (Hammerschlag *et al*., 1987). In 1989, da Câmara Machado *et al*. gave patterns for bud generation using leaf discs and micro-sticks from some apple, pear and peach cultivars to address *Agrobacterium*-mediated genetic transformation of these species. Results from such studies showed just slight callusing leading to rare budding in the cutting edges of the explants with no regeneration. In the same period, other works reported poor bud generation from *P. persica* explants such as immature endosperms (Meng and Zou, 1981), zygotic embryo-derived calli (Hammerschlag *et al*., 1985; Bhansali *et al*., 1990; Scorza *et al*., 1990a), immature (Mante *et al*., 1989) and mature (Pooler and Scorza, 1995) cotyledons.

Most of the studies describing genetic transformation in peach have described the use of *A. tumefaciens* infection in either immature or mature explants (Hammerschlag *et al*., 1989; Scorza and Sherman, 1996; Pérez-Clemente *et al*., 2004; Padilla *et al*., 2006); in general, targeting on immature tissues leads over the use of mature explants in woody species (da Câmara Machado *et al*., 1992; Ye *et al*., 1994). In those experiments, the use of cytokinines (BAP, kinetin, zeatin or isopentyladenine) has been reported in order to induce regeneration of immature cotyledons, although the number of produced buds has been extremely low. Pérez-Clemente *et al*. (2004) reported the use of mature cotyledons with a very low adventitius budding efficiency. No whole plants or stable foreign DNA incorporation into the tissues were described from these reports. In addition, Pérez-Clemente *et al*. (2004) proposed the use of longitudinal embryo segments as a reliable method for regeneration and plant genetic transformation, however, massiveness and reproducibility of this methodology has not been really achieved.

Transversal stem segments isolated from mature plants were infected with the *shooty Agrobacterium* mutant (tms328::Tn5; Hammerschlag *et al*., 1989), which commonly induces budding in tobacco calli. A cytokinine-independent growth was observed in the treated tissues, although no budding was finally observed. In a similar strategy, in which stems were replaced by immature embryos, Smigocki and Hammerschlag (1991) reached regeneration of a few buds from calli generated from embryos; however, a marked trend to rooting of the treated explants and no transgenic status of the obtained buds was reported by the authors. Recently, Padilla *et al.* (2006) described optimal conditions for gene transfer using *Agrobacterium* in different explants types (hypocotyls cylinders, cotyledons, embryonic axis from no germinated seeds, internodal segments isolated from epicotyls obtained from non germinated seeds). Initially, authors established transient expression rates for different DNA constructs and *Agrobacterium* strains, determining that cotyledons and nodal segments were the explants showing the highest β-glucuronidase and green fluorescent protein (GFP)

transient expression. These observed responses were variety dependent and none of these explants leaded to fully regenerated plants.

4.1 Combining trials to obtain genetically modified peaches

As depicted, explants from *P. persica* excepting a couple of examples referred to embryo longitudinal segments and mature cotyledons, the species can be considered as recalcitrant to *in vitro* regeneration. In addition, these successful responses do not represent a reliable process applicable to several other varieties or hybrids.

In our hands, the use of leaves as starting explants in the transformation pipeline for ´O´Henry´ did not produce a consistent number of buds, on the contrary to the reported by Gentile *et al.* (2002). Despite this and after several years of evaluations, the use of immature cotyledons leaded us to propose a combined procedure based on these procedures for regeneration and transformation of this and other commercial cultivars. ´O´Henry´ and ´Rich Lady´ immature cotyledons have been cultivated in LP modified medium (Gentile *et al.*, 2002) generating viable explants that can produce either direct budding in MP modified medium (Gentile *et al.*, 2002) supplemented with BAP (5 µM) and NAA (concentrations between 3 and 5 µM) or lead to formation of white structures (as shown in Figure 7) that, in presence of LP medium and 2,4-D (1 µM), will become into green buds after 60-90 days of culturing.

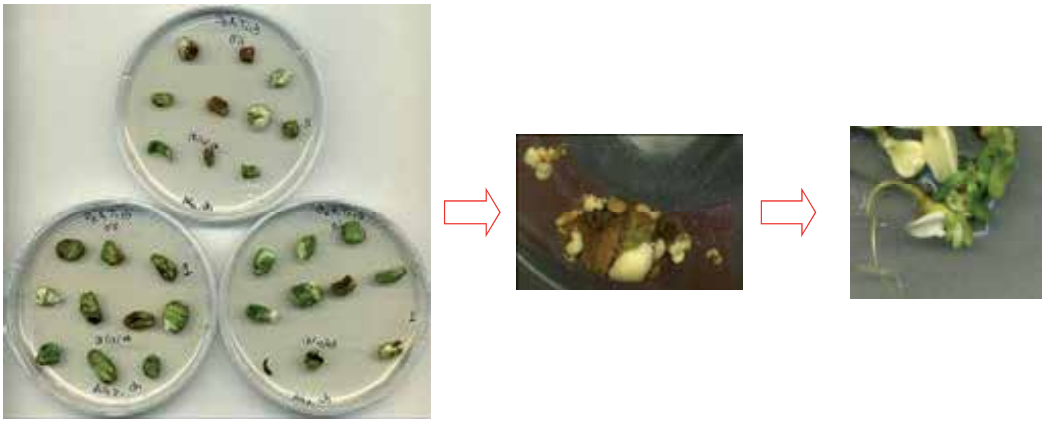

Fig. 7. *Prunus persica* regeneration system. A brief and summarized scheme of the system for peach regeneration using immature cotyledons.

These results are quite similar to those obtained using mature cotyledons from *Prunus avium* and the technical approach is the same (Canli and Tian, 2008). Activated tissues can be then cultured in LP medium supplemented with 2,4-D (1 µM) and BAP (3 µM) for additional 90 days and then transferred into LP salts with no growth regulators to obtain gradually green buds which can be transferred into a LP derivative medium supplemented with BAP (0.5 µM), IBA (0.01 µM) and glutamine (0.2 g/L). Shoots start to appear after eight months of total treatments and generation of plantlets can be reached after 8-12 months. Up to date, in the most responsive cultivars (i.e. 'O'Henry' and 'Rich Lady'), regeneration rates are close to 20% and transformation efficiencies in the regenerated plantlets are close to 2%.

By this methodology, trees expressing GFP have been generated (Figures 8 and 9); interestingly, the behavior of a 35S RNA Cauliflower Mosaic Virus promoter driving the *gfp* gene expression could be analyzed (observed) in peaches by epifluorescence microscopy in leaves, fruit tissues (mesocarps), and radicles (Figure 8). The same qualitative analysis carried out in flowers from these trees (Figure 9), have shown clear differences in the fluorescence obtained when transformed tissues are compared to the corresponding non transgenic controls.

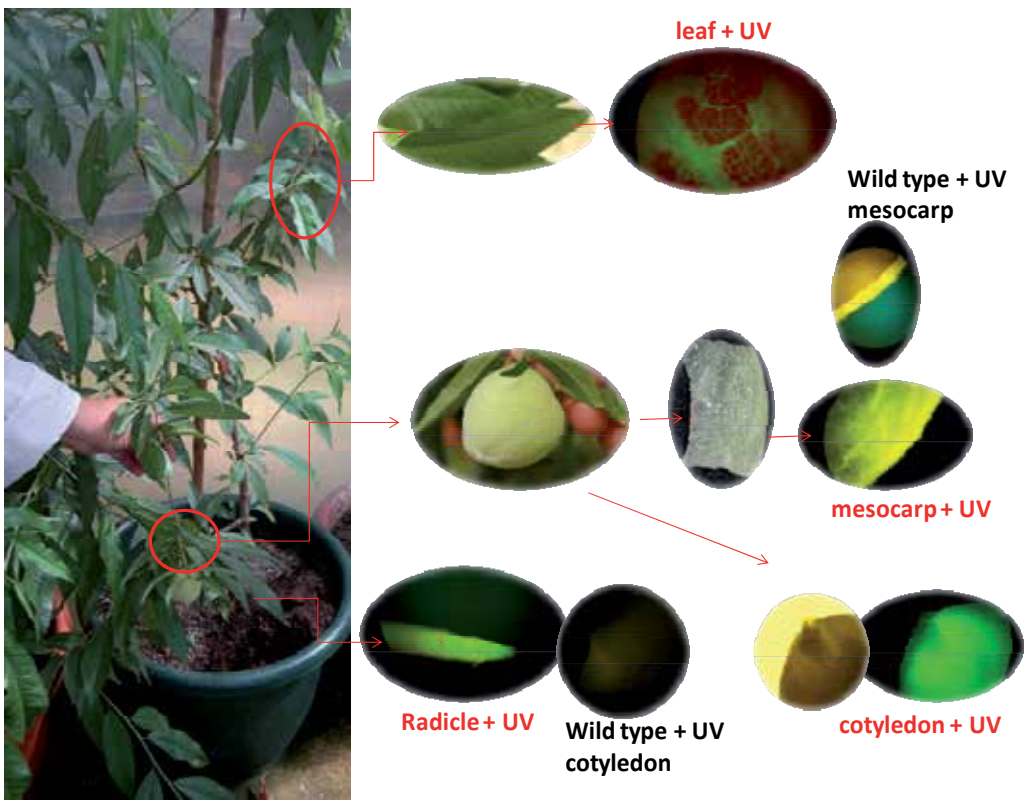

Fig. 8. Genetically modified peach tree expressing GFP.
Epifluorescence microscopy in different tissues from a genetically modified peach tree. Red fonts indicate view of transgenic tissues under UV light (+UV); black fonts show views of the corresponding tissues from non transformed trees used as a control.

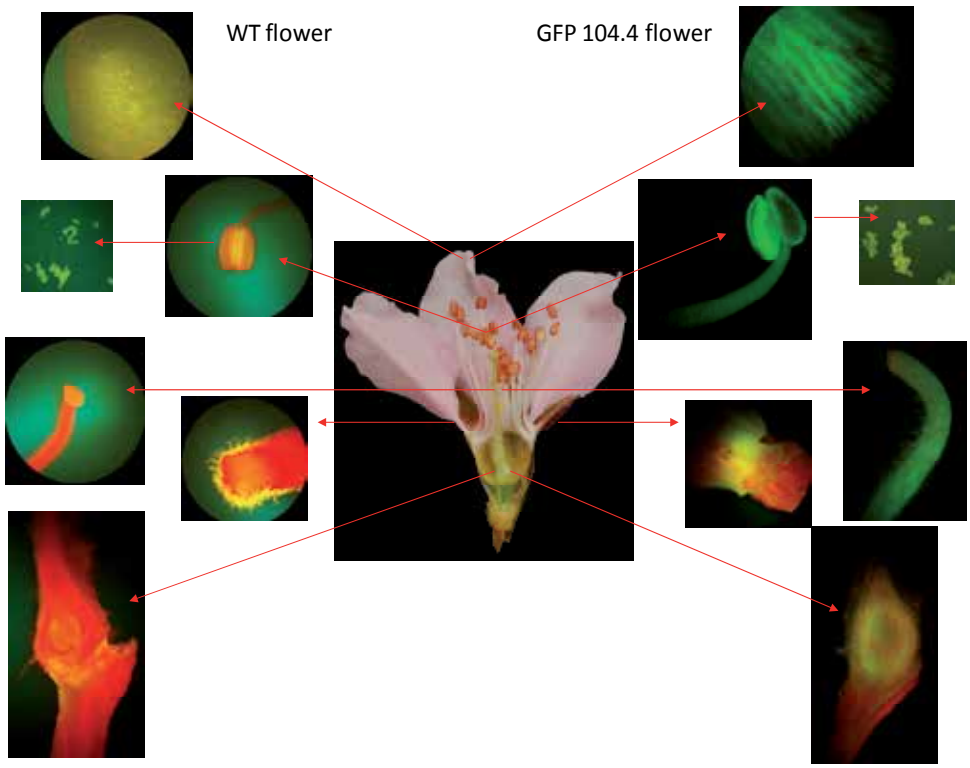

Fig. 9. Schematic representation of different floral organs from a genetically modified peach flower expressing GFP (right, GFP 104.4 flower) compared to wild type tissues (left, WT flower).

5. Conclusions

A compact view about different strategies to reach successful genetic transformation experiments in grapes and stone fruits (plums and peach) has been presented using results from our close experimentation. This time, the focus has been on mixing different ideas from procedures used in both genera and their corresponding results. *Vitis* spp. genetic transformation has mainly relied on the use and availability of SE procedures. Early in the ´70s, regeneration of whole Gloryvine (*V. vinifera* x *V. rupestris*) plants was achieved from somatic embryos previously obtained in this hybrid by anthers tissue culture cultivation. Since then, successful procedures have made possible SE establishment in *V. vinifera*, *V. labruscana* and *V. rotundifolia*. Recently, mixing of solid and liquid media-based procedures, grapevine SE has leaded to the design, characterization and scaled-up production of ´Thompson Seedless´ embryogenesis using an air-lift bioreactor. The system can be expanded to other genotypes. The accumulated information derived from this scaling-up process fused to the characterization of some of the kinetic parameters involved in grapevine SE, have enabled design of new experimentation focused on the development of SE protocols for genotypes such as rootstocks (´Harmony´, ´Freedom´ or ´Salt Creek´) and more recalcitrant cultivars such as ´Red Globe´. The results indicate that grape genetic transformation can be considered as a model system in which efficiency is not necessarily an issue and the possibility for high through-put candidate gene evaluation is plausible.

In case of *Prunus* spp., European and Japanese plums have been the more successful rosaceous fruit models to be regenerated and transformed in our hands (plums and peach). Under the trade name HoneySweet and deregulated in 2009, the PPV-resistant C5 event established a baseline for regeneration protocols using seed-derived tissue explants; these were successfully dissected and evaluated on *P. salicina* using four relatively close media. The regenerative responses, with shooting on about 12% on the total cultured explants in two varieties (´Larry Ann´ and ´Angeleno), leaded to the generation of stable genetically modified lines. The research "baseline" in plums transformation has later conducted to improving regeneration and transformation efficiencies in both species, from which new PPV-resistant plant materials have been produced. Hairpin dsRNA inducing constructs are currently under evaluation for silencing different PPV genes, from which CP has been here illustrated. Finally, these achievements have been recently optimized, reaching the successful transformation of European plum leaf explants in some genotypes. Plum can be considered as an attainable model system for candidate gene evaluation in stone fruits, with hexaploid and diploid versions for such studies. At the same time, plum genetic transformation can be judged as a proof of concept for peaches.

For *P. persica*, genetic transformation seems attainable although not reproducible. Several protocols have worked just in the place when they were generated. In our hands, the use of immature cotyledons subjected to modifications in the workflow described for leaves regeneration has allowed for generation of GFP expressing peaches. Consistently, this platform has leaded to the production of new transgenic lines and constructs already evaluated in Japanese plum (i.e. PPV silencing), are now used for this species. The peach case reinforces the concept that one previous development is a necessary step leading to the next one.

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7. References

Ammirato, P. (1983). Embryogenesis. In: *Handbook of Plant Cell Cultures, Techniques for Propagation and Breeding*. D.A. Evans, W.R. Sharp, P.V. Ammirato, & Y. Yamada (Eds.), McMillan, ISBN 0-02-949230-0, New York, USA.

Araya, S., Prieto, H. & Hinrichsen, P. (2008). An efficient buds culture method for the regeneration via somatic embryogenesis of table grapes cvs. Red Globe and Flame Seedless. *Vitis*, Vol. 47, No. 4, 251-252, ISSN 00427500.

Archambault, J., Williams, R., Lavoie, L., Pepin, M. & Chavarie, C. (1994). Production of somatic embryos in a helical ribbon impeller bioreactor. *Biotechnology and Bioengineering*, Vol. 44, No. 8, 930–943, ISSN 0006-3592.

Bhansali, R., Drive, J. & Durzan, D. (1990). Rapid multiplication of adventitious somatic embryos in peach and nectarine by secondary embryogenesis. *Plant Cell Reports*, Vol. 9, No. 5, 280-284, ISSN 0721-7714.

Canli, F. & Tian, L. (2008). *In vitro* shoot regeneration from stored mature cotyledons of sweet cherry (*Prunus avium* L.) cultivars. *Scientia Horticulturae*, Vol. 116, No. 1, 34–40, ISSN 0304-4238.

Da Câmara Machado, M., Da Câmara Machado, A., Hanzer, V., Mattanovich, D., Himier, G. & Katinger, H. (1989). Regeneration of shoots from leaf discs and stem micro-cuttings of fruit trees as a tool for transformation. *Proceedings of the XIV International Symposium on Fruit Tree Virus Diseases - Acta Horticulturae* 235, 85-92, ISBN 978-90-66053-33-5, Thessaloniki, Greece, June 12-18, 1988.

Da Câmara Machado, M., Da Câmara Machado, A., Hanzer, V., Weiss, H., Regner, F., Steinkeliner, H., Mattanovich, D., Plail, R., Knapp, E., Kalthoff, B. & Katinger, H. (1992). Regeneration of transgenic plants of *Prunus armeniaca* containing the coat protein gene of *Plum Pox Virus*. *Plant Cell Reports*, Vol. 11, No. 1, 25-29, ISSN 0721-7714.

Dhekney, S., Li, Z., Compton, M. & Gray, D. (2009). Optimizing initiation and maintenance of *Vitis* embryogenic cultures. *HortScience* 44, 5, 1400–1406, ISSN 0018-5345.

Dolgov, S., Mikhailov, R. & Shulga, O. (2005). Genetic engineering approach in stone fruits breeding. *Proceedings of the International Symposium on Biotechnology of Temperate Fruit Crops and Tropical Species*, ISSN 0567-7572, Daytona Beach, Florida, USA, October 10-14, 2005.

Dolgov, S., Mikhaylov, R., Serova, T., Shulga, O. & Firsov, A. (2010). Pathogen-derived methods for improving resistance of transgenic plums (*Prunus domestica* L.) for *Plum pox virus* infection. *Proceedings of the 21st International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops - Julius-Kühn-Archiv*, 133-140, ISBN 978-3-930037-67-4, Neustadt, Germany, July 5-10, 2009.

Driver, J. & Kuniyuki, A. (1984). *In vitro* propagation of Paradox walnut rootstock. *HortScience*, Vol. 19, No. 4, 507–509, ISSN 0018-5345.

Gamborg, O., Miller, R. & Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, Vol. 50, No. 1, 151–158, 0014-4827.

Gentile, A., Monticelli, S. & Damiano, C. (2002). Adventitious shoot regeneration in peach (*Prunus persica* (L.) Batsch). *Plant Cell Reports*, Vol. 20, No. 11, 1011-1016, ISSN 0721-7714.

González-Padilla, I., Webb, K. & Scorza, R. (2003). Early antibiotic selection and efficient rooting and acclimatization improved the production of transgenic plum plants (*Prunus domestica* L.). *Plant Cell Reports*, Vol. 22, No. 1, 38-45, ISSN 0721-7714.

Gray, D. (1995). Somatic embryogenesis in grape. In: *Somatic embryogenesis in woody plants – vol 2*, P. Gupta, S. Jain, & R. Newton (Eds.), 191-217, Kluwer Academic, ISBN 0-7923-3070-6, Dordrecht, The Netherlands.

Hammerschlag, F. (1982). Factors affecting establishment and growth of peach shoots *in vitro*. *HortScience*, Vol. 17, No. 1, 85-86, ISSN 0018-5345.

Hammerschlag, F., Bauchan, G. & Scorza, R. (1985). Regeneration of peach plants from callus derived from immature embryos. *Theoretical and Applied Genetics*, Vol. 70, No. 3, 248-251, ISSN 0040-5752.

Hammerschlag, F., Bauchan, G. & Scorza, R. (1987). Factors influencing *in vitro* multiplication and rooting of peach cultivars. *Plant Cell Tissue Organ Culture*, Vol. 8, No. 3, 235-242, ISSN 0167-6857.

Hammerschlag, F., Oowns, L. & Smigocki, A. (1989). *Agrobacterium* mediated transformation of Peach cells derived from mature plants that were propagated *in vitro*. *Journal of the American Society for Horticultural Science*, Vol. 114, No. 3, 508-510, ISSN 003-1062.

Hily, J., Scorza, R., Malinowski, T., Zawadzka, B. & Ravelonandro, M. (2004). Stability of gene silencing-based resistance to *Plum pox virus* in transgenic plum (*Prunus domestica* L.) under field conditions. *Transgenic Research*, Vol. 13, No. 5, 427-436, ISSN 0962-8819.

Hily, J., Scorza, R., Webb, K. & Ravelonandro, M. (2005). Accumulation of the long class of siRNA is associated with resistance to *Plum pox virus* in a transgenic woody perennial plum tree. *Molecular Plant Microbe Interaction*, Vol. 18, No. 8, 794-799, ISSN 0894-0282.

Hinrichsen, P., Reyes, M., Castro, A., Blanchard, E., Araya, S., Garnier, M., Reyes, F., Dell'Orto, P., Moynihan, M., Prieto, H. & Muñoz, C. (2005). Genetic transformation of grapevines with *Trichoderma harzianum* and antimicrobial peptide genes for improvement of fungal tolerance. *Proceddings of the VII International Symposium on Grapevine Physiology and Biotechnology - Acta Horticulturae*, Vol. 689, 469-474, ISBN 978-90-66057-18-0, Davis, California, USA, June 21-25, 2004.

Iocco, O., Franks, T. & Thomas, M. (2001). Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. *Transgenic Research*, Vol. 10, No. 2, 105-112, ISSN 0962-8819.

Jaillon, O., Aury, J., Noel, B., Policriti, A., Clepet, C., Casagrande, A., Choisne, N., Aubourg, S., Vitulo, N., Jubin, C., Vezzi, A., Legeai, F., Hugueney, P., Dasilva, C., Horner, D., Mica, E., Jublot, D., Poulain, J., Bruyère, C., Billault, A., Segurens, B., Gouyvenoux, M., Ugarte, E., Cattonaro, F., Anthouard, V., Vico, V., Del Fabbro, C., Alaux, M., Di Gaspero, G., Dumas, V., Felice, N., Paillard, S., Juman, I., Moroldo, M., Scalabrin, S., Canaguier, A., Le Clainche, I., Malacrida, G., Durand, E., Pesole, G., Laucou, V., Chatelet, P., Merdinoglu, D., Delledonne, M., Pezzotti, M., Lecharny, A., Scarpelli, C., Artiguenave, F., Pè, M.E., Valle, G., Morgante, M., Caboche, M., Adam-Blondon, A.F., Weissenbach, J., Quétier, F. & Wincker, P. (2007). French-Italian Public Consortium for Grapevine Genome Characterization. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature*, Vol. 449, No. 7161, 463-467, 0028-0836.

Jayasankar, S., Bondada, B., Li, Z. & Gray, D. (2003). Comparative anatomy and morphology of *Vitis vinifera* (Vitaceae) somatic embryos from solid- and liquid-culture-derived proembryogenic masses. *American Journal of Botany*, Vol. 90, No. 90,973–979, ISSN 0002-9122.

Kundu, J., P. Briard, J. Hily, M. Ravelonandro & Scorza, R. (2008). Role of the 25–26 nt siRNA in the resistance of transgenic *Prunus domestica* graft inoculated with *Plum pox virus*. *Virus Genes*¸Vol. 36, No. 1, 215-220, ISSN 0920-8569.

Li, A., Jayasankar, S. & Gray, D. (2001). Expression of a bifunctional green fluorescent protein (GFP) fusion marker under the control of three constitutive promoters and enhanced derivatives in transgenic grape (*Vitis vinifera*). *Plant Science*, Vol. 160, No. 5, 877-887, ISSN 0168-9452.

Li, Z., Dhekney, S., Dutt, M. & Gray, D. (2008). An improved protocol for *Agrobacterium*-mediated transformation of grapevine (*Vitis vinifera* L.). *Plant Cell Tissue and Organ Culture*, Vol. 93, No. 3, 311–321, ISSN 0167-6857.

Malinowski, T., Cambra, M., Capote, N., Zawadzka, B., Gorris, M., Scorza, R. & Ravelonandro, M. (2006). Field trials of plum clones transformed with the *Plum Pox Virus* coat protein (PPV-CP) gene. *Plant Disease*, Vol. 90, No. 8, 1012-1018, ISSN 0191-2917.

Mante, S., Scorza, R. & Cordts, J. (1989). Plant regeneration from cotyledons of *Prunus persica*, *Prunus domestica*, and *Prunus cerasus*. *Plant Cell Tissue and Organ Culture*, Vol. 19, No. 1, 1-11, ISSN 0167-6857.

Mante, S., Morgens, P., Scorza, R., Cordts, J. & Callahan, A. (1991). *Agrobacterium*-mediated transformation of plum (*Prunus domestica*) hypocotyls slices and regeneration of transgenic plants. *Nature Biotechnology*, Vol. 9, No. 9, 853-857, ISSN 1087-0156.

Martinelli, L., Bragagna, P., Poletti, V. & Scienza, A. (1993). Somatic embryogenesis from leaf- and petiole-derived callus of *Vitis rupestris*. *Plant Cell Reports*, Vol. 12, No. 4, 207–210. ISSN 0721-7714

Martinelli, L. & Gribaudo, I. (2001). Somatic embryogenesis in grapevine. In: *Molecular Biology and Biotechnology of the Grapevine*. K.A. Roubelakis-Angelakis (Ed.), 393-410, Kluwer Academic Publishers, ISBN 0792369491, Dordrecht, The Netherlands.

Martinelli, L., Candioli, E., Costa, D. & Poletti, V. (2001). Morphogenic competence of *Vitis rupestris* secondary somatic embryos with a long culture history. *Plant Cell Reports*, Vol. 20, No. 4, 279-284, ISSN 0721-7714.

Martinelli, L., Gribaudo, I., Bertoldi, D., Candioli, E. & Poletti, V. (2001a). High efficiency somatic embryogenesis and plant germination in grapevine cultivars Chardonnay and Brachetto a grappolo lungo. *Vitis*, Vol. 40, No. 3, 111-115, ISSN 0042-7500.

Martinelli, L. & Gribaudo, I. (2009). Strategies for effective somatic embryogenesis in grapevine: an appraisal. In: *Grapevine molecular physiology and biotechnology, Second Edition*, K. A. Roubelakis-Angelakis (Ed.), 461-494, Springer, ISBN, 978-90-481-2304-9, Dordrecht, The Netherlands.

Meng, X. & Zhou, W. (1981). Induction of embroid and production of plantlets *in vitro* from endosperm of peach. *Acta Agriculturae Universitatis Pekinensis*, Vol. 7, 95-98, ISSN 0479-8007.

Mikhaylov, R. & Dolgov, S.V. (2007). Transgenic plum (*Prunus domestica* L.) plants obtained by *Agrobacterium*-mediated transformation of leaf explants with various selective agents. *Proceedings of the International Symposium on Biotechnology of Temperate Fruit Crops and Tropical Species - Acta Horticulturae*, Vol. 738, 613-623, ISBN 978-90-66052-19-2, Daytona Beach, Florida, USA, October 10-14, 2005.

Mullins, M. & Srinivasan, C. (1976). Somatic embryos and plantlets from an ancient clone of grapevine (cv. Cabernet-Sauvignon) by apomixes *in vitro*. *Journal of Experimental Botany*, Vol. 27, No. 100, 1022–1030, ISSN 0022-0957.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum*, Vol. 15, No. 43, 473-497, ISSN 0031-9317.

Padilla, I., Golis, A., Gentile, A., Damiano, C. & Scorza, R. (2006). Evaluation of transformation in peach Prunus persica explants using green fluorescent protein (GFP) and beta-glucuronidase (GUS) reporter genes. *Plant Cell, Tissue and Organ Culture*, Vol. 84, No. 3, 309-314, ISSN 0167-6857.

Pennisi, E. (2010). Sowing the seeds for the ideal crop. *Science*, Vol. 327, No. 5967, 802-803, ISSN 0036-8075.

Pérez-Clemente, R., Pérez-Sanjuán, A., García-Férriz, L., Beltrán, J. & Cañas, L. (2004). Transgenic peach plants (*Prunus persica* L.) produced by genetic transformation of embryo sections using the green fluorescent protein (GFP) as an *in vivo* marker. *Molecular Breeding*, Vol. 14, No. 4,419–427, ISSN 1380-3743.

Polák, J., Pívalová, J., Kundu, M., Jokes, M., Scorza, R. & Ravelonandro, M. (2008). Behaviour of transgenic *Plum pox virus*-resistant *Prunus domestica* L. Clone C5 grown in the open field under a high and permanent infection pressure of the PPV-rec strain. *Journal of Plant Patholology*, Vol. 90, No 1-Supplement, S1.33-S1.36, ISSN 1125-4653.

Pooler, M. & Scorza, R. (1995). Regeneration of peach (*Prunus persica* (L.) Batsch) rootstock cultivars from cotyledons of mature stored seed. *HortScience*, Vol. 30, No 2, 355-356, ISSN 0018-5345.

Qu, J., Ye, J. & Fang, R., (2007). Artificial microRNA-mediated virus resistance in plants. *Journal of Virology*, Vol. 81, No 12, 6690-6699, ISSN 0022-538X.

Rajasekaran, K. & Mullins, M., (1983). Influence of genotype and sex-expression on formation of plantlets by cultured anthers of grapevines. *Agronomie*, Vol. 3, No. 3, 233–238, ISSN 0249-5627.

Salunkhe, C., Rao, P. & Mhatre, M. (1997). Induction of somatic embryogenesis and plantlets in tendrils of *Vitis vinifera* L. *Plant Cell Reports*, Vol. 17, No. 1, 65-67. ISSN 0721-7714

Scorza, R., Cordts, J. & Mante, S. (1990). Long-Term somatic embryo production and regeneration from embryo-derived peach callus. *Proceeding of the I International Symposium on In Vitro Culture and Horticultural Breeding - Acta Horticulturae*, Vol. 280, 183-190, ISBN 978-90-66052-14-7, Bologna, Cesena, Italy, May 30-June 3, 1989.

Scorza, R., Cordts, J., Gray, D., Emershad, R. & Ramming, D. (1996). Producing transgenic ´Thompson Seedless´ grape (*Vitis vinifera* L.) plants. *Journal of American Society for Horticultural Science*, Vol. 121, No. 4, 616-619, ISSN 0003-1062.

Scorza, R., A. Callahan, L. Levy, V. Damsteegt, K. Webb & M. Ravelonandro (2001). Post-transcriptional gene silencing in *Plum pox virus* resistant transgenic European plum containing the Plum pox potyvirus coat protein gene. *Transgenic Research*, Vol. 10, No. 3, 201-209, ISSN 0962-8819.

Scorza, R., Georgi, L., Callahan, A., Petri, C., Hily, J., Dardick, C., Damsteegt, V. & Ravelonandro, M. (2010). Hairpin *Plum Pox Virus* coat protein (hpPPV-CP) structure in 'HoneySweet' C5 plum provides PPV resistance when genetically engineered into plum (*Prunus domestica*) seedlings. *Proceeding of the 21st International Conference on Virus and Other Graft Transmissible Diseases of Fruit Crops - Julius-Kühn-Archiv* Vol. 427, 141-146, ISSN 1868-9892, Neustadt, Germany July 5 - 10, 2009.

Scorza, R. & Sherman, W. (1996). Peaches. In: *Fruit Breeding vol. I: Tree and Tropical Fruits*. J. Wiley & J. Moore (Eds.), Wiley, 047131014X, New York, USA.

Smigocki, A. & Hammerschlag, F. (1991). Regeneration of plants from peach embryo cells infected with a shooty mutant strain of *Agrobacterium*. *Journal of the American Society for Horticultural Science*, Vol. 116, No. 6, 1092-1097, ISSN 0003-1062.

Stamp, J. & Meredith, C. (1988). Somatic embryogenesis from leaves and anthers of grapevine. *Scientia Horticulturae*, Vol. 35, Nos. 3-4, 235-250, ISSN 0304-4238.

Tapia, E., Sequeida, A., Castro, A, Montes, C., Zamora, P. & Prieto, P. (2009). Development of grapevine somatic embryogenesis using an air-lift bioreactor as an efficient tool in the generation of transgenic plants. *Journal of Biotechnology*, Vol. 139, No 1, 95-101, ISSN 0168-1656.

Teycheney, P.Y., G. Tavert, R. Delbos, M. Ravelonandro & J. Dunez (1989). The complete nucleotide sequence of *Plum Pox Virus* RNA (strain D). *Nucleic Acids Research*, Vol. 17, No. 23, 10115-10116, ISSN 0305-1048.

Tian, L., Wen, Y., Jayasankar, S. & Sibbald, S. (2007). Regeneration of *Prunus salicina* Lindl (Japanese plum) from hypocotyls of mature seeds. *In Vitro Cellular and Developmental Biology – Plant*, Vol. 43, No. 4, 343-347, ISSN 1054-5476.

Torregrosa, L., Iocco, P. & Thomas, M. (2002). Influence of Agrobacterium strain, culture medium, and cultivar on the transformation efficiency of *Vitis vinifera* L. *American Journal of Enology and Viticulture*, Vol. 53, No. 3, 183-190, ISSN 0002-9254.

Urtubia, C., Devia, J., Castro, A., Zamora, P., Aguirre, C., Tapia, E., Barba, P., Dell`Orto, P., Moynihan, M., Petri, C., Scorza, R. & Prieto, H. (2008). *Agrobacterium*-mediated genetic transformation of *Prunus salicina*. *Plant Cell Reports*, Vol. 27, No. 9, 1333-1340, ISSN 0721-7714.

Velasco, R., Zharkikh, A., Troggio, M., Cartwright, D., Cestaro, A., Pruss, D., Pindo, M., Fitzgerald, L., Vezzulli, S., Reid, J., Malacarne, G., Iliev, D., Coppola, G., Wardell, B., Micheletti, D., Macalma, T., Facci, M., Mitchell, J., Perazzolli, M., Eldredge, G., Gatto, P., Oyzerski, R., Moretto, M., Gutin, N., Stefanini, M., Chen, Y., Segala, C., Davenport, C., Demattè, L., Mraz, A., Battilana, J., Stormo, K., Costa, F., Tao, Q., Si-Ammour, A., Harkins, T., Lackey, A., Perbost, C., Taillon, B., Stella, A., Solovyev, V., Fawcett, J.A., Sterck, L., Vandepoele, K., Grando, S.M., Toppo, S., Moser, C., Lanchbury, J., Bogden, R., Skolnick, M., Sgaramella, V., Bhatnagar, S.K., Fontana, P., Gutin, A., Van de Peer, Y., Salamini, F. & Viola, R., (2007). A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. *PLoS ONE*, Vol. 2, No. 12, e1326, ISSN 1932-6203.

Watson, J., Fusaro, A., Wang, M. & Waterhouse, P. (2005). RNA silencing platforms in plants. *FEBS Letters*, Vol. 579, No. 26, 5982–5987, ISSN 0014-5793.

Wesley, S., Helliwell, C., Smith, N., Wang, M., Rouse, D., Liu, Q., Gooding, P., Singh, S., Abbott, D., Stoutjesdijk, P., Robinson, S., Gleave, A., Green, A. & Waterhouse, P. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. *The Plant Journal*, Vol. 27, No. 6,581-590, ISSN 0960-7412.

Wong W., Barba P., Álvarez C., Castro A., Acuña M., Zamora P., Rosales M., Dell'Orto P., Moynihan M., Scorza, R. & Prieto H. (2010) Evaluation of the resistance of transgenic C5 plum (*Prunus domestica* l.) Against four Chilean *Plum pox virus* isolates through micro-grafting. *Chilean Journal of Agricultural Research*, Vol. 70, No. 3, 372 – 380, ISSN 0365-2807.

Yancheva, S., Druart, Ph. & Watillon, B. (2002). *Agrobacterium*-mediated transformation of plum (*Prunus domestica* L.). *Proceeding of the VII International Symposium on Plum and Prune Genetics, Breeding and Pomology - Acta Horticulturae*, Vol. 577, 215-217, ISBN 978-90-66058-95-8, Plovdiv, Bulgaria, August 20-24, 2001.

Ye, X., Brown, S. & Scorza, R. (1994). Genetic transformation of peach tissue by particle bombardment. *Journal of the American Society for Horticultural Science*, Vol. 119, No. 2, 367-373, ISSN 0003-1062.

5

Citrus Transformation: Challenges and Prospects

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1. Introduction

Citrus is an important commodity worldwide and is produced in tropical and subtropical regions around the world. Annually, the total citrus fruit production is estimated to be more than 124.5 million tonnes worldwide, with China, Brazil, the United States, Mexico and India the main producers (FAO, 2011). Oranges, lemons, tangerines and grapefruits are among the most commonly grown citrus types and they are traded as fresh fruit, juice, or as concentrate. Growers, however, face important challenges for maintaining or improving yield: disease, drought, cold and soil salinity are some of the factors that can limit production and can have an important economic impact on growers. Traditional breeding methods have been used successfully over the years to improve citrus; however this is done with difficulty due to the slow growth and maturation of this crop, incompatibility, polyembryony, parthenocarpy, etc. Because traditional breeding takes such a long time the fast incorporation of desirable traits is not possible. In other instances, certain desirable traits are not present in cultivated citrus types. This has been made more evident in the battle against diseases. Diseases can appear in a region and within a few years spread and become limiting factors for production and have a major economical impact because of yield reduction and/or increased production costs. Therefore, genetic engineering via citrus transformation is an alternative method used to incorporate desirable traits into citrus genotypes.

2. Citrus transformation: generalities

The genetic transformation procedure involves two major processes. The first is the incorporation of the foreign gene of interest into the plant genome while the second entails the regeneration of the transformed cells into whole transgenic plants (Singh & Rajam, 2009). The success of the genetic transformation technique depends on an effective and reliable procedure as efficiencies are often low. Several techniques such as polyethylene glycol (PEG)-mediated direct uptake of DNA by protoplast (Kobayashi & Uchimaya, 1989), particle bombardment (Yao et al., 1996) and *Agrobacterium*-mediated transformations (Hidaka & Omura, 1993) have been developed and used with various *Citrus* spp. However, the latter transformation system is now the most commonly used method because it has been proven most successful with higher transformation efficiencies resulting in the production of transgenic plants (Peña et al., 2007; Singh & Rajam, 2009; Yu et al., 2002).

2.1 Protoplast transformation

Although, *Agrobacterium*-mediated transformation is considered the best overall method, direct uptake of DNA by protoplasts and particle bombardment have their advantages over the former method. Protoplast transformation is mostly used with commercially important citrus genotypes that are either seedless or contain very few seeds, which is required in most *Agrobacterium*-mediated transformation procedures (Fleming et al., 2000). Here, the citrus plant is regenerated from the protoplast via somatic embryogenesis and additionally it can eliminate the need for the use of antibiotics either for plant selection or bacterial inhibition (Fleming et al., 2000). This method also allows the improvement of citrus genotypes that are sexually incompatible by producing superior scion or rootstock somatic hybrids (Fleming et al., 2000; Grosser et al., 1998a; Grosser et al., 1998b). Regeneration using this system has been used with many citrus species, including lemons [*C. limon* (L.) Burm. F.], limes [*C. aurantifolia* (Cristm.) Swingle], mandarins (*C. reticulata* Blanco), grapefruits (*C. paradisi* Macf.), sweet orange (*C. sinensis* Osbeck) and sour orange (*C. aurantium* L.). Although, limited success has previously been reported using protoplast transformation with sweet orange, rough lemon (*C. jambhiri* Lush.) and 'Ponkan' mandarin (Hidaka & Omura, 1993; Kobayashi & Uchimaya, 1989; Vardi et al., 1990). Fleming et al. (2000) have reported success in recovering transgenic sweet orange plantlets by an optimized version of this method.

2.2 Particle bombardment

Particle bombardment involves the direct delivery of DNA coated onto microprojectiles into intact cells or organized tissue via a gene gun or a biolistic particle delivery system (Yao et al., 1996). This method is used alternatively in cases where citrus genotypes are recalcitrant to *Agrobacterium* infection. A reason for this is that citrus is not a natural host for the bacteria (Khan, 2007). A problem that arises from this method is the low regeneration frequency of stably transformed cells from calli as was observed with tangelo (*C. reticulata* x *C. paradisi*) (Yao et al., 1996). Nevertheless, transformation efficiencies of 93%, based on transient expression experiments, have been reported with citrange (*C. sinensis* x *P. trifoliata*) when particle bombardment is carried out using thin epicotyl segments (Bespalhok et al., 2003).

2.3 *Agrobacterium*-mediated transformation

This system uses the ability of the *Agrobacterium*-plant interaction to transfer and integrate genetic information into the plant's genome. The bacteria, depending on the species, contain either a rhizogenic (Ri) or a tumor-inducing (Ti) plasmid which includes a T-region or transferred DNA region (T-DNA). This T-DNA region is manipulated by genetic engineering to include the gene of interest for transfer in the transformation process. The T-DNA movement from *Agrobacterium* occurs only onto wounded plant cells (Gelvin, 2003; Messens et al., 1990). The initiation of this transfer depends on the induction of the virulence (*vir*) region located in the Ti plasmid. There are 6 *vir* genes *virA-virE* and *virG* that make up this 35 kilobase pairs (kb) region between the left and right borders of the T-DNA. Wounded plant cells produce *vir* inducing compounds such as acetosyringone and α-hydroxyacetosyringone that induce the expression of these *vir* genes initiating the T-DNA transfer and thus transformation of the plant cells (Gelvin, 2003; Messens et al., 1990). *Agrobacterium*-mediated transformation experiments have been carried out with numerous hybrids and species of citrus, such as grapefruit, sour orange, sweet orange, trifoliate orange (*Poncirus trifoliata* Raf.), 'Carrizo' citrange, 'Mexican' lime, 'Swingle' citrumelo (*C. paradisi* x *P. trifoliata*), 'Cleopatra' mandarin, and alemow (*C. macrophylla* Wester) (Dominguez et al.,

2000; Ghorbel et al., 2000; Gutierrez-E et al., 1997; Luth & Moore, 1999; Molinari et al., 2004; Moore et al., 1992; Peña et al., 2004, 2007). Transformation of other economically important citrus cultivars with the existing protocols has not yet been successful.
Generally, transformation efficiencies obtained by using *Agrobacterium* with most citrus cultivars can range from 0 to 45%. This is due to a number of limiting factors that can affect the transformation process. These include: species or cultivar specificity, age and type of explant used, competence of the citrus cells or tissues, *Agrobacterium* strains used and inoculation procedure, co-cultivation and pre-culturing conditions, adequate selection conditions and recovery of transgenic shoots (Bond & Roose, 1998; Costa et al., 2002; Peña et al., 2007; Yu et al., 2002).

2.3.1 Species or cultivar specificity

Data from early studies indicated that the type of citrus species and cultivar used in transformation experiments affect transformation efficiencies. Bond & Roose (1998) showed that when 7 citrus cultivars, 'Washington navel' and 'Olinda Valencia' oranges, 'Lisbon' lemon, 'Rio Red' grapefruit, 'Carrizo' citrange, mandarin and 'Mexican' lime were transformed with *Agrobacterium* only 'Washington navel' and 'Carrizo', resulted in GUS-positive shoots. These results were indicative of the receptiveness of these cultivars to this type of transformation protocol compared to the others. Although very little diversity exists between the sweet orange cultivars, 'Washington navel' and 'Olinda Valencia', the difference that exists was sufficient to affect the transformation efficiency. As a result, different protocols have been developed for different citrus species and cultivars (Bond & Roose, 1998; Costa et al., 2002; Peña et al., 1997).

2.3.2 Age and type of explant used

Studies have also shown that lower transformation efficiencies are obtained with older segments (Moore et al., 1992; Peña et al., 1995a). Transformations of three week old 'Washington navel' orange epicotyl segments resulted in efficiencies of up to 87%, while 5 to 8 week old epicotyl segments gave lower efficiencies of 5 to 40% (Bond & Roose, 1998). This reduction in transformation efficiency is presumed to be the result of older epicotyl segments having a lower number of actively dividing cells and consequently less susceptible to T-DNA integration and the regeneration of shoots (Bond & Roose, 1998; Villemont et al., 1997). In addition, it is regarded that older epicotyl segments have different wound exudates or cell wall components that result in a reduction in bacterial binding or the activation of the virulence genes (Bond & Roose, 1998).
Various types of explants such as, callus, leaf sections, seeds, epicotyl nodal and inter-nodal stem segments are often used, with varying results (Hidaka & Omura, 1993; Kaneyoshi et al., 1994; Moore et al., 1992). For instance, higher transformation efficiencies are obtained from citrus callus of 'Ponkan' mandarin. The advantages of using callus as explants are that a larger number of transgenic plants are produced, there is rapid proliferation and chimeras are rarely observed during the regeneration process (Li et al., 2002). However, drawbacks to using this system are that some citrus varieties do not possess embryogenic potential and the regenerated plants are juvenile, resulting in a long waiting period for the evaluation of the traits of interest and, additionally, it increases the risk of somaclonal variation which results in abnormal plant morphologies (Cervera et al., 2000; Li et al., 2002). *Agrobacterium*-mediated transformation involving epicotyl and internodal stem segments are the

predominantly used explants for regeneration of transgenic citrus plants. These types of explant are the most widely used in citrus transformation experiments and appear to be the most responsive. The disadvantage of using these types of explants is that the process is very laborious and takes a long time. Alternatively, another efficient system uses cotyledons from ungerminated mature seeds, followed by shoot regeneration via direct organogenesis (Khawale et al., 2006; de Oliveira, Fisher and Moore unpublished). The advantage of this method is that it is less time consuming and laborious. It involves the use of mature seeds that are sterilized, subsequently the seed coat is removed and the cotyledons are directly inoculated with the *Agrobacterium* suspension and later transferred to the appropriate selection media. The use of this type of explant eliminates the time required for germination of seedlings to produce epicotyl segments and we have obtained higher transformation and regeneration frequencies with grapefruit and sweet orange. Although GUS expression was observed, we have not yet carried out the evaluation for stable integration of the transgene in the putative transgenic plants generated by this method. However, Khawale et al. (2006) proved the stability of this transformation method in 'Nagpur' mandarin.

2.3.3 Competence of the citrus cells or tissues

Cell division and dedifferentiation of plant cells are responsible for the explants' competent state and result in callus proliferation (Peña et al., 1997, 2004). Observations of transformed citrus inter-nodal and epicotyl segments showed that resulting transgenic cells were localized in callus tissue and are of cambial origin. It is also suggested that certain treatments such as the inclusion of auxins, which promote active cell division and dedifferentiation of plant cells, correlated with higher transformation efficiencies (Peña et al., 2004).

2.3.4 *Agrobacterium* strains used and inoculation procedure

A study involving the use of three different strains of *Agrobacterium* (C58 C1, EHA101-5 and LB4404) to transform seven citrus cultivars showed varying transformation efficiencies (Bond & Roose, 1998). In four separate experiments, strain C58 C1 had the highest transformation efficiency of 45%, while strains EHA101-5 and LB4404 resulted in transformation efficiencies of 29% and 0%, respectively (Bond & Roose, 1998).

The inoculation of the citrus explants with the *Agrobacterium* culture typically requires incubation periods of 1 to 30 minutes (Bond & Roose, 1998; Costa et al., 2002; Luth & Moore, 1999; Peña et al., 1997). However, incubation periods greater than 10 minutes have led to the increase in regeneration of escape shoots and a reduction in transformation efficiency (Costa et al., 2002).

The optimal *Agrobacterium* culture concentrations that have been determined for the effective inoculation and transformation of citrus are $5x10^8$ and $4x10^7$ cfu/ml, and are dependent on the citrus cultivar being transformed (Bond & Roose, 1998; Cervera et al., 1998b; Costa et al., 2002; Dominguez et al., 2000; Luth & Moore, 1999; Peña et al., 1995a; Yu et al., 2002). A limited source of bacterial cells reduces the frequency of T-DNA transfer while excess bacteria stress the plant cells (Costa et al., 2002; Yu et al., 2002).

2.3.5 Co-cultivation and pre-culturing conditions

Co-cultivation involves incubating both the explants and *Agrobacterium* on media containing no selective agent for the transformed cells or against the bacteria, for a period of time. An increase in the co-cultivation period has been associated with a higher number of

regenerated and transformed shoots (Costa et al., 2002). Transformation frequency increased when the co-cultivation period was increased from 1 to 5 days at which it reached a maximum (Cervera et al., 1998a). However, prolonged co-cultivation periods often lead to an overgrowth of *Agrobacterium* which reduces the regeneration frequency of transformed shoots (Cervera et al., 1998b; Costa et al., 2002). As a result, most transformation protocols routinely use a 2 to 3 days co-cultivation period (Cervera et al., 1998b; Costa et al., 2002; Luth & Moore, 1999; Peña et al., 1997).

The composition of the co-cultivation medium also affects the transformation process. The presence of auxins such as 2,4 dichlorophenoxyacetic acid (2,4-D), in co-cultivation medium has resulted in higher transformation frequencies in comparison to co-cultivation medium containing a filter paper layer, tomato cell suspension or a cell feed layer alone (Cervera et al., 1998b; Costa et al., 2002). The use of tomato cell feeder layers with high auxin concentrations has also improved citrus transformation (Costa et al., 2002).

The principle of pre-culturing the explants on co-cultivation medium before inoculation with *Agrobacterium* is to promote the production of vir-inducing cell components by metabolically active cells, which enhances the transformation process (Costa et al., 2002; Spencer & Towers, 1991). However, some studies have shown that pre-culturing citrus explants has a negative effect on the transformation efficiency (Cervera et al., 1998b; Costa et al., 2002). Explants without pre-culture gave a reported 8.4-fold higher level in transformation efficiency compared to those that were pre-cultured (Costa et al., 2002). Most transformation experiments have bypassed this pre-culturing stage and have instead used acetosyringone (Cervera et al., 1998b). In nature, this phenolic compound is produced in wounded plant cells and is responsible for the activation of the *vir* genes. This has been shown to increase transformation efficiencies when added to the *Agrobacterium* inoculum and the co-cultivation medium by promoting transcription of *A. tumefaciens* virulence genes (Cervera et al., 1998b; Kaneyoshi et al., 1994); however, in our personal experience working with grapefruit the addition of acetosyringone does not have much of an effect on the transformation efficiencies.

2.3.6 Adequate selection conditions

Finding suitable selective agents to recover transformed cells is critical in citrus transformation in order to eliminate the high numbers of chimeras and escapes that can be obtained during the process (Gutierrez-E et al., 1997; Moore et al., 1992; Peña et al., 1995a). Hence, an effective selective agent is required to improve transformation recovery. Selection is usually based on antibiotic or herbicide resistance. Kanamycin is one of the most widely used selective antibiotics in transformation processes and is most effective when used in concentrations of up to 100 mg/L. However, shoot regeneration may be inhibited at this concentration. Other antibiotics such as geneticin and hygromycin have also been used, but are not as effective as kanamycin (Costa et al., 2002; Peña et al., 1997). The selective antibiotic can be ineffective in situations where residual *Agrobacterium* cells are present or neighboring transformed cells result in the break down or neutralization of the antibiotic. Invariably, non-transformed plant cells, i.e. escapes, strive in the absence of selective pressure (Cervera et al., 1998b). Other non-toxic selective genes, for instance *manA*, which encodes for the enzyme phosphomannose-isomerase (PMI), have been successfully used in the transformation of sweet orange (Boscariol et al., 2003). The principle is based on the ability of the transformed cells to metabolize mannose as a carbon source present in the selective

medium. Additionally, the use of non-metabolizable genes instead of antibiotic and herbicide resistance genes as selective agents provides a suitable alternative and would satisfy public concerns about their dissemination into the environment and potential effect to consumers. This PMI positive selection system has been shown to be more effective than using kanamycin in many plant transformation protocols (Sundar & Sakthivel, 2008) but this did not seem to be the case in citrus.

2.3.7 Recovery of transgenic shoots

Recovering whole transgenic plants from transformation experiments is often difficult. Typically, most regenerated transformed shoots are either placed directly in soil containing rooting hormone or on rooting media containing varying levels (0 to 1.0 mg/l) of the auxin naphthaleneacetic acid (NAA) which promotes root development (de Oliveira et al., 2009; Gutierrez-E et al., 1997; Luth & Moore, 1999; Moore et al., 1992). Some researchers have gotten better results by first transferring the shoots to hormone-free media to eliminate the cytokinin benzyl aminopurine (BA) from the regeneration media before placing on NAA containing media. Different combinations of BA, NAA and another auxin, indole 3-butyric acid (IBA), NAA and IBA only or just IBA and BA in the rooting medium have been tested so as to improve rooting efficiency in citrus cultivars such as mandarin, lemon, 'Troyer' citrange and lime (Al-Bahrany, 2002; Jajoo, 2010; Moreira-Dias et al., 2000; Singh et al., 1994). Again, the concentrations of these phytohormones vary depending on the citrus genotype. High rooting efficiencies of transgenic shoots have been obtained with citrus types, such as grapefruit, 'Carrizo' citrange and *P. trifoliata* (Peña et al., 2007), but with other citrus types, the rooting efficiency is very low. This problem is overcome by shoot-tip micrografting the transgenic shoot onto a decapitated rootstock seedling (Peña et al., 1995a, 1995b).

3. Genetic engineering and disease control in citrus

Recent advances in genomics, both in citrus and other species, have made available an abundance of genes that can be easily cloned and used in transformation. This is particularly useful in the genetic engineering process as characterized gene(s) derived from known sources can be incorporated into the genome of a recipient plant to obtain desirable traits. Because of its economic impact, disease control is often the objective of plant improvement programs. Hence, resistance and defense genes isolated from well studied plant species have been successfully incorporated into other species to generate pathogen-resistant plants. Another successful strategy in the control of diseases has been the transformation of genes derived from pathogens which can also result in resistant plants.

According to the USDA economic research service, genetically engineered (GE) crops have been widely adopted since their introduction in 1996 (USDA, 2010). Herbicide-tolerant genetically engineered soybeans and cotton have been the most extensively and rapidly adopted GE crops in the U.S., followed by insect-resistant cotton and corn (Cao et al., 2010). The positive impact of these GE crops was due to lower labor and production costs, and gains in profitability, in addition to their increased environmental benefits. In the particular case of citrus, although a variety of transgenic types have been reported in the literature, none has reached commercialization. However, field trials, including our own, are underway. Below we describe some recent and relevant cases of transgenics in citrus.

3.1 Pathogen-derived genes

Some of the earliest success stories in the control of diseases by genetic engineering were using pathogen-derived genes from viruses (Abel et al., 1986). When certain viral genes, particularly the capsid protein (CP), were transformed into plants they showed resistance or immunity against closely related viral strains. A well-known case in a perennial species is the control of *Papaya ringspot virus* by the insertion of its CP into the papaya genome. This effort virtually saved this industry in Hawaii (Gonsalves, 1998). The control mechanism that prevents viral replication in the transgenic plants was initially denominated co-suppression but it is currently referred to as RNA interference or RNA silencing.

Several studies have transformed sequences from a variety of economically important viruses into different citrus types to attempt to produce resistant plants. One of such viral diseases is caused by *Citrus tristeza virus* (CTV). Severe strains of CTV can dramatically reduce production and in some instances lead to tree death in a relatively short period of time (Moreno et al., 2008). In some areas of the world CTV is an important or the most important limiting factor in citrus production and incorporation of resistance by traditional breeding techniques is not possible. For this reason many laboratories have tried to genetically engineer different CTV sequences into citrus as a way to control this important pathogen. However, these attempts have never been completely successful. For example, transforming the major CP (p25) into 'Mexican' lime had two types of response to viral challenge. In replicate plants, propagated from the same line (i.e. genetically identical), 10 to 33% were resistant to CTV while the rest developed typical symptoms, despite a significant delay in virus accumulation (Domínguez et al., 2002). Similar results were obtained in 'Duncan' grapefruit when translatable and untranslatable versions of the major CP were transformed (Febres et al., 2003, 2008). Various forms (full length, hairpins) of the p23 gene, located in the 3' end of the viral genome, have also been transformed into citrus genotypes. In 'Mexican' lime expression of the p23 protein produced viral symptoms in some plants (Fagoaga et al., 2005). Lines with normal phenotype (no symptoms) were further propagated and tested for CTV resistance and again the results were mixed with some plants completely immune to the virus while others from the same line had delayed symptom development and virus accumulation (Fagoaga et al., 2006; López et al., 2010). The use of the 3' region of the p23 and the contiguous 3'-untranslated region (UTR), either as a hairpin or as single copy, has also been transformed into 'Duncan', 'Flame', 'Marsh', and 'Ruby Red' grapefruit and alemow plants with similar results as described above in which some plants derived from a particular line were fully resistant and others were not (Ananthakrishnan et al., 2007; Batuman et al., 2006; Febres et al., 2008). Only in one line full resistance was observed (Febres et al., 2008). This line is currently being evaluated in the field for its horticultural value and durability of the resistance under natural conditions. Other CTV genes have been used but either no transgenic plants were regenerated (p20 and minor CP/p27) or they did not show resistance (RdRp gene) (Febres et al., 2003, 2008).

Resistance to another important viral disease, *Citrus psorosis virus* (CPsV), has been reported in transgenic sweet orange plants transformed with intron-hairpin constructs (ihp) corresponding to the viral CP, the 54K or the 24K genes (Reyes et al., 2011). After challenge with the virus, the CP transgenic plants were more effective in controlling the CPsV and consistently showed lower virus levels and no symptom development compared to 54K and 24K transgenic plants. The study reported that the observed CPsV resistance was due to pre-activated RNA silencing rather than the siRNA accumulation levels in the *ihp-CP* transgenic sweet orange plants prior to virus challenge (Reyes et al., 2011).

Pathogen-derived genes have also been used to control bacterial diseases. Citrus canker, caused by *Xanthomonas axonopodis* pv. *citri* is an economically important disease, especially for the fresh fruit market. The pthA protein is involved in the pathogenesis and symptom development of this bacterial pathogen and the C-terminus contains three nuclear localizing signals (NLS) critical for the interaction with a host protein, translocation to the nucleus and function (Yang et al., 2011). By using a truncated version of the *pthA* gene, coding only for the C-terminus portion of the protein, it was theorized that the resulting protein would interrupt binding and function of the native bacterial pthA during infection and prevent symptom development and pathogen growth. Indeed transgenic sweet orange plants that expressed the truncated protein showed lower disease incidence and symptom development compared to wild type plants, demonstrating a certain degree of resistance (Yang et al., 2011). The authors are currently conducting field experiments to determine the effectiveness of this strategy under natural conditions.

In another strategy, also to control citrus canker, a *hrpN* gene derived from *Erwinia amylovora* was transformed into 'Hamlin' sweet orange plants. The *hrpN* encodes a harpin protein that elicits the hypersensitive response (HR) and systemic acquired resistance (SAR) in plants. The *hrpN* gene was inserted in a construct made up of *gst1*, a pathogen-inducible promoter (so the gene would not be expressed constitutively and hence the SAR response would only be induced in the presence of the pathogen), a signal peptide for protein secretion to the apoplast (the canker bacterium does not penetrate the cell and remains apoplastic). Several of the *hrpN* transgenic lines showed reduction in their susceptibility to citrus canker as compared to wild type plants, and one line in particular displayed very high resistance to the pathogen (up to 79% reduction in disease severity) (Barbosa-Mendes et al., 2009).

Fungal pathogens also affect citrus production. In particular, *Phytophthora* spp can cause root rot and gummosis in mature trees and damping-off in seedlings. For the control of *Phytophthora nicotianae* Azevedo et al (2006) used a bacterio-opsin (*bO*) gene to transform 'Rangpur' lime. The *bO* gene is derived from *Halobacteria halobium* and can spontaneously activate programmed cell death and enhance broad-spectrum disease resistance accompanied by pathogenesis-related (PR) protein accumulation. In two of the transgenic lines, higher levels of tolerance to this pathogen with significantly smaller lesions were observed; however, these lines also exhibited HR-like lesions in the absence of pathogen (Azevedo et al., 2006). It remains to be seen if this strategy will work under field conditions given the fact that the transgenic plants develop spontaneous lesions.

3.2 Plant defense genes

Upon recognition of a potential pathogen plants naturally respond by triggering defense mechanisms that can, in some instances, halt pathogen colonization. One such defense mechanism is SAR, a form of inducible defense in which infection by a pathogen leads to an enhanced defense state that is durable and provides resistance or tolerance to a wide range of pathogens in subsequent challenges (Durrant & Dong, 2004).

A gene that has been identified as critical in the establishment of SAR is the *NON-EXPRESSOR OF PATHOGENESIS RELATED 1* (*NPR1*). NPR1 is a transcription co-activator and plays a key role in regulating defense gene transcription and signal transduction pathways that lead to SAR (Despres et al., 2000; Zhang et al., 1999). Under normal conditions the NPR1 protein does not induce SAR, however in the presence of a pathogen and increased levels of salicylic acid (SA) NPR1 is translocated to the nucleus where it

interacts with transcription factors that ultimately induce the expression of SAR-associated genes (Kinkema et al., 2000).
A number of studies have demonstrated that the over-expression of the *Arabidopsis* NPR1 provides a broad-spectrum enhanced resistance to various pathogens (Cao et al., 1998; Lin et al., 2004). Our laboratory and others have invested a considerable amount of time and effort investigating the nature of SAR in citrus and the full length sequences of five citrus *NPR1*-like genes has been cloned and sequenced. Their expression levels are differentially affected by pathogen and other treatments (Febres and Khalaf, unpublished results).
Zhang et al. (2010) reported transforming the *Arabidopsis NPR1* gene into 'Duncan' grapefruit and 'Hamlin' sweet orange. The over-expression of this gene increased resistance to citrus canker and the observed resistance correlated with the expression levels of the transgene. Our results of transgenic 'Carrizo' citrange plants, also transformed with the *Arabidopsis NPR1* gene indicated that the transgenic lines were as well more tolerant to citrus canker (slower lesion development) and had higher levels of pathogenesis-related (PR) genes than wild type plants (Febres, unpublished).

3.3 Additional strategies

As mentioned above, attempts to use pathogen-derived sequences for the control of CTV have not rendered consistent results. A different approach has recently been tested (Cervera et al., 2010) by using single-chain variable fragments (scFv) from two monoclonal antibodies that in combination seem to detect the major CP from most CTV isolates. 'Mexican' lime plants were transformed with each scFv either individually or in combination. Essentially all constructs conferred some level of protection when the plants were challenged with a severe strain of CTV. Between 40 to 60% of the plants tested did not get infected, compared to 95% infection in control plants. In addition a delay and attenuation in symptom development was also observed. Although complete resistance was not observed in this case either it is still a promising approach that needs further investigation.

4. Emerging technologies

The production of new varieties via transformation in citrus and many other woody perennials poses a challenge not found in the breeding of annuals and other fast-growing plants. Due to combinations of long juvenile periods, biological barriers to crossing, and the difficulty of reconstituting favored types, such as the complex hybrids sweet orange and grapefruit in citrus, new cultivars will probably have to be selected from T0 transformants. There are several implications to this, discussed below.
One of the greatest challenges of producing and testing transgenic *Citrus* plants is the long juvenile periods observed in this genus. As discussed above, most citrus transformation techniques utilize explants derived from juvenile tissue, and the transgenic plants must be grown for many years, in most cases, for their horticultural attributes to be evaluated. Two approaches are being investigated to overcome this problem. The first is efforts to decrease the juvenile periods of transgenic plants. There are both historical work and ongoing efforts to use horticultural methods to bring citrus plants into bearing earlier. Another alternative for shortening the juvenile period is to produce transgenic plants that over-express a flower meristem identity gene that causes them to flower earlier. The *Arabidopsis LEAFY* and *APETALA1* genes have been over-expressed in 'Carrizo' citrange (Peña et al., 2001) and transgenic *Poncirus* plants over-expressing a citrus orthologue of *Arabidopsis FLOWERING*

LOCUS T (FT) have been produced (Endo et al., 2005; Nishikawa et al., 2010). However, in most cases, the expression of these genes, while dramatically reducing time to flowering, also conferred deleterious morphological phenotypes to the transgenic plants. Thus this approach may benefit citrus breeding efforts and early testing of traits designed to be evinced in fruit, it may not produce T0 transgenic plants that could directly be used in production. However, all possibilities of this approach have not been explored. For instance, citrus genomes contain at least three orthologues of *FT* that produce quite different phenotypes when overexpressed in transgenic tobacco (Kamps and Moore, unpublished). Also, Carrizo plants transformed with *APETALA1* displayed normal morphology (Peña & Séguin, 2001).

The second approach for overcoming juvenility is to use explants from mature plants for transformation. However, taking explants directly from mature trees is not likely to be successful due to the low regeneration potential of such explants and perhaps also of lower competence for transformation. Success has been achieved by reinvigorating mature citrus types by grafting mature buds on vigorous juvenile rootstocks and using the first flushes for *Agrobacterium*-mediated transformation (Cervera et al., 1998a, 2005). However, this is a technically demanding approach. The plant material must be in excellent condition, which is particularly difficult to achieve in humid climates, where the pathogen load on tissue, even when grown under greenhouse conditions, may make disinfection of explants difficult. Even then, only a relatively small number of explants can be obtained from the first flush or two of the grafted plant. In some genotypes, a lack of bud uniformity in sprouting and morphology is problematic (Cervera et al., 2008). In other cases, culture requirements for regeneration may be quite different for even closely related citrus types (Almeida et al., 2003; Rodríguez et al., 2008). Kobayashi (2003) circumvented some of these problems by using already grafted 'Pera' sweet orange nursery plants for harvest of explants and by thin segments (1 to 2 mm) of stems as explants. In all cases, transgenic plants in the greenhouse began to flower after 14 months or less after micrografting the transgenic scions on rootstock. Experiments are underway in several laboratories to improve still further on the production of transgenic plants from mature tissue. The importance of the cambium in producing transgenic tissue in many of the above reports and the recent description of the cambium cells of several plants as analogous to vascular stem cells (Lee et al., 2010) suggest that one research direction could be exploration of other types of explants where the cambium cells are maximally exposed to *Agrobacterium* and subsequent growth hormones in the culture medium.

Another problem with using T0 plants is that the gene insertion site(s) is unknown. This can affect the expression of the transgene and could lead to altered morphology that was not intended. However, genomic changes that are not selected for also may happen during conventional breeding due to, for instance, transposon activity or irradiation and mutation breeding.

Of course there are also advantages to utilizing T0 transformants in perennials. With the explosion in genomic information, the functions of more and more genes are being elucidated (Talon & Gmitter Jr, 2008), so choosing a transgene that will impart a particular trait should be more targeted in the future. It has also been found in both conventional and molecular breeding that valuable genes or alleles are found in plant relatives or wild species. In such cases using T0 transgenics circumvents the problem of linkage drag that may result from the transfer of unknown and undesirable genes that are linked to the desirable gene or allele from the donor parent. It might also be possible to "stack" valuable genes or alleles in

a desirable citrus type via multiple transformations or multiple genes inserted in a single transformation.

Another important area of research has been to increasing the cold hardiness of citrus. This could potentially extend production areas to new regions where pathogens or other limiting factors are not present. As in the case with disease resistance there are some citrus relatives that can endure freezing temperatures. While most commercially important citrus varieties are susceptible to freezing, *P. trifoliata* for instance can tolerate temperatures well bellow freezing if cold acclimated prior to the exposure (Talon & Gmitter Jr, 2008).

Genes associated with cold acclimation have been identified in citrus as an initial milestone in a multistep approach to ultimately incorporate some of these genes in the genome of selected citrus varieties that are naturally susceptible to freezing. Our laboratory and others have studied the effect of cold stress or freezing on gene expression. For instance, in an attempt to minimize the chilling injury during citrus fruits storage, a genome-wide transcriptional profiling analysis was performed (Maul et al., 2008). Grapefruit flavedo RNA was used to study the responses of citrus fruit to low temperatures. The study applied a pre-storage conditioning treatment of 16°C for 7 days and utilized an Affymetrix Citrus GeneChip microarray. While the applied treatment seemed to have halted the expression of general cellular metabolic activity, it induced changes in the expression of transcripts related to membranes, lipid, sterol and carbohydrate metabolism, stress stimuli, hormone biosynthesis, and modifications in DNA binding and transcription factors.

Our laboratory provided the first evidence of an association in citrus between C-repeat binding factors (CBF) expression levels and the extent of cold tolerance (Champ et al., 2007). CBFs have been identified in many species and they function as transcriptional activators regulating the expression levels of a number of genes that impart cold and stress tolerance. *P. trifoliata*, a *Citrus* relative, can survive freezes of -20°C when fully cold acclimated. On the other hand, grapefruit cannot withstand temperatures lower than 0°C. In *P. trifoliata* transcripts of *CBF1* and *CORc115* (a cold-induced group II LEA gene, and a likely target of CBF1) accumulate both earlier and to higher levels than in grapefruit when exposed to cold temperatures. Additionally, using subtractive hybridization we identified a number of new, differentially cold-regulated genes from *P. trifoliata* (Sahin-Cevik & Moore, 2006). Although several of the genes identified were unique sequences, many were homologous to cold and environmental stress-induced genes from other species. Taken together, our results indicate that similar pathways are present and activated during cold acclimation in diverse plant species.

In a more recent study (Crifo et al., 2011) performed a transcriptome analysis based on subtractive hybridization to study cold stress response of pigmented sweet oranges (blood oranges) in order to study the overall induction in gene expression after the exposure to low temperatures. On the whole, the expression of transcripts related to defense, oxidative damage, osmo-regulation, lipid desaturation and primary and secondary metabolism were induced. In addition, cold stress induced flavonoid biosynthesis, including those reactions involved in anthocyanin biosynthesis and metabolic pathways supplying it. Several transcription factors were identified for the first time as cold responsive genes in plants.

In summary, cold stress has been linked to signaling pathways where gene expression can further interrelate with additional stress related pathways. The entire signaling network throughout the plant affects its response(s) to biotic or abiotic stress. Along with the mentioned gene annotations, additional functional analyses are crucial to study the nature of the expected phenotype before we can introduce new genes into the *Citrus* genome using transformation techniques.

Antimicrobial peptides (AMPs) are currently the subject of intense research for the control of diseases in citrus, particularly canker and huanglongbing (HLB) or citrus greening. There is no known resistance in *Citrus* to HLB (caused by *Candidatus* Liberibacter spp); however, it can have devastating effects by reducing overall production. Infected trees have smaller fruits with less juice, the flavor of the juice is changed and it eventually leads to micronutrient deficiencies, defoliation and tree death. It has been known for years that AMPs play a vital role in plant defense. Plant AMPs are monomer or oligomer building units that have mostly three-dimensional or tertiary structures of either amphipathic or amphiphylic nature (Sitaram & Nagaraj, 1999, 2002). The latter characteristic and folding are essential for the peptides antibacterial activity (Epand & Vogel, 1999). Different scenarios for their function have been suggested but they all agree on the fact that these AMPs operate by the formation of membrane pores that ultimately cause the disruption of the membrane and subsequently cell death through ion and metabolite leakage (Yeamn & Yount, 2003). A number of studies have confirmed the inhibitory effect of these peptides to fungal and bacterial pathogens when expressed in different plant species such as rice, wheat, and tomato fruits (Jha & Chattoo, 2010; Jha et al., 2009; Ramamoorthy et al., 2007). In a recent study, two AMP genes, *Shiva A* and *Cecropin B*, were transformed into 'Jincheng' and 'Newhall' sweet orange. Subsequently, the transgenic plants were challenged with *Xanthomonas axonopodis* pv. *citri*, the causal agent of citrus canker. In both greenhouse and field experiments with artificial or natural inoculation, respectively, some transgenic lines were highly resistant to canker and either did not develop canker lesions or the number of lesions was significantly reduced compared to wild types. The plants were also phenotypically normal, flowered after two years (grafted on *Poncirus*), borne fruit and the juice was no different in solid and sugar content and acidity from non-transgenic plants (He et al., 2011).

4.1 Transformation vs. transient expression

Transient expression systems are beneficial for some purposes, such as rapidly and easily assaying promoter function or gene expression under some conditions. Although it has been surprisingly difficult to implement transient expression in citrus leaves it has been possible to transiently express genes in the fruit, particularly young fruit (Ahmad & Mirza, 2005; Spolaore et al., 2001).

Finally, a vector based on CTV has been developed (Folimonov et al., 2007). Such vectors have been used in herbaceous plants to study gene function, expression, and silencing, but have not been available for woody plants. This can be seen as a hybrid strategy between transient expression and stable transformation. Although the virus vector nucleic acid is not incorporated into the genome of the citrus host, Folimonov et al. (2007) reported that expression of GFP continued for up to four years after introduction of the scorable marker into CTV vectors.

5. Conclusions: The future of citrus transformation

Ultimately the use of genetic engineering is just another tool in the improvement of citrus. Genetic transformation has the advantage of potentially reducing breeding time, particularly important in the case of a perennial crop such as citrus with a long juvenile period, and also facilitating the introduction of traits not readily available in the particular species. Breeding programs take into consideration the needs of both farmers and

consumers. Production of genetically modified citrus should also take into consideration the needs of both; however, genetically modified organisms (GMOs) tend to be more controversial and subjected to more public scrutiny than traditionally produced varieties. For instance, a recent European survey indicated that among respondents GMOs were considered unnatural (70%), made them feel uneasy (61%), harmed the environment (59%) and were unsafe for people's health (59%) (European Comission, 2010). Regardless of whether these concerns are just perceived or real they will have to be addressed in order to fully implement the benefits of genetic engineering in solving real and important problems for citrus farmers and at the same time delivering desirable products to consumers.

Two major concerns regarding GMOs are: 1) impact to the environment, in the form of the transgene 'escaping' and transferring to wild species and thus eroding the biodiversity of wild relatives of the crop or, on the other hand, creating 'super weeds' of species that acquire the transgene and become better fitted and difficult to control (Azevedo & Araujo, 2003; Parrott, 2010; Sweet, 2009); and 2) impact to human health by a potentially toxic or allergenic transgenic protein (Domingo & Gine Bordonaba, 2011).

In the particular case of citrus there are ways to mitigate these concerns. Essentially all presently grown GMOs are *transgenic* in nature, with "trans" referring to genetic sequences that come from organisms that are not crossable with the plant in question, such as sequences from viruses or bacteria or even from a plant species that is not crossable, for instance the insertion of an *Arabidopsis* gene into a citrus plant. This has led to many countries and groups being resistant to the growth and consumption of GMOs. Thus, there are proponents of producing GMOs that are *cisgenic*, where all of the inserted genetic material comes from the original plant or a crossable type (Jacobsen & Schouten, 2008). Such genes could be perceived by the public as more "natural" and could potentially be less likely to be toxic or allergenic (although this would have to be tested experimentally on a case by case basis). Plants transformed this way do not appear to raise the fear and ethical concerns that the production of transgenic plants inspires (Conner et al., 2006; Rommens et al., 2007). However, this approach would rule out the use of most commonly used selectable and scorable marker genes, as well as the most commonly used promoters and termination sequences and the necessary T-DNA borders for *Agrobacterium*-mediated transformation. A cisgene consists of a native gene with its native promoter and termination. In these discussions, there is also mention of *intragenes* in which gene parts can originate from different genes as long as the donor is a crossable type (Jacobsen & Schouten, 2008). Many laboratories are now looking for plant DNA sequences that are homologous to the bacterial sequences present in T-DNA borders and for methods to produce genetically modified plants where selectable and scorable genes can be either removed after transformation or are of plant origin (Rommens et al., 2007).

There has been a small amount of research of this kind in citrus. Fleming et al. (2000) transformed sweet orange protoplasts with a construct containing the GFP scorable gene using a PEG method. Transformed regenerating somatic embryos were identified by their GFP expression and physically separated from nontransformed tissues, resulting in transgenic plants. No *Agrobacterium* was involved and there was no selective agent applied. Ballester et al. (2008) compared the most common citrus transformation and selection system, using kanamycin selection and scorable GUS staining to three methods that did not utilize antibiotic selection, in 'Carrizo' citrange and 'Pineapple' sweet orange. The alternative methods included scoring for GUS staining without applying selection, transforming explants with a multi-autotransformation (MAT) vector, combining an

inducible recombinase-specific recombination system (R/RS) with transgenic-shoot selection through expression of isopentenyl transferase (ipt) and indoleacetamide hydrolase/tryptophan monooxygenase (iaaM/H) marker genes, and selection with the PMI/mannose conditional positive selection system (Boscariol et al., 2003). Transgenic plants were obtained from all treatments, but selection for *nptII* expression was by far the most efficient. The authors preferred the MAT vector, because with it they could obtain transformed plants where the selectable marker would recombine out (Ballester et al., 2007). However, all of the transgenic plants still contained some sequences of bacterial origin.

Another approach is the use of promoters that do not express the transgene in the edible parts (fruits). Again this would potentially reduce the possibility of becoming harmful to human health. Several groups are actively searching for such promoters in citrus, including inducible promoters that would be turned on at will by chemical application, etc. As explained before the genomic information currently available should facilitate this endeavor. A third strategy we are exploring is the use of transgenic rootstocks that could confer the desired trait to the wild type (non transgenic) scion, without the need of incorporating and expressing transgenes in the scion and edible parts of the plant. This would prevent or at least reduce the chances of spreading transgenic pollen into the wild. There is evidence for the transfer of genetic material between rootstock and scion but this seems to be limited to the graft union region (Stegemann & Bock, 2009). However, it is unlikely that this grafting approach would work with all transgenes since not all expressed proteins are translocated and/or have a systemic effect. One case in which it could work in citrus is the reduction of juvenility using the FT protein. Transgenic FT is capable of inducing flowering through graft unions (Notaguchi et al., 2008; Notaguchi et al., 2009). Induction of pathogen defense could potentially be tackled this way as well since some of the proteins activate systemic signaling (Xia et al., 2004).

These approaches take into consideration consumer's perception about GMOs, educated concerns about the release of GMOs and the needs of citrus farmers for better, disease resistant crops. Citrus production faces important challenges due to climate change and disease and genetic engineering has the potential, as has been the case in other crops, of becoming an important weapon in the arsenal against these major challenges.

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7. References

Abel P. P.; Nelson R. S.; De B.; Hoffmann N.; Rogers S. G.; Fraley R. T. & Beachy R. N. (1986). Delay of Disease Development in Transgenic Plants That Express the *Tobacco Mosaic Virus* Coat Protein Gene. *Science,*Vol.232, No.4751, pp 738-743

Ahmad M. & Mirza B. (2005). An Efficient Protocol for Transient Transformation of Intact Fruit and Transgene Expression in Citrus. *Plant Molecular Biology Reporter,*Vol.23, No.4, pp 419-420

Al-Bahrany A. M. (2002). Effect of Phytohormones on in Vitro Shoot Multiplication and Rooting of Lime *Citrus aurantifolia* (Christm.) Swing. *Scientia Horticulturae,*Vol.95, No.4, pp 285-295

Almeida W. A. B.; Mourao F. A. A.; Pino L. E.; Boscariol R. L.; Rodriguez A. P. M. & Mendes B. M. J. (2003). Genetic Transformation and Plant Recovery from Mature Tissues of *Citrus sinensis* L. Osbeck. *Plant Science,*Vol.164, No.2, pp 203-211

Ananthakrishnan G.; Orbovic V.; Pasquali G.; Calovic M. & Grosser J. W. (2007). Transfer of *Citrus Tristeza Virus* (CTV)-Derived Resistance Candidate Sequences to Four Grapefruit Cultivars through *Agrobacterium*-Mediated Genetic Transformation. *In Vitro Cellular & Developmental Biology-Plant,*Vol.43, No.6, pp 593-601

Azevedo F. A.; Mourao F. A. A.; Mendes B. M. J.; Almeida W. A. B.; Schinor E. H.; Pio R.; Barbosa J. M.; Guidetti-Gonzalez S.; Carrer H. & Lam E. (2006). Genetic Transformation of Rangpur Lime (*Citrus limonia* Osbeck) with the *B0* (Bacterio-Opsin) Gene and Its Initial Evaluation for *Phytophthora nicotianae* Resistance. *Plant Molecular Biology Reporter,*Vol.24, No.2, pp 185-196

Azevedo J. L. & Araujo W. L. (2003). Genetically Modified Crops: Environmental and Human Health Concerns. *Mutation research,*Vol.544, No.2-3, pp 223-233

Ballester A.; Cervera M. & Pena L. (2008). Evaluation of Selection Strategies Alternative to NPTII in Genetic Transformation of Citrus. *Plant Cell Reports,*Vol.27, No.6, pp 1005-1015

Ballester A.; Cervera M. & Peña L. (2007). Efficient Production of Transgenic Citrus Plants Using Isopentenyl Transferase Positive Selection and Removal of the Marker Gene by Site-Specific Recombination. *Plant Cell Reports,*Vol.26, No.1, pp 39-45

Barbosa-Mendes J. M.; Mourao F. D. A.; Bergamin A.; Harakava R.; Beer S. V. & Mendes B. M. J. (2009). Genetic Transformation of *Citrus sinensis* cv. Hamlin with *Hrpn* Gene from *Erwinia amylovora* and Evaluation of the Transgenic Lines for Resistance to Citrus Canker. *Scientia Horticulturae,*Vol.122, No.1, pp 109-115

Batuman O.; Mawassi M. & Bar-Joseph M. (2006). Transgenes Consisting of a dsRNA of an RNAi Suppressor Plus the 3' UTR Provide Resistance to *Citrus Tristeza Virus* Sequences in *Nicotiana benthamiana* but Not in Citrus. *Virus genes,*Vol.33, No.3, pp 319-327

Bespalhok F. J. C.; Kobayashi A. K.; Pereira L. F. P.; Galvao R. M. & Vieira L. G. E. (2003). Transient Gene Expression of β-Glucuronidase in Citrus Thin Epicotyl Transversal Sections Using Particle Bombardment. *Brazilian Archives of Biology and Technology,*Vol.46, No.1, pp 1-6

Bond J. E. & Roose M. L. (1998). *Agrobacterium*-Mediated Transformation of the Commercially Important Citrus Cultivar Washington Navel Orange. *Plant Cell Reports,*Vol.18, No.3-4, pp 229-234

Boscariol R. L.; Almeida W. A. B.; Derbyshire M. T. V. C.; Mourão Filho F. A. A. & Mendes B. M. J. (2003). The Use of the PMI/Mannose Selection System to Recover Transgenic Sweet Orange Plants (*Citrus Sinensis* L. Osbeck). *Plant Cell Reports,*Vol.22, No.2, pp 122-128

Cao H.; Li X. & Dong X. N. (1998). Generation of Broad-Spectrum Disease Resistance by Overexpression of an Essential Regulatory Gene in Systemic Acquired Resistance. *Proceedings of the National Academy of Sciences of the United States of America,*Vol.95, No.11, pp 6531-6536

Cao M.; Sato S. J.; Behrens M.; Jiang W. Z.; Clemente T. E. & Weeks D. P. (2010). Genetic Engineering of Maize (*Zea mays*) for High-Level Tolerance to Treatment with the Herbicide Dicamba. *Journal of Agricultural and Food Chemistry.* 10.1021/jf104233h

Cervera M.; Esteban O.; Gil M.; Gorris M. T.; Martinez M. C.; Pena L. & Cambra M. (2010). Transgenic Expression in Citrus of Single-Chain Antibody Fragments Specific to *Citrus Tristeza Virus* Confers Virus Resistance. *Transgenic Research,*Vol.19, No.6, pp 1001-1015

Cervera M.; Juarez J.; Navarro A.; Pina J. A.; Duran-Vila N.; Navarro L. & Peña L. (1998a). Genetic Transformation and Regeneration of Mature Tissues of Woody Fruit Plants Bypassing the Juvenile Stage. in *Transgenic Research* (Springer Netherlands), pp 51-59

Cervera M.; Juarez J.; Navarro L. & Pena L. (2005). Genetic Transformation of Mature Citrus Plants. *Methods in Molecular Biology,*Vol.286, No.III, pp 177-188

Cervera M.; Navarro A.; Navarro L. & Peña L. (2008). Production of Transgenic Adult Plants from Clementine Mandarin by Enhancing Cell Competence for Transformation and Regeneration. *Tree Physiology,*Vol.28, No.1, pp 55-66

Cervera M.; Pina J. A.; Juarez J.; Navarro L. & Pena L. (2000). A Broad Exploration of a Transgenic Population of Citrus: Stability of Gene Expression and Phenotype. *Theoretical and Applied Genetics,*Vol.100, No.5, pp 670-677

Cervera M.; Pina J. A.; Juarez J.; Navarro L. & Peña L. (1998b). *Agrobacterium*-Mediated Transformation of Citrange: Factors Affecting Transformation and Regeneration. *Plant Cell Reports,*Vol.18, No.3-4, pp 271-278

Champ K. I.; Febres V. J. & Moore G. A. (2007). The Role of CBF Transcriptional Activators in Two Citrus Species (*Poncirus* and *Citrus*) with Contrasting Levels of Freezing Tolerance. *Physiologia Plantarum,*Vol.129, No.3, pp 529-541

Conner A. J.; Barrell P. J.; Baldwin S. J.; Lokerse A. S.; Cooper P. a.; Erasmuson A. K.; Nap J.-P. & Jacobs J. M. E. (2006). Intragenic Vectors for Gene Transfer without Foreign DNA. *Euphytica,*Vol.154, No.3, pp 341-353

Costa M. G. C.; Otoni W. C. & Moore G. A. (2002). An Evaluation of Factors Affecting the Efficiency of *Agrobacterium*-Mediated Transformation of *Citrus paradisi* (Macf.) and Production of Transgenic Plants Containing Carotenoid Biosynthetic Genes. *Plant Cell Reports,*Vol.21, No.4, pp 365-373

Crifo T.; Puglisi I.; Petrone G.; Recupero G. R. & Lo Piero A. R. (2011). Expression Analysis in Response to Low Temperature Stress in Blood Oranges: Implication of the Flavonoid Biosynthetic Pathway. *Gene,*Vol.476, No.1-2, pp 1-9

de Oliveira M. L.; Febres V. J.; Costa M. G.; Moore G. A. & Otoni W. C. (2009). High-Efficiency *Agrobacterium*-Mediated Transformation of Citrus Via Sonication and Vacuum Infiltration. *Plant Cell Reports,*Vol.28, No.3, pp 387-395

Despres C.; DeLong C.; Glaze S.; Liu E. & Fobert P. R. (2000). The Arabidopsis NPR1/NIM1 Protein Enhances the DNA Binding Activity of a Subgroup of the TGA Family of Bzip Transcription Factors. *The Plant Cell,*Vol.12, No.2, pp 279-290

Domingo J. L. & Gine Bordonaba J. (2011). A Literature Review on the Safety Assessment of Genetically Modified Plants. *Environment International,* Vol. 37, No. 4, pp 734-742

Domínguez A.; de Mendoza A. H.; Guerri J.; Cambra M.; Navarro L.; Moreno P. & Peña L. (2002). Pathogen-Derived Resistance to *Citrus Tristeza Virus* (CTV) in Transgenic

Mexican Lime (*Citrus aurantifolia* (Christ.) Swing.) Plants Expressing Its *P25*; Coat Protein Gene. *Molecular Breeding,*Vol.10, No.1, pp 1-10

Dominguez A.; Guerri J.; Cambra M.; Navarro L.; Moreno P. & Peña L. (2000). Efficient Production of Transgenic Citrus Plants Expressing the Coat Protein Gene of *Citrus Tristeza Virus*. *Plant Cell Reports,*Vol.19, No.4, pp 427-433

Durrant W. E. & Dong X. (2004). Systemic Acquired Resistance. *Annual Review of Phytopathology,*Vol.42, pp 185-209

Endo T.; Shimada T.; Fujii H.; Kobayashi Y.; Araki T. & Omura M. (2005). Ectopic Expression of an FT Homolog from Citrus Confers an Early Flowering Phenotype on Trifoliate Orange (*Poncirus trifoliata* L. Raf.). *Transgenic Research,*Vol.14, No.5, pp 703-712

Epand R. M. & Vogel H. J. (1999). Diversity of Antimicrobial Peptides and Their Mechanisms of Action. *Biochimica et Biophysica Acta (BBA) - Biomembranes,*Vol.1462, No.1-2, pp 11-28

European Comission (2010). Special Eurobarometer-Biotechnology. *Eurobarometer.* 341 (Wave 73.1): pp 385.
http://ec.europa.eu/public_opinion/archives/ebs/ebs_341_en.pdf

Fagoaga C.; Lopez C.; de Mendoza A. H.; Moreno P.; Navarro L.; Flores R. & Pena L. (2006). Post-Transcriptional Gene Silencing of the P23 Silencing Suppressor of *Citrus Tristeza Virus* Confers Resistance to the Virus in Transgenic Mexican Lime. *Plant Molecular Biology,*Vol.60, No.2, pp 153-165

Fagoaga C.; Lopez C.; Moreno P.; Navarro L.; Flores R. & Pena L. (2005). Viral-Like Symptoms Induced by the Ectopic Expression of the P23 Gene of *Citrus Tristeza Virus* Are Citrus Specific and Do Not Correlate with the Pathogenicity of the Virus Strain. *Molecular Plant-Microbe Interactions,*Vol.18, No.5, pp 435-445

FAO (2011). Citrus Fruit Production. *Food And Agriculture Organization (FAO) Of The United Nations, FAOSTAT.* 2009. http://faostat.fao.org

Febres V. J.; Lee R. F. & Moore G. A. (2008). Transgenic Resistance to *Citrus Tristeza Virus* in Grapefruit. *Plant Cell Reports,*Vol.27, No.1, pp 93-104

Febres V. J.; Niblett C. L.; Lee R. F. & Moore G. A. (2003). Characterization of Grapefruit Plants (*Citrus paradisi* Macf.) Transformed with *Citrus Tristeza Closterovirus* Genes. *Plant Cell Reports,*Vol.21, No.5, pp 421-428

Fleming G. H.; Olivares-Fuster O.; Del-Bosco S. F. & Grosser J. W. (2000). An Alternative Method for the Genetic Transformation of Sweet Orange. *In Vitro Cellular & Developmental Biology - Plant,*Vol.36, No.6, pp 450-455

Folimonov A. S.; Folimonova S. Y.; Bar-Joseph M. & Dawson W. O. (2007). A Stable RNA Virus-Based Vector for Citrus Trees. *Virology,*Vol.368, No.1, pp 205-216

Gelvin S. B. (2003). Agrobacterium-Mediated Plant Transformation: The Biology Behind the "Gene-Jockeying" Tool. *Microbiology and Molecular Biology Reviews,*Vol.67, No.1, pp 16-37

Ghorbel R.; Domínguez A.; Navarro L. & Peña L. (2000). High Efficiency Genetic Transformation of Sour Orange (*Citrus aurantium*) and Production of Transgenic Trees Containing the Coat Protein Gene of *Citrus Tristeza Virus*. *Tree Physiology,*Vol.20, No.17, pp 1183-1189

Gonsalves D. (1998). Control of *Papaya Ringspot Virus* in Papaya: A Case Study. *Annual Review of Phytopathology,*Vol.36, pp 415-437

Grosser J. W.; Jiang J.; Louzada E. S.; Chandler J. L. & Gmitter F. G. (1998a). Somatic Hybridization, an Integral Component of Citrus Cultivar Improvement: II. Rootstock Improvement. *Hortscience,*Vol.33, No.6, pp 1060-1061

Grosser J. W.; Jiang J.; Mourao F. D. A.; Louzada E. S.; Baergen K.; Chandler J. L. & Gmitter F. G. (1998b). Somatic Hybridization, an Integral Component of Citrus Cultivar Improvement: I. Scion Improvement. *Hortscience,*Vol.33, No.6, pp 1057-1059

Gutierrez-E M. A.; Luth D. & Moore G. A. (1997). Factors Affecting *Agrobacterium*-Mediated Transformation in Citrus and Production of Sour Orange (*Citrus aurantium* L.) Plants Expressing the Coat Protein Gene of *Citrus Tristeza Virus*. *Plant Cell Reports,*Vol.16, No.11, pp 745-753

He Y. R.; Chen S. C.; Peng A. H.; Zou X. P.; Xu L. Z.; Lei T. G.; Liu X. F. & Yao L. X. (2011). Production and Evaluation of Transgenic Sweet Orange (*Citrus sinensis* Osbeck) Containing Bivalent Antibacterial Peptide Genes (*Shiva A* and *Cecropin B*) Via a Novel *Agrobacterium*-Mediated Transformation of Mature Axillary Buds. *Scientia Horticulturae,*Vol.128, No.2, pp 99-107

Hidaka T. & Omura M. (1993). *Agrobacterium*-Mediated Transformation and Regeneration of *Citrus Spp*. From Suspension Cells. *Journal of the Japanese Society for Horticultural Science,*Vol.62, No.2, pp 371-376

Jacobsen E. & Schouten H. J. (2008). Cisgenesis, a New Tool for Traditional Plant Breeding, Should Be Exempted from the Regulation on Genetically Modified Organisms in a Step by Step Approach. *Potato Research,*Vol.51, No.1, pp 75-88

Jajoo A. (2010). *In Vitro* Propagation of *Citrus limonia* Osbeck through Nucellar Embryo Culture. *Current Research Journal of Biological Sciences,*Vol.2 No.1, pp 6-8

Jha S. & Chattoo B. (2010). Expression of a Plant Defensin in Rice Confers Resistance to Fungal Phytopathogens. *Transgenic Research,*Vol.19, No.3, pp 373-384

Jha S.; Tank H.; Prasad B. & Chattoo B. (2009). Expression of *Dm-Amp1* in Rice Confers Resistance to *Magnaporthe oryzae* and *Rhizoctonia solani*. *Transgenic Research,*Vol.18, No.1, pp 59-69

Kaneyoshi J.; Kobayashi S.; Nakamura Y.; Shigemoto N. & Doi Y. (1994). A Simple and Efficient Gene-Transfer System of Trifoliate Orange (*Poncirus trifoliata* Raf). *Plant Cell Reports,*Vol.13, No.10, pp 541-545

Khan I. A. (2007). *Citrus Genetics, Breeding and Biotechnology* (CABI, Wallingford, UK ; Cambridge, MA) 370 p.

Khawale R. N.; Singh S. K.; Garg G.; Baranwal V. K. & Ajirlo S. A. (2006). *Agrobacterium*-Mediated Genetic Transformation of Nagpur Mandarin (*Citrus reticulata* Blanco). *Current Science,*Vol.91, No.12, pp 1700-1705

Kinkema M.; Fan W. & Dong X. (2000). Nuclear Localization of NPR1 Is Required for Activation of PR Gene Expression. *The Plant Cell,*Vol.12, No.12, pp 2339-2350

Kobayashi A. K. (2003). Plant Regeneration of Sweet Orange (*Citrus sinensis*) from Thin Sections of Mature Stem Segments. *In Vitro,*Vol.74, No.1, pp 99-102

Kobayashi S. & Uchimaya H. (1989). Expression and Integration of a Foreign Gene in Orange (*Citrus sinensis* Osb.) Protoplasts by Direct DNA Transfer. *Japanese Journal of Genetics,*Vol.64, No.2, pp 91-97

Lee E. K.; Jin Y. W.; Park J. H.; Yoo Y. M.; Hong S. M.; Amir R.; Yan Z.; Kwon E.; Elfick A.; Tomlinson S.; Halbritter F.; Waibel T.; Yun B. W. & Loake G. J. (2010). Cultured

Cambial Meristematic Cells as a Source of Plant Natural Products. *Nature Biotechnology,*Vol.28, No.11, pp 1213-1217

Li D. D.; Shi W. & Deng X. X. (2002). *Agrobacterium*-Mediated Transformation of Embryogenic Calluses of Ponkan Mandarin and the Regeneration of Plants Containing the Chimeric Ribonuclease Gene. *Plant Cell Reports,*Vol.21, No.2, pp 153-156

Lin W. C.; Lu C. F.; Wu J. W.; Cheng M. L.; Lin Y. M.; Yang N. S.; Black L.; Green S. K.; Wang J. F. & Cheng C. P. (2004). Transgenic Tomato Plants Expressing the *Arabidopsis NPR1* Gene Display Enhanced Resistance to a Spectrum of Fungal and Bacterial Diseases. *Transgenic Research,*Vol.13, No.6, pp 567-581

López C.; Cervera M.; Fagoaga C.; Moreno P.; Navarro L.; Flores R. & Peña L. (2010). Accumulation of Transgene-Derived Sirnas Is Not Sufficient for RNAi-Mediated Protection against *Citrus Tristeza Virus* in Transgenic Mexican Lime. *Molecular plant pathology,*Vol.11, No.1, pp 33-41

Luth D. & Moore G. A. (1999). Transgenic Grapefruit Plants Obtained by *Agrobacterium tumefaciens*-Mediated Transformation. *Plant Cell, Tissue and Organ Culture,*Vol.57, No.3, pp 219-222

Maul P.; McCollum G. T.; Popp M.; Guy C. L. & Porat R. (2008). Transcriptome Profiling of Grapefruit Flavedo Following Exposure to Low Temperature and Conditioning Treatments Uncovers Principal Molecular Components Involved in Chilling Tolerance and Susceptibility. *Plant Cell Environ,*Vol.31, No.6, pp 752-768

Messens E.; Dekeyser R. & Stachel S. E. (1990). A Nontransformable *Triticum monococcum* Monocotyledonous Culture Produces the Potent *Agrobacterium* Vir-Inducing Compound Ethyl Ferulate. *Proceedings of the National Academy of Sciences of the United States of America,*Vol.87, pp 4368-4372

Molinari H.; Bespalhok J. C.; Kobayashi A. K.; Pereira L. F. P. & Vieira L. G. E. (2004). Agrobacterium Tumefaciens-Mediated Transformation of Swingle Citrumelo (*Citrus paradisi* Macf.× *Poncirus trifoliata* L. Raf.) Using Thin Epicotyl Sections. *Scientia Horticulturae,*Vol.99, No.3-4, pp 379-385

Moore G. A.; Jacono C. C.; Neidigh J. L.; Lawrence S. D. & Cline K. (1992). *Agrobacterium*-Mediated Transformation of Citrus Stem Segments and Regeneration of Transgenic Plants. *Plant Cell Reports,*Vol.11, No.5-6, pp 238-242

Moreira-Dias J. M.; Molina R. V.; Bordón Y.; Guardiola J. L. & García-Luis A. (2000). Direct and Indirect Shoot Organogenic Pathways in Epicotyl Cuttings of Troyer Citrange Differ in Hormone Requirements and in Their Response to Light. *Annals of Botany,*Vol.85, No.1, pp 103-110

Moreno P.; Ambros S.; Albiach-Marti M. R.; Guerri J. & Pena L. (2008). *Citrus Tristeza Virus*: A Pathogen That Changed the Course of the Citrus Industry. *Molecular Plant Pathology,*Vol.9, No.2, pp 251-268

Nishikawa F.; Endo T.; Shimada T.; Fujii H.; Shimizu T.; Kobayashi Y.; Araki T. & Omura M. (2010). Transcriptional Changes in Cift-Introduced Transgenic Trifoliate Orange (*Poncirus trifoliata* L. Raf.). *Tree Physiology,*Vol.30, No.3, pp 431-439

Notaguchi M.; Abe M.; Kimura T.; Daimon Y.; Kobayashi T.; Yamaguchi A.; Tomita Y.; Dohi K.; Mori M. & Araki T. (2008). Long-Distance, Graft-Transmissible Action of

Arabidopsis Flowering Locus T Protein to Promote Flowering. *Plant & Cell Physiology,*Vol.49, No.11, pp 1645-1658

Notaguchi M.; Daimon Y.; Abe M. & Araki T. (2009). Graft-Transmissible Action of Arabidopsis Flowering Locus T Protein to Promote Flowering. *Plant Signaling & Behavior,*Vol.4, No.2, pp 123-125

Parrott W. (2010). Genetically Modified Myths and Realities. *New Biotechnology,*Vol.27, No.5, pp 545-551

Peña L.; Cervera M.; Fagoaga C.; Romero J.; Juárez J.; Pina J. A. & Navarro L. (2007). Citrus. *Biotechnology in Agriculture and Forrestry* Vol.60, pp 35-50.

Peña L.; Cervera M.; Juarez J.; Navarro A.; Pina J. A.; Duranvila N. & Navarro L. (1995a). *Agrobacterium*-Mediated Transformation of Sweet Orange and Regeneration of Transgenic Plants. *Plant Cell Reports,*Vol.14, No.10, pp 616-619

Peña L.; Cervera M.; Juarez J.; Navarro A.; Pina J. A. & Navarro L. (1997). Genetic Transformation of Lime (*Citrus aurantifolia* Swing): Factors Affecting Transformation and Regeneration. *Plant Cell Reports,*Vol.16, No.11, pp 731-737

Peña L.; Cervera M.; Juarez J.; Ortega C.; Pina J. A.; Duran-Vila N. & Navarro L. (1995b). High Efficiency *Agrobacterium*-Mediated Transformation and Regeneration of Citrus. *Plant Science,*Vol.104, No.2, pp 183-191

Peña L.; Martin-Trillo M.; Juarez J.; Pina J. A.; Navarro L. & Martinez-Zapater J. M. (2001). Constitutive Expression of *Arabidopsis Leafy* or *Apetala1* Genes in Citrus Reduces Their Generation Time. *Nature Biotechnology,*Vol.19, No.3, pp 263-267

Peña L.; Perez R. M.; Cervera M.; Juarez J. A. & Navarro L. (2004). Early Events in *Agrobacterium*-Mediated Genetic Transformation of Citrus Explants. *Annals of Botany,*Vol.94, No.1, pp 67-74

Peña L. & Séguin a. (2001). Recent Advances in the Genetic Transformation of Trees. *Trends in Biotechnology,*Vol.19, No.12, pp 500-506

Ramamoorthy V.; Cahoon E. B.; Li J.; Thokala M.; Minto R. E. & Shah D. M. (2007). Glucosylceramide Synthase Is Essential for Alfalfa Defensin-Mediated Growth Inhibition but Not for Pathogenicity of *Fusarium graminearum*. *Molecular Microbiology,*Vol.66, No.3, pp 771-786

Reyes C. A.; De Francesco A.; Pena E. J.; Costa N.; Plata M. I.; Sendin L.; Castagnaro A. P. & Garcia M. L. (2011). Resistance to *Citrus Psorosis Virus* in Transgenic Sweet Orange Plants Is Triggered by Coat Protein-Rna Silencing. *Journal of Biotechnology,*Vol.151, No.1, pp 151-158

Rodríguez A.; Cervera M.; Peris J. E. & Peña L. (2008). The Same Treatment for Transgenic Shoot Regeneration Elicits the Opposite Effect in Mature Explants from Two Closely Related Sweet Orange (*Citrus sinensis* (L.) Osb.) Genotypes. *Plant Cell, Tissue and Organ Culture,*Vol.93, No.1, pp 97-106

Rommens C. M.; Haring M. A.; Swords K.; Davies H. V. & Belknap W. R. (2007). The Intragenic Approach as a New Extension to Traditional Plant Breeding. *Trends in Plant Science,*Vol.12, No.9, pp 397-403

Sahin-Cevik M. & Moore G. A. (2006). Identification and Expression Analysis of Cold-Regulated Genes from the Cold-Hardy Citrus Relative *Poncirus trifoliate* (L.) Raf. *Plant Molecular Biology,*Vol.62, No.1-2, pp 83-97

Singh S. & Rajam M. V. (2009). Citrus Biotechnology: Achievements, Limitations and Future Directions. *Physiology and Molecular Biology of Plants,*Vol.15, No.1, pp 3-22

Singh S.; Ray B. K.; Bhattacharyya S. & Deka P. C. (1994). *In Vitro* Propagation of *Citrus reticulata* Blanco and *Citrus limon* Burm.F. *Hortscience,*Vol.29, No.3 pp 214-216

Sitaram N. & Nagaraj R. (1999). Interaction of Antimicrobial Peptides with Biological and Model Membranes: Structural and Charge Requirements for Activity. *Biochimica et Biophysica Acta (BBA) - Biomembranes,*Vol.1462, No.1-2, pp 29-54

Sitaram N. & Nagaraj R. (2002). Host-Defense Antimicrobial Peptides: Importance of Structure for Activity. *Current Pharmaceutical Design,*Vol.8, No.9, pp 1381-6128

Spencer P. A. & Towers G. H. N. (1991). Restricted Occurrence of Acetophenone Signal Compounds. *Phytochemistry,*Vol.30, No.9, pp 2933-2937

Spolaore S.; Trainotti L. & Casadoro G. (2001). A Simple Protocol for Transient Gene Expression in Ripe Fleshy Fruit Mediated by *Agrobacterium*. *Journal of experimental botany,*Vol.52, No.357, pp 845-850

Stegemann S. & Bock R. (2009). Exchange of Genetic Material between Cells in Plant Tissue Grafts. *Science,*Vol.324, No.5927, pp 649-651

Sundar I. K. & Sakthivel N. (2008). Advances in Selectable Marker Genes for Plant Transformation. *Journal of Plant Physiology,*Vol.165, No.16, pp 1698-1716

Sweet J. (2009). The 10th International Symposium on the Biosafety of Genetically Modified Organisms (Isbgmo), Wellington, New Zealand, November 2008. *Environmental Biosafety Research,*Vol.8, No.3, pp 161-181

Talon M. & Gmitter Jr F. G. (2008). Citrus Genomics. *International Journal of Plant Genomics,*Vol.2008, pp Article ID 528361

USDA (2010). Adoption of Genetically Engineered Crops in the U.S. *Economic Research Service, Data Set.* http://www.ers.usda.gov/Data/BiotechCrops/

Vardi A.; Bleichman S. & Aviv D. (1990). Genetic Transformation of *Citrus* Protoplasts and Regeneration of Transgenic Plants. *Plant Science,*Vol.69, No.2, pp 199-206

Villemont E.; Dubois F.; Sangwan R. S.; Vasseur G.; Bourgeois Y. & SangwanNorreel B. S. (1997). Role of the Host Cell Cycle in the *Agrobacterium*-Mediated Genetic Transformation of Petunia: Evidence of an S-Phase Control Mechanism for T-DNA Transfer. *Planta,*Vol.201, No.2, pp 160-172

Xia Y.; Suzuki H.; Borevitz J.; Blount J.; Guo Z.; Patel K.; Dixon R. A. & Lamb C. (2004). An Extracellular Aspartic Protease Functions in *Arabidopsis* Disease Resistance Signaling. *The EMBO journal,*Vol.23, No.4, pp 980-988

Yang L.; Hu C.; Li N.; Zhang J.; Yan J. & Deng Z. (2011). Transformation of Sweet Orange [*Citrus sinensis* (L.) Osbeck] with PthA-nls for Acquiring Resistance to Citrus Canker Disease. *Plant Molecular Biology,*Vol.75, No.1-2, pp 11-23

Yao J. L.; Wu J. H.; Gleave A. P. & Morris B. A. M. (1996). Transformation of Citrus Embryogenic Cells Using Particle Bombardment and Production of Transgenic Embryos. *Plant Science,*Vol.113, No.2, pp 175-183

Yeamn M. R. & Yount N. Y. (2003). Mechanisms of Atnimicrobial Peptide Action and Resistance. *Pharmacoloy Reviews,*Vol.55, No.1, pp 27-55

Yu C. H.; Huang S.; Chen C. X.; Deng Z. N.; Ling P. & Gmitter F. G. (2002). Factors Affecting *Agrobacterium*-Mediated Transformation and Regeneration of Sweet Orange and Citrange. *Plant Cell Tissue and Organ Culture,*Vol.71, No.2, pp 147-155

Zhang X. D.; Francis M. I.; Dawson W. O.; Graham J. H.; Orbovic V.; Triplett E. W. & Mou Z. L. (2010). Over-Expression of the Arabidopsis *NPR1* Gene in Citrus Increases Resistance to Citrus Canker. *European Journal of Plant Pathology,*Vol.128, No.1, pp 91-100

Zhang Y. L.; Fan W. H.; Kinkema M.; Li X. & Dong X. N. (1999). Interaction of NPR1 with Basic Leucine Zipper Protein Transcription Factors That Bind Sequences Required for Salicylic Acid Induction of the *PR-1* Gene. *Proceedings of the National Academy of Sciences of the United States of America,*Vol.96, No.11, pp 6523-6528

6

Evaluation of Factors Affecting European Plum (*Prunus domestica* L.) Genetic Transformation

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1. Introduction

With the advancement of genomics research, many genes have been identified and cloned from various plants. Transfer of these genes into plants for gene function studies and for plant improvement is important in the post-genomics era. At this time, the lack of efficient, effective, and high throughput genetic transformation systems in many crops and varieties is a major barrier and a challenge in functional genomics research and for plant trait improvement via biotechnology (Petri and Burgos 2005). Studies and understanding of different aspects and factors in plant transformation are important and are a prerequisite in the development of effective and efficient transformation technologies for various crops and varieties (Gill et al. 2004; Petri and Burgos 2005).

European plum (*Prunus domestica* L.) is an economically important fruit crop and is widely grown across the world (Hartmann 1994; Okie and Ramming 1999; Kaufmane et al. 2002). There have been a number of technology reports of European plum genetic transformation via *Agrobacterium tumefaciens* using hypocotyls as explants (Mante et al. 1991; Padilla et al. 2003; Petri et al. 2008). These studies were actually all conducted in a single research laboratory. Several studies have reported use of transgenic plum for disease resistance (Scorza et al. 1994; 2001; Ravelonandro et al. 2000; Hily et al. 2004; Malimowski et al. 1998, 2006; Capote et al. 2008), however, transgenic plum plants used in these studies were apparently generated in the same research laboratory indicated before. Several other laboratories have reported plum transformation but only putative transformants were reported and Southern blot and other related analyses, which were essential for confirmation of transformation, were not provided (Da Camara-Machado et al. 1994; Yancheva et al. 2002). Use of leaves as explants for plum transformation was described recently but again Southern blot analysis was not provided to confirm transformation

(Kikhailov et al., 2008). Indeed, European plum genetic transformation has only been successful in a few laboratories. Tian et al. (2006) evaluated in vitro regeneration of European plum germplasms and varieties adapted to high latitude and developed genetic transformation technology for these types of plum plants via hopcotyl regeneration (Tian et al. 2009). Nevertheless, at this time, wide and practical use of plum genetic transformation technology in many other laboratories and studies is still not feasible and is difficult. In addition, transformation of European plum has been reported in only a few varieties and the efficiency is low. Development of transformation technologies for many commercial plum varieties and improvement of transformation technology for high efficiency are still major tasks and also important for the germplasm improvement of European plum via biotechnology.

Plant transformation is a complicated process which involves various factors, such as plant genotype and variety, regeneration efficiency, culture medium and condition, selectable marker, infection condition, gene construct and *Agrobacterium* strain. Any of these factors can be important in the success of transformation. Studying, understanding and optimizing various factors are important for the development of transformation technologies for different germplasms and varieties and for technology improvement (Gill et al. 2004; Petri and Burgos 2005).

The objective of this research was to study important aspects of *Agrobacterium*-mediated genetic transformation in European plum via hypocotyl regeneration system. The research results contribute to the knowledge advancement of plum transformation and are useful for the development and improvement of transformation technologies for different varieties for European plum improvement, especially for the genotypes and germplasms adapted to high latitude.

2. Materials and methods

2.1 Plant materials

Plum (*Prunus domestica* L.) plants adapted to high latitude were used in this study. These types of plum germplasms and varieties have been developed by Canadian *Prunus* breeding program over the past years. These genotypes and varieties are more resistant to cold weather, the fruit development and maturation of these genotypes are relative slow, and the fruit ripening is also relatively late in the season (Dr. J. Submaranian, *Prunus* tree breeding program, person. communication). Our previous studies indicate that these plums have low response to *in vitro* regeneration (Tian et al. 2006) and the genetic transformation efficiency is also low (Tian et al. 2009).

Plum fruits, two weeks prior to maturation, were collected from plum trees in Vineland, Ontario, Canada. Endocarps were cracked open and the seeds were sterilized in 10% commercial bleach solution. The seeds were then rinsed three times with sterile distilled water in the laminar flow hood and were imbibed in final rinsing water overnight. After a removal of the seed coat, the embryonic axis was excised from the cotyledons. Embryonic axis was cut into five sections with one radicle, three hypocotyls and one epicotyl. Hypocotyl and epicotyl segments were employed in transient gene expression studies while the radicle was discarded. For stable transformation studies, only hypocotyl slices were used in the experiments. Transformation was conducted using hypocotyls as explants as described by Mante et al. (1991) and Padilla et al. (2003) with modifications by Tian et al. (2009) and in this study.

2.2 *Agrobacterium* and vectors

Three *Agrobacterium tumefaciens* strains, namely EHA105 (Hood et al.1993), LBA4404 (Hoekema et al. 1983) and GV3101 (Holster et al. 1980) and five vectors were included in the research. The vector pCAMBIA2301 (pC2301) has the GUS (*uidA*) reporter gene coding for β-glucuronidase and the *nptII* gene coding for neomycin phosphotransferase under the control of the 35S promoters in the following order: 35S- *uidA* -35S-*nptII*. The plasmid pCAMBIA1301 (pC1301) carries the GUS reporter gene and the *hpt* gene coding for hygromycin B phosphotransferase under the control of the 35S promoters in the following order: 35S- *uidA* -35S-*hpt*. The two vectors are the same except for the selectable marker. The GUS gene in these pCAMBIA vectors contains a plant specific intron which can only be recognized in plant cells and thus cannot express in *Agrobacterium*. The constructs pPV1, pPV2, and pPV3 carry the genes of interest (not shown, unpublished constructs) other than the *nptII* marker gene. These genes were cloned using proper restriction enzymes into the pCaMter X vector and the constructs were introduced in *Agrobacterium* strains LBA4404 and GV3101, respectively.

2.3 *Agrobacterium* infection and plant transformation

Agrobacterium was grown in LB medium with appropriate antibiotics to optimal OD_{600} reading. Explants were immersed in *Agrobacterium* solution for 30 minutes and were blotted dry on sterile Whatman filter paper. The explants were then transferred on co-culture MS medium. The co-culture medium consisted of MS salts (Murashige and Skoog 1962) supplemented with 2.5 µM indolebutyric acid (IBA), 555 µM myo-inositol, 1.2 µM thiamine HCl, 1.4 µM nicotinic acid, 2.4 µM pyridoxine HCl, 25 g L^{-1} sucrose, and 7 g L^{-1} Bactogar. The pH of the medium was adjusted to 5.9 prior to autoclaving. Thidiazuron (TDZ, 7.5 µM) was added to the medium after autoclaving. Different antibiotics and other chemicals were added to the culture medium as needed. The culture was maintained at 25±1°C with 16 hour photoperiod supplied by fluorescent Sylvania "Cool White" light with a Photosynthetic Photon Flux of about 50µmol·m^{-2}·s^{-1}. The explants were collected five days after the infection for transient expression studies. For stable transformation, explants were maintained on co-cultivation MS medium for one week. After co-cultivation, the explants were transferred onto the shoot induction medium. Shoot induction media was the same as the co-cultivation medium but contained 75 mg L^{-1} kanamycin or 5 mg L^{-1} hygromycin depending on the transformation vector used and 300 mg L^{-1} timentin was added to the media. The explants were sub-cultured on fresh induction medium every three weeks. For evaluation of medium type on transformation, B5 medium (Gamborg et al. 1968) was included in the research and other chemicals were added in to B5 medium as in MS medium.

Regenerated shoots at about 0.5 -1 cm in length from antibiotic selections were excised from the explants and transferred to fresh shoot induction medium containing the same antibiotics as well as timetin. After another 2-3 subcultures, well established and developed shoots in antibiotic-containing medium were placed in Magenta boxes containing rooting medium. The rooting medium consisted of 1/2-strength MS salts (Murashige and Skoog 1962), 5 µM naphthalene acetic acid (NAA), 0.01 µM kinetin, vitamins (555 µM myo-inositol, 1.2 µM thiamine HCl, 1.4 µM nicotinic acid, 2.4 µM pyridoxine HCl), 10 g L^{-1} sucrose, and 7 g L^{-1} Bactoagar. Plants developed in magenta containers were transferred to soil and plants were established in a greenhouse. Plants were analyzed for transformation using different approaches as described in previous studies (Tian et al. 2009).

2.4 Histochemical and Fluorogenic GUS expression assay

Five days after *Agrobacterium* infection, explants were collected from co-cultivation media and incubated in 5-bromo-4-chloro-indolyl β-D-glucuronide (X-Glu) in 100 mM sodium phosphate buffer, pH 7.0 overnight at 37°C. Histochemical GUS analysis followed the procedure described in Jefferson et al. (1987). The GUS expression was scaled from 1 - 3 depending on the intensity of GUS staining, with 1 the minimum and 3 the maximum.

For fluorometric GUS expression, plant tissues five days after *Agrobacterium* infection were ground in liquid nitrogen with a pestle and a mortar. A volume of 50 µL of the crude extract was incubated at 37°C with 1 mM 4-methylumbelliferyl glucuronide (MUG) in 0.3 mL of GUS assay buffer (50 mM NaPO4, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 10 mM β-mercaptoethanol). At different time periods of incubation, 0.1 mL aliquot was removed and added to 1.9 ml of 0.2 M Na_2CO_3 to terminate the reaction. Protein standard curve was made by Bradford protein assay and GUS activity was expressed as picomoles of 4-methylumbelliferone (MU) per milligram of protein per hour.

3. Results and discussion

Efficient infection of *Agrobacterium* to plant cells and the subsequent transfer of T-DNA from *Agrobacterium* into plant cells are the first and also essential steps in the stable transformation process. Transient reporter gene expression can be used to evaluate *Agrobacterium* infection and gene transfer into plants cells. The relationship between transient and stable transformation is complicated and varies among species. Studies must be conducted for a particular species to understand how these two aspects are related. If a positive relation can be found and established for a plant species, study of transient reporter gene expression can be very useful for evaluation of various factors for development and optimization of genetic transformation technologies (Chen et al. 1998; Petri et al. 2004).

The plum explants were infected with different *Agrobacterium* strains containing construct pC2301 or pC1301. These constructs carry the GUS-intron design and GUS expression is only activated in plant cells and the GUS expression cannot be due to the presence of *Agrobacterium* cells. Histochemical assay was first conducted to evaluate transient GUS expression in explants infected by different *Agrobacterium* strains and plum varieties Stanley and Vanette were used in the experiments. Results showed that GUS expression was significantly higher when *Agrobacterium* strains LBA4404 and EHA105 were used (Fig. 1, Fig. 2). Enzymatic assay was then conducted in plum Stanley and the results showed that GUS expression in explants was significantly higher using *Agrobacterium* strains LBA4404 and EHA105 than using GV3101 (Fig.3). Research was also conducted to evaluate transient GUS expression using some additional plum varieties including V72511, Veeblue, and Italian. Results showed that explants infected with *Agrobacterium* strains LBA4404 and EHA105 in overall exhibited higher levels of GUS enzyme activities than GV3101 (not shown).

Stable transformation were conducted with either kanamycin selection or hygromycin selection depending on the vectors used. It appeared that transient reporter gene expression was well related to the effectiveness of stable transformation in plum (Table 1, Fig. 2&3). Specifically, higher levels of transient GUS expression after EHA105 and LBA4404 infection led to the effectiveness of stable transformation and consistently generated transgenic lines (Table 1). On the other hand, lower transient GUS expression using strain GV3101 resulted in ineffectiveness of stable transformation (Table 1, Fig. 2&3). Such relation is consistent

using different constructs and with either the kanamycin selection or the hygromycin selection (Table 1).

	Plant Variety and Vector Type		
***Agrobacterium* Strain**	**Stanley-2301**	**Stanley-1301**	**Vanette-1301**
EHA105			
LBA4404			
GV3101			
Control			

Fig. 1. Transient GUS expression in European plum (*Prunus domestica* L.) after infection by *Agrobacterium* strains LBA4404, EHA105 and GV3101 containing either pCAMBIA2301 or pCAMBIA1301 plasmids.

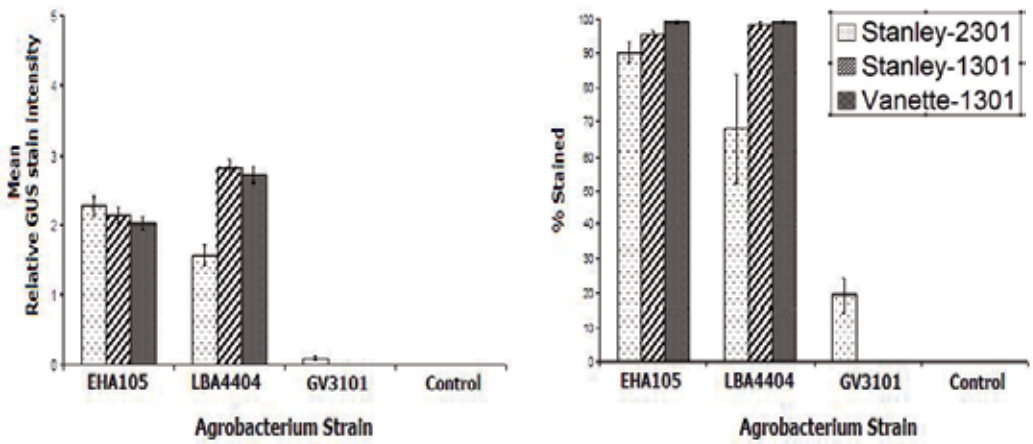

Fig. 2. Histochemical analysis of GUS gene expression in Stanley and Vanette varieties infected by different *Agrobacterium* strains containing either pCAMBIA2301 or pCAMBIA1301 vector.

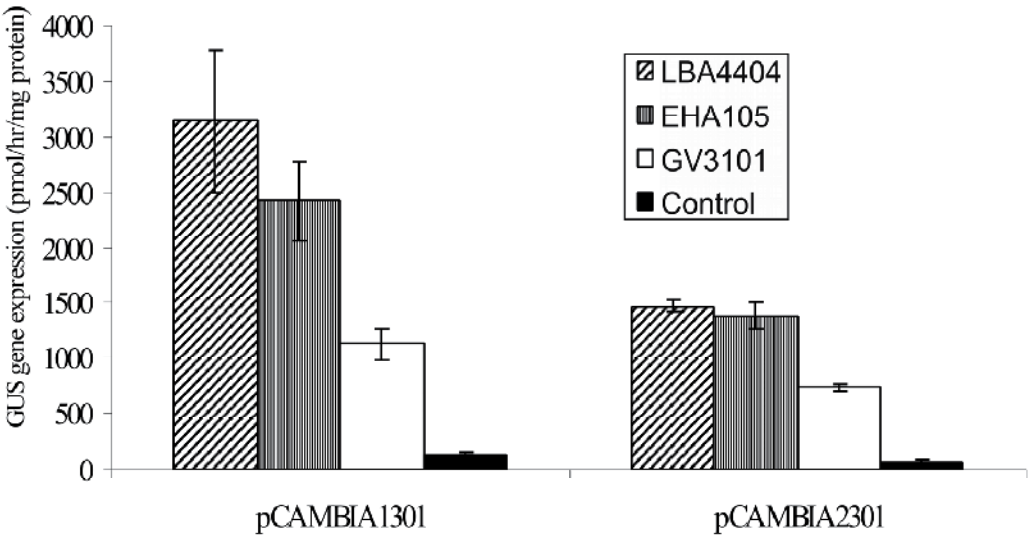

Fig. 3. GUS gene expression via fluorometric assay with Stanley after infection by different *Agrobacterium* strains containing either pCAMBIA2301 or pCAMBIA1301 vector.

Vector	*Agrobacterium* Strain	Selection scheme	No of Explants	No. of Transformants	Transformation Efficiency
	EHA105	Kanamycin	272	6	2.2%
pC2301	LBA4404	Kanamycin	270	2	0.7%
	GV3101	Kanamycin	272	0	0%
	EHA105	Hygromycin	272	3	1.1%
pC1301	LBA4404	Hygromycin	271	2	0.7%
	GV3101	Hygromycin	272	0	0%

Table 1. Stable transformation of European plum using and *Agrobacterium* strains LBA4404, EHA105 and GV3101 containing either pCAMBIA2301 or pCAMBIA1301 vector.

L-cysteine (mg/L)	Number of explants	% of explants transiently expressing GUS gene	Number of transgenic line	Transformation efficiency
0	78	80.0	2	2.6%
900	78	22.7	0	0

Table 2. Transient GUS expression and stable transformation of European plum using L-cysteine in culture medium.

Our previous studies have indicated that use of culture medium including L-cysteine, which was used in media for transformation improvement (Olhoft et al. 2001), could affect transient GUS gene expression in plum (non-published results). We conducted research to study how transient gene expression was related to stable transformation using medium containing L-cysteine. The results showed that explants cultured in medium with L-cysteine resulted in significantly low levels of transient GUS gene expression (Table 2) as found in previous studies. No transformation was obtained from the explants cultured in the presence of L-cysteine. On the other hand, high level of transient GUS expression was observed in explants without L-cysteine treatment and stable transformation was routinely recovered (Table 2). This study further indicated that transient reporter gene expression was related to stable transformation in European plum. The positive relationship between transient reporter gene expression and stable transformation in plum could be important for studying and evaluating various factors and conditions for transformation, which can be useful in the development and improvement of stable transformation technologies in different plum varieties.

Construct	Agro strain	No. of total explants	Lines recovered	Efficiency
pPV-1	GV3101	444	0	0.0%
	LBA4404	1019	17	1.7%
pPV-2	GV3101	330	1	0.3%
	LBA4404	601	11	1.8%
pPV-3	GV3101	283	0	0.0%
	LBA4404	769	20	2.6%
Summary	GV3101	1057	1	0.09%
	LBA4404	2389	48	2.0%

Table 3. Stable genetic transformation of European plum using *Agrobacterium* strains LBA4404 and GV3101 containing different transformation vectors with the genes of interest

Medium	Number of explants	Number of transgenic lines	Transformation efficiency (%)
MS	300	6	2.0
B5	310	19	6.1

Table 4. Plum genetic transformation using B5 and MS co-cultivation and shoot induction media.

Agrobacterium strain is a major factor in plant transformation. Numerous studies have indicated that the effectiveness of transformation via different strains of *Agrobacterium* can be significantly different (e.g., De Bondt et al. 1994; Le Gall et al. 1994; Bond and Rose 1998; Cervera et al. 1998; Gill et al. 2004; Petri et al. 2004; Joyce et al. 2010). Several *Agrobacterium* strains, including LBA4404, EHA101, EHA105, GV3101, have been used in plum transformation previously (Mante et al. 1991; Padilla et al. 2003; Petri et al. 2008). No transformation efficiency difference was found using EHA105 and LBA4404 (Padilla et al 2003). Petri et al. (2008) conducted plum transformation using EHA105 and GV3101. The study showed that use of the same *Agrobacterium* strains carrying different transformation constructs resulted in significant difference of transformation efficiency (Petri et al. 2008). This difference in the transformation efficiency could be due to the presence of the different constructs. Till date, the effect of *Agrobacterium* strains on plum transformation is still not well understood. In this research we studied two *Agrobacterium* strains, LBA4404 and GV3101, which have never been directly compared in plum transformation. These two strains, in contrast to the study by Petri et al (2008), carried the identical constructs and transformation was conducted using a large number of explants. No stable transformation was achieved with constructs pPV-1 and pPV-3 when the strain GV3101 was used, whereas with strain LBA4404, the transformation efficiencies with these constructs were 1.7% and 2.6%, respectively (Table 3). Of the three constructs, only one transformant was recovered using GV3101 (Table 3). Combining the results of all three constructs and all experiments, the transformation efficiency with LAB4401 was 22 times higher than that using GV3101 (Table 3). The different transformation efficiencies of *Agrobacterium* strains can also be observed in the study of transient gene expression and stable transformation described before (Fig. 2,3; Table 1). The results showed that *Agrobacterium* strain LBA4404 was much more effective and was more suitable than GV3101 in plum transformation. The study suggests that *Agrobacterium* strains can be an important factor in plum transformation and should be carefully considered for various studies and for transformation of different plum varieties. Effective plum transformation using *Agrobacterium* strain LBA4404 is illustrated in Fig. 4.

Plant genetic transformation is usually conducted *via* tissue culture systems. The culture medium is the platform and the fundamental base of transformation. Medium can be an important factor for plant transformation (Joyce et al. 2010). There is not report regarding the effect of different culture media on plum genetic transformation. We conducted research on this aspect. Two commonly used culture media, B5 medium and MS medium, and construct pCAMBIA2301 were used in the research. Explants, after *Agrobacterium* infection, were cultured on MS and B5 co-cultivation media as well as regeneration media respectively. The transformation efficiency using MS medium was 2.0% and the efficiency with B5 medium was 6.1% (Table 4). Use of B5 medium was three times more efficient in plum transformation. Use of different media apparently had a major impact on plum transformation. The B5 medium might have promoted more transformed cells to develop and regenerate into plants, resulting in higher transformation efficiency. A recent study has showed that adding 2, 4-D to culture medium can significantly increase plum transformation efficiency (Petri et al. 2008). This addition of a plant growth regulator probably increased recovery of more transformed cells as discussed in this study. We would conduct experiments to evaluate the effect of 2, 4-D on the transformation of plum genotypes adapted to high latitude.

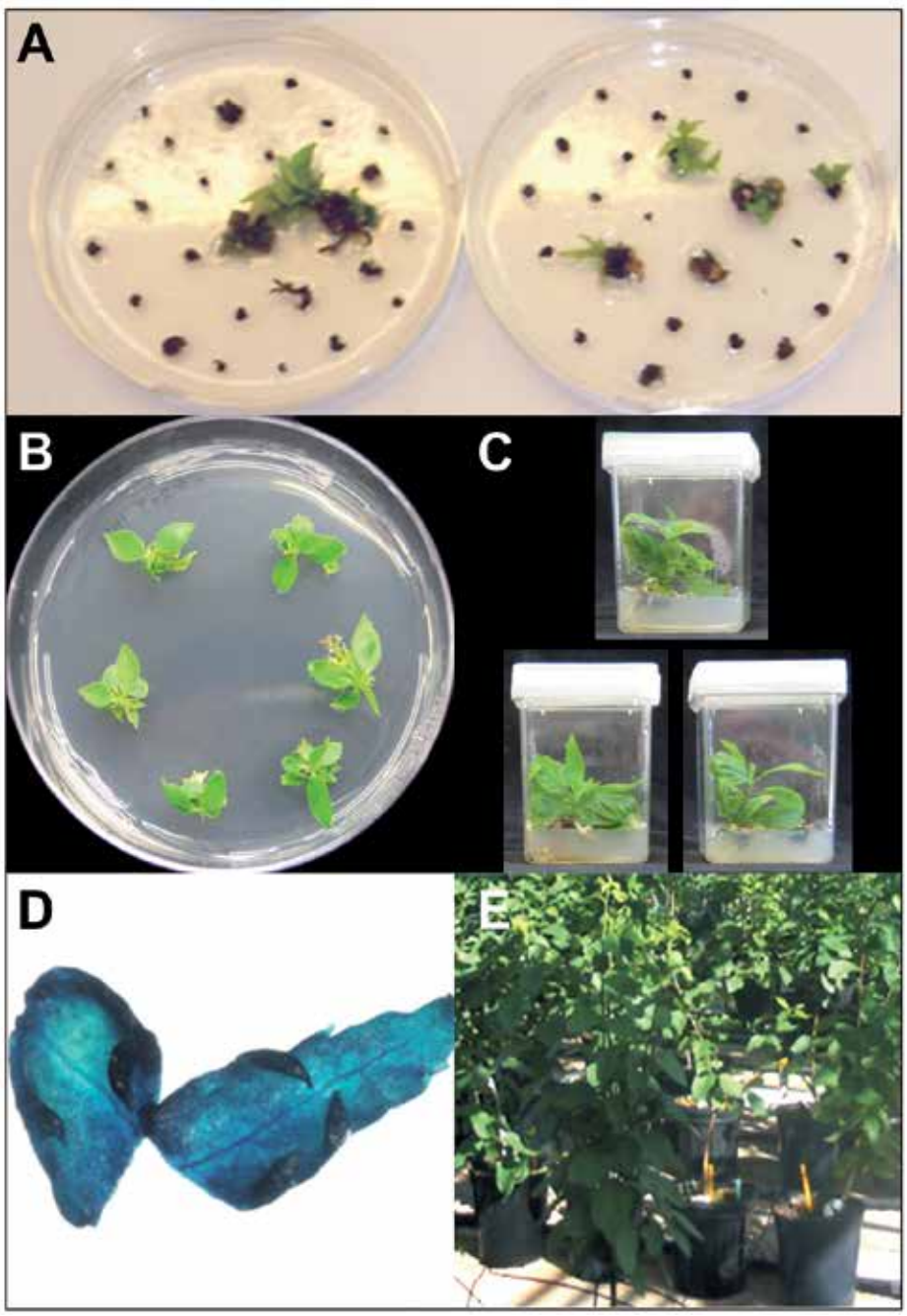

Fig. 4. European plum using *Agrobacterium* LBA4404. (A) Development of transgenic shoots on selection medium containing 75 mg·L^{-1} kanamycin. (B) Shoots excised from explants grew vigorously upon subculturing to the same selection medium. (C) Development of transgenic plants on rooting medium in Magenta boxes. (D) GUS expression in leaves of transgenic plum plants. (E) Transgenic plants in the greenhouse.

4. Conclusion

Genetic transformation efficiency in *Prunus* crops is significantly lower and the technology is much less developed compared to some other fruit crops. We have studied some aspects of plum transformation which have not been explored before. The study shows that the transient gene expression is in general well related to stable transformation in plum. This is important for studying and optimizing various conditions and factors for stable transformation of plum varieties. The study also shows that certain *Agrobacterium* strains strongly affect European plum genetic transformation. While *Agrobacterium* strains LBA4404 can lead to successful plum transformation, the strain GV3101 is ineffective in generating transgenic lines. Moreover, use of different types of media can significantly affect stable transformation. The results obtained from this research would contribute to the knowledge advancement and to the development of more efficient and effective transformation technologies for plum fruit crop, especially for germplasms and varieties adapted to high latitude.

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6. References

Bond, J. E. and Roose, M. L. 1998. *Agrobacterium*-mediated transformation of the commercially important citrus cultivar Washington navel orange. Plant Cell Rep. 18:229-234.

Capote, N., Pérez-Panadés, J., Monzó, C., Carbonell, E., Urbaneja, A., Scorza, R., Ravelonandro, M. and Cambra, M. 2008. Assessment of the diversity and dynamics of Plum pox virus and aphid populations in transgenic European plums under Mediterranean conditions. Transgenic Res. 17:367-377.

Cervera, M., Lopez, M. M., Navarro, L. and Pena, L. 1998. Virulence and supervirulence of *Agrobacterium Tumefaciens* in woody fruit plants. Physiological and Molecular Plant Pathology 52:67-78.

Chen, L., Zhang, S., Beachy, R. N. and Fauquet, C. M. 1998. A protocol for consistent, large-scale production of fertile transgenic rice plants. Plant Cell Rep. 18:25-31.

da Câmara Machado, A., Katinger, H. and Laimer da Câmara Machado, M. 1994. Coat protein-mediated protection against plum pox virus in herbaceous model plants and transformation of apricot and plum. Euphytica 77:129-134.

De Bondt, A., Eggermont, K., Druart, P., De Vil, M., Goderis, I., Vanderleyden, J. and Broekaert, W. F. 1994. *Agrobacterium*-mediated transformation of apple (*Malus* x *domestica* Borkh.): An assessment of factors affecting gene transfer efficiency during early transformation steps. Plant Cell Rep. 13:587-593.

Gamborg, O. L., Miller, R. A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cell. Exp. Cell Research 50:151-158.

Gill, M. I. S., Zora, S. and Agrez, V. 2004. Factors affecting *Agrobacterium*-mediated genetic transformation in fruit and nut crops - an overview. J. Food, Agriculture & Environment 2:327-347.

Hartmann, W. 1994. Plum production in Germany. Acta Hort 359:17-25.

Hily, J. M., Scorza, R., Malinowski, T., Zawadzka, B. and Ravelonandro, M. 2004. Stability of gene silencing-based resistance to Plum pox virus in transgenic plum (*Prunus domestica* L.) under field conditions. Transgenic Res. 13:427-436.

Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J. and Schilperoort, R. A. 1983. A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179-180.

Holsters, M., Silva, B., Van Vliet, F., Genetello, C., De Block, M., Dhaese, P., Depicker, A., Inzé, D., Engler, G., Villarroel, R. and others. 1980. The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. Plasmid 3:212-230.

Hood, E. E., Gelvin, S. B., Melchers, L. S. and Hoekema, A. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res. 2:208-218.

Jefferson, R. A., Kavanaugh, T. A. and Bevan, M. W. 1987. GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.

Joyce, P., Kuwahata, M., Turner, N. and Lakshmanan, P. 2010. Selection system and co-cultivation medium are important determinants of *Agrobacterium*-mediated transformation of sugarcane. Plant Cell Rep. 29:173-183.

Kaufmane, E., Ikase, L., Trajkovski, V. and Lacis, G. 2002. Evaluation and characterization of plum genetic resources in Sweden and Latvia. Acta Hort. 577:207-213.

Kikhailov R.V,Shuga O.A., and Dolgov, S. V. 2008. Genetic transformation os somatic tissues and molecular biology approach for improving resistance to sharka disease of plum (Prunuc domestica L.) varieties. First International Symposium on Biotechnology of Fruit Species. September 1-5, 2008. Dreston, Germany.

Le Gall, O., Torregrosa, L., Danglot, Y., Candresse, T. and Bouquet, A. 1994. *Agrobacterium*-mediated genetic transformation of grapevine somatic embryos and regeneration of transgenic plants expressing the coat protein of grapevine chrome mosaic nepovirus (GCMV). Plant Sci. 102:161-170.

Malinowski, T., Zawadzka, B, Ravelonandro, M. and Scorza, R. 1998 Preliminary report on the apparent breaking of resistance of a transgenic plum by chip bud inoculation of *Plum pox virus* PPV-S. Acta Virol. 42:241–243

Malinowski, T., Cambra, M., Capote, N., Zawadzka, B, Gorris, M. T., Scorza, R. and Ravelonandro, M. 2006. Field trials of plum clones transformed with the Plum pox virus coat protein (PPV-CT) gene. Planr Disease 90: 1012-1018.

Mante, S., Morgens, P. H., Scorza, R., Cordts, J. M. and Callahan, A. M. 1991. *Agrobacterium*-mediated transformation of plum (*Prunus domestica* L.) hypocotyl slices and regeneration of transgenic plants. Bio/Technol 9:853-857.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Okie, W. R. and Ramming, D. W. 1999. Plum breeding worldwide. HortTechnology 9:162-176.

Olhoft, P. M. and Somers, D. A. 2001. L-Cysteine increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells. Plant Cell Rep. 20:706-711.

Padilla, I. M. G., Webb, K. and Scorza, R. 2003. Early antibiotic selection and efficient rooting and acclimatization improve the production of transgenic plum plants (*Prunus Domestica* L.). Plant Cell Rep. 22:38-45.

Petri, C., Alburquerque, N., GarcÃa-Castillo, S., Egea, J. and Burgos, L. 2004. Factors affecting gene transfer efficiency to apricot leaves during early *Agrobacterium*-mediated transformation steps. Journal of Hort. Sci. Biotech. 79:704-712.

Petri, C. and Burgos, L. 2005. Transformation of fruit trees. Useful breeding tool or continued future prospect? Transgenic Res. 14:15-26.

Petri, C., Webb, K., Hily, J. M., Dardick, C. and Scorza, R. 2008. High transformation efficiency in plum (*Prunus domestica* L.): A new tool for functional genomics studies in *Prunus* spp. Mole. Breed. 22:581-591.

Ravelonandro, M., Scorza, R., Callahan, A., Levy, L., Jacquet, C., Monsion, M. and Damsteegt, V. 2000. The Use of Transgenic Fruit Trees as a Resistance Strategy for Virus Epidemics: the Plum Pox (Sharka) Model. Virus Res. 71:63-69.

Scorza, R., Ravelonandro, M., Callahan, A. M., Cordts, J. M., Fuchs, M., Dunez, J. and Gonsalves, D. 1994. Transgenic plums (*Prunus domestica* L.) express the plum pox virus coat protein gene. Plant Cell Rep. 14:18-22.

Scorza R, Callahan A, Levy L, Damsteegt V, Webb K, Ravelonandro M 2001 Post-transcriptional gene silencing in Plum pox virus resistant transgenic European plum containing the Plum pox potyvirus coat protein gene. Transgenic Res.10:201–209.

Tian, L., Sibbald, S., Subramanian, J and Svircev, S. 2006. Characterization of *Prunus domestica* L. *in vitro* regeneration via hypocotyls. Sci. Hort.. 112:462-466.

Tian, L., Canli, F. A., Wang, X. and Sibbald, S. 2009. Genetic transformation of *Prunus domestica* L. using the *hpt* gene coding for hygromycin resistance as the selectable marker. Sci. Hort. 119:339-343.

Yancheva, S. D., Druart, P. and Watillon, B. 2002. *Agrobacterium* - nediated transformation of plum (*Prunus domestica* L.). Acta Hort. 577:215-217.

7

Genetic Transformation of Wheat: Advances in the Transformation Method and Applications for Obtaining Lines with Improved Bread-Making Quality and Low Toxicity in Relation to Celiac Disease

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1. Introduction

Wheat is one of the most important crops and is counted among the "big three" cereal crops (rice, wheat and maize), with an annual world production of around 680 million tonnes in 2009. Wheat is also one of the main sources of calories and proteins in the human diet. However, in spite of its global importance, wheat has been one of the last crops being transformed and it was not until 1992 when Vasil et al. (1992) obtained the first fertile transgenic plant of wheat. Nowadays, wheat transformation still presents more difficulties than transformation of other cereals, such as rice and maize, with lower transformation efficiencies and greater genotype dependence (Shewry & Jones, 2005). Particle bombardment is the most widely used method for genetic transformation of wheat, presenting higher transformation efficiencies than *Agrobacterium*-mediated transformation (Lazzeri & Jones, 2009). However, particle bombardment causes physical damage to the scutellar tissues used for transformation, negatively affecting the embryogenesis, *in vitro* regeneration of the explants and therefore the transformation efficiency. Osmotic treatment is thought to offer protection to bombarded material by minimising cytoplasm leakage from target cells (Vain et al., 1993), so it is of great importance to optimise the duration and moment of application of the osmotic treatment to the explants.

Among the applications of genetic transformation, gene over-expression and post-transcriptional gene silencing (PTGS) are two strategies successfully used to enhance the wheat quality. In particular, the baking quality of wheat, largely determined by the high molecular weight glutenin subunits (HMW-GS), is one of the most important targets for genetic transformation. Transgenic wheat lines expressing additional copies of the 1Ax1, 1Dx5, 1Dy10 HMW-GS genes were obtained by particle bombardment by León et al. (2009) (Fig. 1 A). In addition, new lines combining the three transgenic events were obtained by conventional crossing (León et al., 2010) (Fig. 1 B). Therefore, a set of

transgenic wheat lines expressing one or two extra HMW-GS was generated. These lines were analysed and changes in the protein and starch composition were studied. In addition, the rheological and pasting properties of dough were substantially improved or altered by expressing those HMW-GS genes as described in León et al. (2009, 2010a, 2010b).

The transgenic line T619, which presents down-regulation of all the HMW-GS, was obtained when aimed to over-express a D hordein from *Hordeum chilense* in *Triticum aestivum* cv Perico. D hordeins from *H. chilense* have a very similar structure and amino acid sequence to the HMW-GS from wheat as reported by Pistón et al. (2007). The result was the specific down-regulation of all the HMW-GS (Fig. 1 C), probably due to a transgene-induced silencing at the transcriptional or post-transcriptional level. These silencing phenomena presumably involve homology-dependent gene silencing (Meyer & Saedler, 1996) and resemble co-suppression in which mutual inactivation of transgenes and homologous genes occurs. Similar silencing effects in the HMW-GS were previously reported by Alvarez et al. (2000) when expressing the 1Ax1 subunit transgene and over-expressing the 1Dx5 gene in wheat.

PTGS by RNA interference (RNAi) is based on sequence-dependent RNA degradation that is triggered by the formation of double-stranded RNA (dsRNA) homologous in sequence to the targeted gene (Baulcombe, 2004). In contrast to other gene silencing methods such as insertional mutagenesis or TILLING (Targeting Induced Local Lesions in Genomes) approaches, RNAi allows silencing of one gene or all members from a multigene family by targeting sequences that are specific or shared by several genes (Miki et al., 2005).

Wheat gliadins account for about 50% of total gluten proteins. Gliadins are divided into three or four groups named α/β-, γ- and ω-gliadins, based on their mobility in an acid polyacrylamide gel electrophoresis (A-PAGE) system. Gliadins are also considered the main factor triggering celiac disease (CD), a common enteropathy induced by ingestion of wheat gluten proteins and related prolamins from oat, rye, and barley in genetically susceptible individuals. When CD patients consume foods containing gluten, their immune systems react by damaging the small intestine, with severe consequences. The only available treatment for the disease is a lifelong gluten-exclusion diet. Therefore, another priority aspect regarding the improvement of wheat quality is the reduction of gluten toxicity for CD patients. Gil-Humanes et al. (2010) reported a high efficiency RNAi hairpin vector that was used in combination with genetic transformation to down-regulate the expression of genes from the three gliadin fractions at the same time in bread wheat. The RNAi approach was very effective in the shutdown of CD-related wheat gliadin T-cell epitopes. Although the suppression of gliadins had a high impact on protein fractions such as gliadins, glutenins, albumins and globulins, it did not affect significantly to the total protein content (Gil-Humanes et al., 2011).

In this work we have optimised the osmotic treatment for wheat transformation using particle bombardment. Immature scutella were exposed to 0.4 M mannitol treatment during 4h or 16h pre- or post-bombardment, and the results obtained have positively contributed to the optimization of the transformation method in wheat, increasing significantly the transformation efficiency. These osmotic treatments are now routinely used in the generation of transgenic plants at high efficiency. We also report the effects of HMW-GS over-expression and silencing, as well as the silencing of all the groups of gliadins, on the content and proportions of protein, starch and carbohydrates in transgenic wheat.

2. Materials and methods

2.1 Plant material and genetic transformation

Three cultivars of bread wheat (*T. aestivum*) were used in this study for genetic transformation: cv Bobwhite, supplied by the CIMMYT1, was used for the osmotic treatment study and gliadin down-regulation; cv Anza was used for HMW over-expression; and cv Perico was used for HMW-GS down-regulation (Fig. 1).

Transgenic lines of *T. aestivum* cv Anza expressing one or two extra HMW-GS genes were described by León et al. (2009, 2010a, 2010b) and are: line T580, line T581 and line T590, which express the subunits 1Ax1, 1Dx5, 1Dy10, respectively (Fig. 1 A); and the lines obtained by conventional crossing of the previous lines: line T606, line T616 and line T617, which express the pairs of subunits 1Ax1+1Dx5, 1Ax1+1Dy10 and 1Dx5+1Dy10, respectively (León et al., 2010b)(Fig. 1 B). Transgenic line T619, with down-regulation of all the HMW-GS, was obtained by transformation of *T. aestivum* cv Perico with a D hordein gene from *H. chilense* (Fig. 1 C). The down-regulation of all the groups of gliadins in *T. aestivum* cv Bobwhite was reported by Gil-Humanes et al. (2010) and for the present work we have used the following lines: D793, D894, E42 and E82. All the transgenic plants were self-pollinated for two to three generations to obtain homozygous lines.

The transformation method used to produce all the lines described in this work was the following: donor plants for genetic transformation were grown in the greenhouse under controlled conditions with supplementary lights providing a day/night regime of 16/8h and 23-25/18-19°C. Sixteen days after anthesis immature caryopses were isolated and sterilised by rinsing in 70% (v/v) aqueous ethanol for 5 min and soaking for 15-20 min in a 1% (v/v) sodium hypochlorite solution. Then, caryopses were washed three times with sterile distilled water. Embryos of approximately 0.5-1.5 mm length were used, since they have been demonstrated to be the most responsive in our conditions. To avoid the precocious germination, immature scutella were isolated from the seed embryos by removing the embryo axis and placed with the scutellum exposed in the induction medium MP4 consisting in MS medium (Murashige & Skoog, 1962) supplemented with 30 gl^{-1} sucrose and 4 mgl^{-1} picloram. Explants were cultured in the dark at 25 ^{o}C for 4 days prior bombardment (see Fig. 2). Osmotic treatment is thought to offer protection to bombarded material by minimising cytoplasm leakage from target cells (Vain et al., 1993), so in the HMW-GS over-expression and gliadins silencing experiments, explants were subjected to a 4h osmotic treatment, before and/or after bombardment. In addition, in order to optimise the transformation conditions, different osmotic treatments (4h and 16h before bombardment and 4h and 16h after bombardment) were evaluated by placing the explants in medium MP40.4M consisting in MP4 solid medium supplemented with 0.4M mannitol (for more details see Table 1). In the transformation experiments for down-regulation of the HMW-GS genes, only 4h osmotic treatment post-bombardment was applied. Particle bombardment and selection of the transformed explants in the *in vitro* culture were as described by León et al. (2009). Modifications in the protocol were introduced in the experiments for optimization of the osmotic treatment as described below.

[1] International maize and wheat improvement center

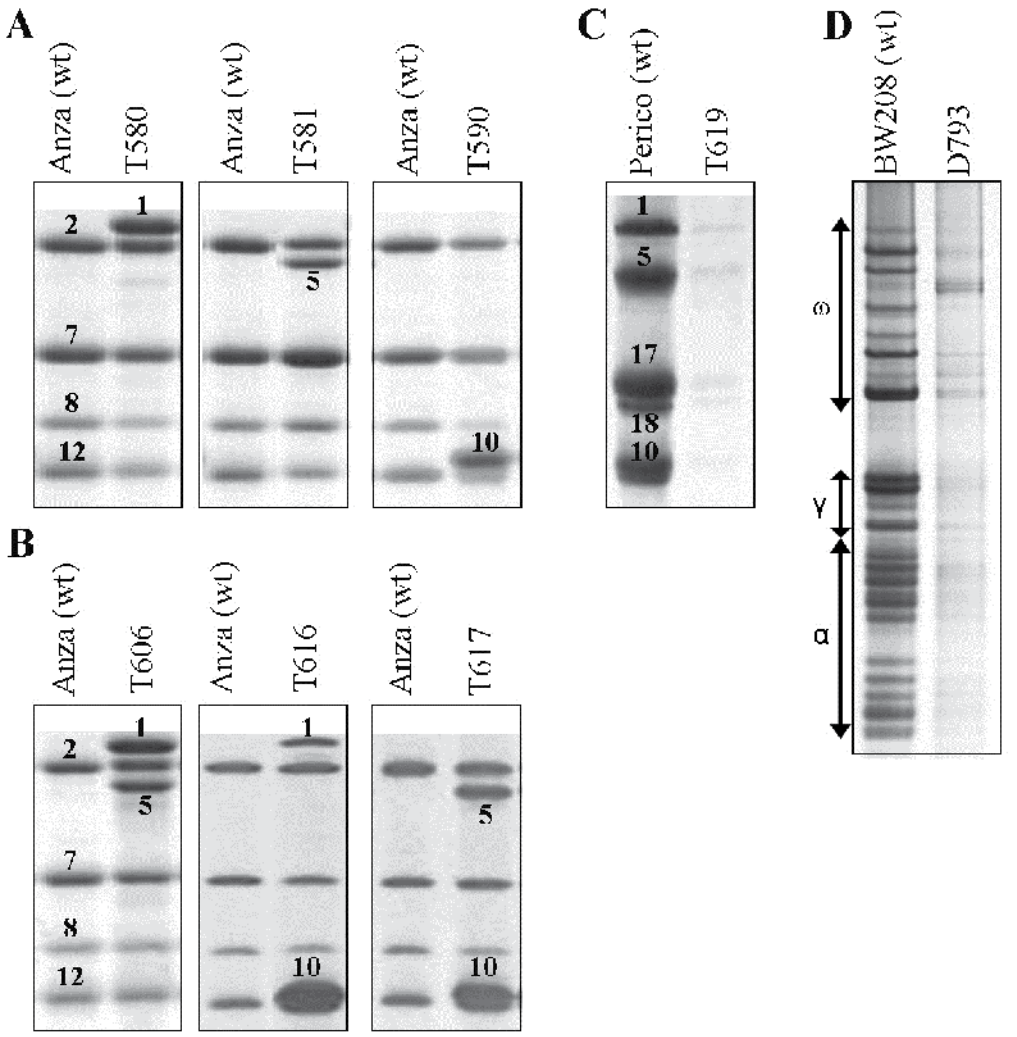

Fig. 1. SDS-PAGE gels of wild type lines and transgenic lines with over-expression of (A) one HMW-GS gene, and (B) two HMW-GS genes; and (C) down-regulation of all the HMW-GS. (D) A-PAGE of wild type and transgenic line D793 with down-regulation of all the gliadin fractions.

2.2 Osmotic treatment

Four different osmotic treatments were evaluated to increase the transformation efficiency of wheat (see Fig. 2). For each treatment, 1500 scutella distributed in 60 Petri dishes (25 scutella per dish) were isolated: 250 scutella were used as non transformed controls and did not receive the osmotic treatment (control-S); explants were transformed with the pAHC25 plasmid (Christensen & Quail, 1996) containing the *uidA* and *bar* genes, and subjected to one of the following osmotic treatments at a ratio of 250 scutella per treatment: 1) no osmotic treatment (control-B), 2) osmotic treatment of 4h prior bombardment, 3) 16h prior bombardment, 4) 4h after bombardment, and 5) 16h after bombardment (Fig. 2).

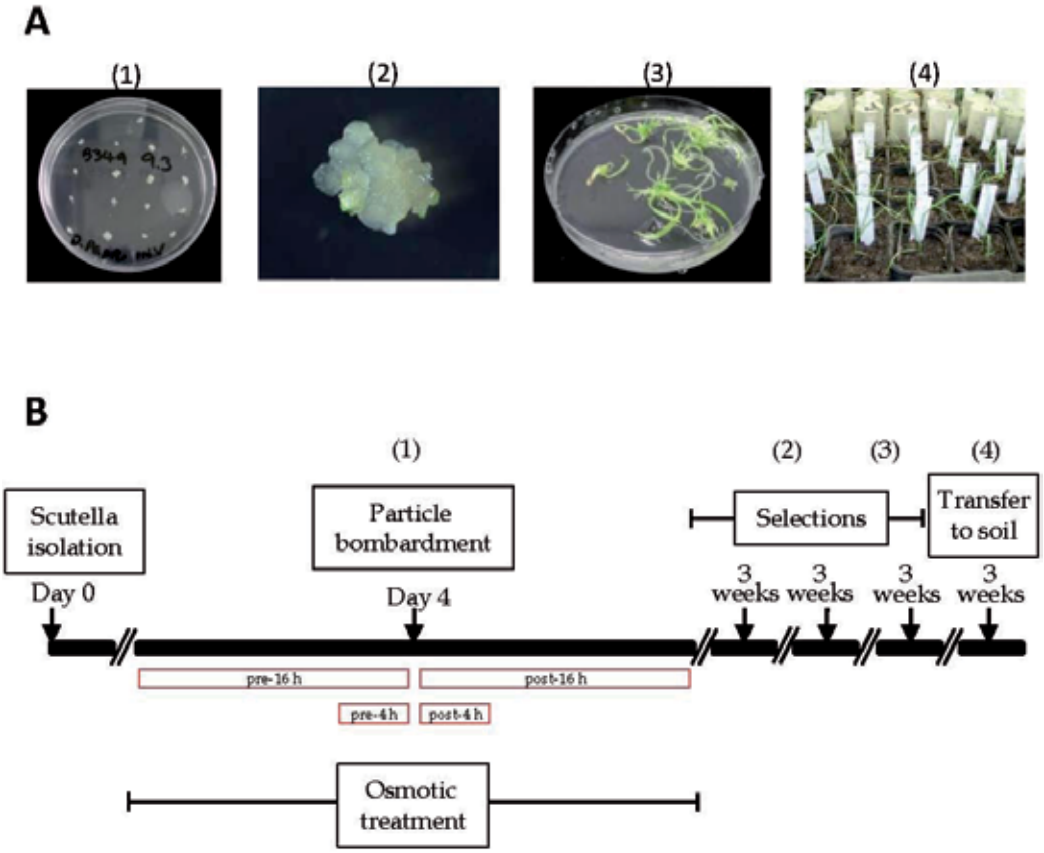

Fig. 2. Scheme of genetic transformation of wheat via particle bombardment. (A) Pictures of (1) the explants used for transformation, (2) callus formation after 3 weeks of cultivation, (3) explants after 8 weeks of cultivation and (4) plantlets after transfer to soil. (B) Time sequence for genetic transformation of wheat: scutella isolation, particle bombardment, selections and transfer to soil.

After the bombardment and the respective osmotic treatments, the explants were cultured in the MP4 induction medium for 3 weeks in the dark at 20-25ºC. Then, the percentage of embryogenesis (% of scutellum surface presenting embryogenic response) was calculated. Embryogenic calluses were transferred for shoot induction to the regeneration medium RZPPT2, consisting in RZ medium, supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), 5 mgl-1 zeatin and 2 mgl^{-1} L-phosphinothricin (L-PPT, the active ingredient of the herbicide BASTA) as described previously by Barro et al. (1998), except the untransformed explants (control-S) that were transferred to the same medium without the selective L-PPT. After 3 weeks of culture in the regeneration medium at 25 ºC in the light, the percentage of regeneration (% of explants presenting shoots) was measured, and shoots were transferred to RPPT2 (R medium supplemented with 2 mgl^{-1} L-PPT) (Barro et al. 1998), where they were cultured for another 3 weeks. Plantlets surviving were sub-cultured for another 3 weeks in RPPT2 medium, and then transferred to soil. Transgenic plants were determined by 1) the histochemical GUS assay and 2) by PCR amplification of a fragment of the *bar* gene. The

transformation efficiency of each treatment was determined as % of transgenic plants transferred to soil over the total number of scutella isolated for each treatment.

2.3 Seed composition and statistical analysis

Six lines over-expressing the HMW-GS (T580, T581, T590, T606, T616 and T617), one line with down-regulation of HMW-GS (T619) and 4 lines with down-regulation of all the groups of gliadins (D793, D894, E42 and E82) were obtained and grown as described above. Mature seeds from all the lines as well as the untransformed controls were collected and crushed into a fine powder. Three independent replicates were made for the determination of each of the following components: total protein, starch, water soluble carbohydrates, fructose, glucose, sucrose and maltose. Total protein was calculated from the Kjeldahl nitrogen content (%N x 5.7). Starch content was determined by polarimetry. Water soluble carbohydrates, as well as fructose, glucose, sucrose and maltose were quantified by HPLC with refractive index detection. Results obtained for all transgenic lines over-expressing one subunit of HMW, two subunits of HMW, and transgenic lines with down-regulation of all the gliadins, were grouped for statistical analysis and named 'HMW1', 'HMW2' and '–Gli', respectively. Line T619 with down-regulation of all the HMW-GS was named '–HMW' (Table 1).

Data were analysed with the statistical software R version 2.12.1 using the Graphical User Interface (GUI) R Commander, and the SPSS version 11.0 statistical software package (SPSS Inc., Somers, NY). Major assumptions of analysis of variance (ANOVA) were confirmed by the Kolmogorov-Smirnov's test for normal distribution and by the Levene's test for homogeneity of variances. ANOVA and two-tailed Dunnett's test for median multiple comparisons were used to analyse the results and compare between transgenic and wild type lines. P values lower than 0.05 were considered significant, and lower than 0.01 were considered highly significant.

3. Results and discussion

The effect of the different osmotic treatments (prior and after particle bombardment) on the embryogenesis as well as on the efficiencies of regeneration and transformation has been studied. The results obtained can help to improve the transformation protocols in cereals.

In addition, the application of the transformation techniques to produce over-expression and silencing of genes encoding HMW-GS and gliadins, as well as the effects on the protein, starch and carbohydrates contents are discussed below.

3.1 Transformation method improvement: osmotic treatment

The ability of the particle bombardment to consistently transform wheat has been previously reported (Lonsdale et al., 1998, Vasil et al., 1992, Witrzens et al., 1998) . However, cereal transformation is still difficult due to the number of parameters involved in the technique, and many research works have been focused on the bombardment conditions such as amount of DNA, amount and size of gold particles, acceleration pressure, bombardment distance or the osmotic condition of tissues (Altpeter et al., 1996, Becker et al., 1994, Li et al., 2003, Rasco Gaunt & Barcelo, 1998). An osmotic treatment of target tissues for stable transformation results in plasmolysis of cells and restricts damages by preventing extrusion of the protoplasm from bombarded cells (Vain et al., 1993). Osmotic treatment

both prior and after particle bombardment has been used in wheat transformation (Altpeter et al., 1996, Brinch-Pedersen et al., 2000, Jordan, 2000, Ortiz et al., 1996, Stoger et al., 1999). In this work we have studied the effect of the osmotic treatment with 0.4 M mannitol prior and after particle bombardment (4 h and 16 h) on the somatic embryogenesis, regeneration capacity and transformation efficiency (Table 1). The highest percentages of embryogenesis, 68.2% and 78.2%, were obtained with the 4 h pre-treatment (4h-pre) and 16 h post-treatment (16h-post), respectively (Table 1). No significant differences were found between these treatments and the not bombarded control (control-S), and the embryogenesis was much higher than the obtained with the bombarded control (control-B). These data indicate that particle bombardment has a negative effect on somatic embryogeneis but the osmotic treatment prevents the damages caused by the particle bombardment. The 16h pre-treatment showed the lowest level of embryogenesis, with only 38.0% of embryogenesis (Table 1).

Treatment	**Embryogenesis** (%)	**Regeneration** (%)	**Plants recovered**	**Transgenic plants**	**Transformation efficiency** (%)
Pre-4h	68,1 ab	78,4 ab	64	3	1,2
Post-4h	57,3 abc	49,2 b	38	17	6,8
Pre-16h	38,0 c	68,3 ab	7	2	0,8
Post-16h	78,2 a	64,1 ab	36	4	1,6
Control-B[a]	41,3 bc	63,2 ab	19	0	0
Control-S[b]	73,1 a	93,4 a	N/A	N/A	N/A

Table 1. Effect of the osmotic treatment with 0.4 M mannitol before and after particle bombardment. [a]Control-B: same conditions of bombardment and herbicide selection, but with no mannitol treatment; [b]Control-S: no bombardment, no mannitol treatment and no herbicide selection.Values within the same column followed by the same letter are not significantly different ($P<0.05$)

The range of regeneration of the explants subjected to the osmotic treatment was between 49.2% and 78.4% for treatments 4h-post and 4h-pre, respectively, while the control-B presented 63.2% of regeneration. The control-S (not bombarded) showed the highest level of regeneration, with 93.4%, a percentage comparable to the 76-86% reported by León et al. (2006) in not treated and not bombarded scutella. Only the 4h-pre treatment was significantly different for the regeneration, and no correlation was found between embryogenesis and regeneration of scutella (data not shown). Overall, the 4h-pre and 16h-post osmotic treatments showed high levels of embryogenesis and regeneration. Twenty-six of the 164 plants regenerated and transferred to soil were confirmed as transgenic by the histochemical assay GUS and by PCR of the *bar* gene. In spite of the high selection pressure, the number of escapes, or regenerated plants that not contained the pAHC25 plasmid, was high in all the treatments. The lowest percentage of escapes was 55.3% in the 4h-post treatment. The percentage of escapes described in previous works was also very high, ranging between 55-88% under selection with bialaphos (Altpeter et al., 1996) and around 80% under selection with L-PPT (Barro et al., 1998). The transformation efficiencies ranged between 0.8% and 6.8% in the 16h-pre and 4h-post, respectively. The 4h-post osmotic treatment also allowed recovering the highest number of positive transgenic plants with 17.

These results indicate that the post-bombardment osmotic treatment is more efficient than the pre-treatment, though long treatments, such as 16h-pre and 16h-post, drastically decreased the transformation efficiency. The transformation efficiency obtained with the 4h-post treatment (6.8%) is higher than the reported by other authors that ranged between 0.15-0.5% (Altpeter et al., 1996, Blechl & Anderson, 1996) and between 0.5-1.5% (Barro et al., 1997, Becker et al., 1994, Vasil et al., 1993). However, the transformation capacity is largely genotype-dependent and controlled by a wide number of factors as indicate the range of variation obtained in the different works.

However, high transformation efficiencies are needed for an increasingly number of applications to crops. In the case of wheat, baking quality and reducing the allergenicity of gluten for a broad sector of the population are priority goals. We have used genetic transformation protocols described in this work to over-express or silence a wide number of genes related to these two goals. The characterization of important traits of the transgenic plants produced is described below.

3.2 Grain components analysis of transgenic wheat lines with over- and down-regulated gluten proteins

Wheat flour consists mainly of starch, water and proteins. In addition, non-starch polysaccharides, in particular arabinoxylans and lipids are important minor flour constituents relevant for bread production and quality (Goesaert et al., 2005). Quantity, composition (quality), type and viscoelastic properties of wheat gluten proteins are important for bread-making (Finney & Barmore, 1948, Shewry & Halford, 2002). Wheat gluten can be divided into two protein families: the glutenins and the gliadins. The glutenins comprise high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) fractions, whereas the gliadins can be divided into three structural types: α/β-, γ- and ω-gliadins (Shewry & Halford, 2002). Although gliadins and glutenins are related with the bread-making quality, the HMW-GS are considered major determinants of quality in wheat flour (Payne, 1987, Shewry et al., 2003). In wheat grain, starch is deposited in partially crystalline granules that vary in morphology and structure between and within plant species. Starch owes much of its functionality in foods to the characteristics of the two constituent glucose polymers, amylose and amylopectin, and to the physical organization of these macromolecules into the granular structure (Annison & Topping, 1994). Changes in the above components provoked by the over-expression or silencing of glutenin and/or gliadin genes are important that they be determined and quantified, as they can largely influence the quality of the new transgenic lines of wheat.

3.2.1 HMW-GS over-expression

Table 2 shows the grain characteristics of the over-expression of HMW-GS genes on transgenic lines of bread wheat in comparison to its wild type (cv Anza). These transgenic lines contain one and two additional transgenic HMW-GS, and are named HMW1 and HMW2, respectively. The lines over-expressing one and two HMW-GS showed a significant increase of total protein content relative to its wild type. The total protein content showed no significant differences between HMW1 and HMW2 transgenic lines. Most works involving over-expression of HMW-GS in wheat seeds have not reported significant changes in total protein content between transgenic lines and their controls (León et al., 2010b, Rakszegi et al., 2005, Yue et al., 2008). Nevertheless, Rooke et al. (1999) showed that a

transgenic wheat line containing an additional gene encoding the HMW subunit 1Dx5, resulted in a slightly increase in the total grain nitrogen. Although some of the samples used in this study had much higher protein contents than those of the non-transformed, these levels were not reproducible in other greenhouse or field trials (León et al., 2010a), which could be because the total protein content is a highly environment-dependent trait.

	HMW over-expression			HMW down-regulation		Gliadins down-regulation	
	wt[a]	**HMW1[b]**	**HMW2[c]**	**wt**	**-HMW[d]**	**wt**	**-Gli[e]**
Total protein	10.4	**12.8** **	**12.4** *	12.1	**11.0** *	12.8	12.6
Starch	58.0	**45.6** **	57.7	55.8	54.7	58.7	57.6
WSC[f]	15.6	17.0	**19.5** **	18.8	20.5	18.1	20.2
Fructose	0.37	**0.73** **	**0.68** **	0.49	0.64	0.40	0.39
Glucose	0.53	**0.93** **	**0.90** **	0.81	0.85	0.51	0.55
Sucrose	0.90	**0.27** **	**0.22** **	0.86	0.86	1.50	2.12
Maltose	0.76	0.33	0.38	1.07	1.19	0.95	1.02

Table 2. Seed composition of untransformed wild types and mean of transgenic lines for HMW-GS over-expression, HMW-GS down-regulation and gliadins down-regulation. All parameters are expressed as percentage (%) of the total weight. [a]wt: wild type; [b]HMW1: over-expression of one subunit of HMW; [c]HMW2: overexpression of two HMW-GS; [d]-HMW: down-regulation of all the HMW-GS; [e]-Gli: down-regulation of all the gliadins; [f]WSC: water soluble carbohidrates

Only transgenic lines HMW1 showed a high reduction of starch percentage, significantly different to the wild type and to the HMW2 transgenic lines. The low starch content of transgenic lines HMW1 could be related to the high percentage of total protein. Likewise, starch:protein ratio was significantly lower in both HMW transgenic lines (Fig. 3). It resulted in an increase of total protein in the over-expressing HMW lines and in a negative correlation between the starch and protein contents. This negative correlation was reported by other researchers (Choct et al., 1995, Kim et al., 2003, Parsaie et al., 2006, Wiseman & Inborr, 1990).
Water soluble carbohydrates (WSC) were higher in HMW2 transgenic lines, but not in HMW1 transgenic lines in comparison with the control (Table 2). On the other hand, the simple carbohydrates contents showed a significant increase in both HMW over-expression transgenic lines, excepting the maltose content, which did not show differences between wild type and transgenic lines.
The gliadin and glutenin content were measured by RP-HPLC and the ratio gliadin:glutenin (Gli:Glu) is showed in Figure 4. Both lines HMW1 and HMW2, showed a significant decrease of Gli:Glu ratio, which was mainly due to the expression of additional HMW-GS. The decrease of the Gli:Glu ratio when an additional HMW-GS is added by genetic transformation has been previously reported by other authors (León et al., 2009, Rakszegi et al., 2005).

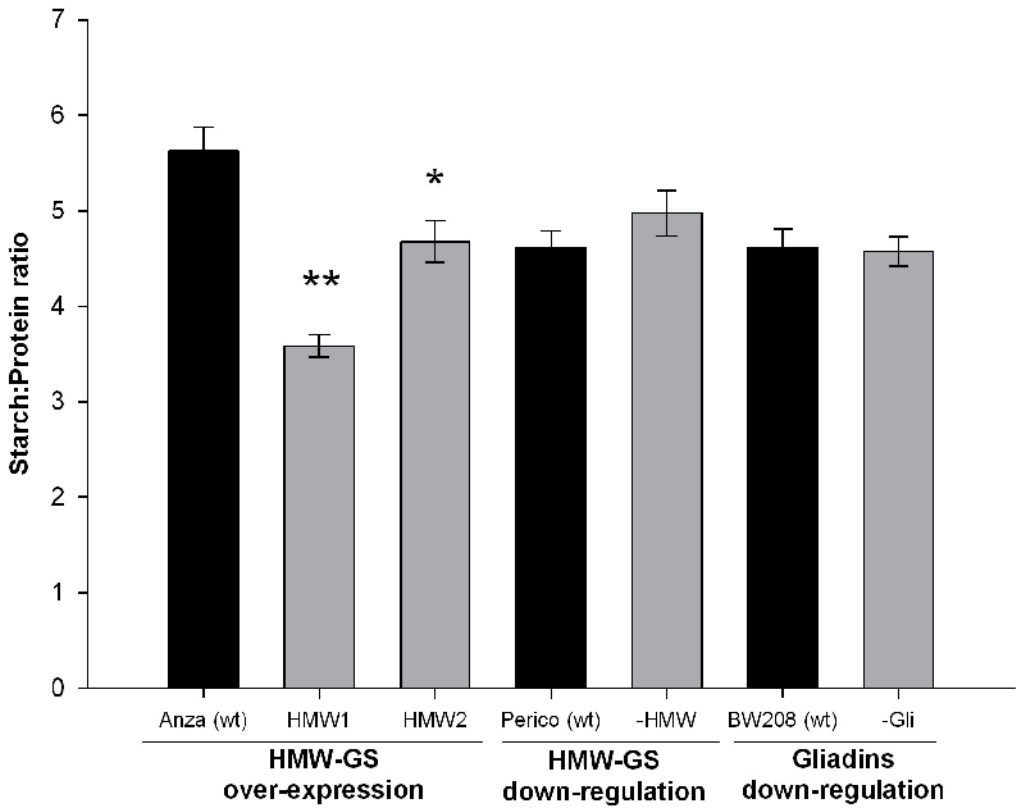

Fig. 3. Starch:protein ratio of untransformed wild types (black) and mean of transgenic lines (grey) for HMW-GS over-expression (HMW1 and HMW2), HMW-GS down-regulation (-HMW) and gliadins down-regulation (-Gli). Asterisks indicate significant differences between transgenics and the corresponding wild type line as determined by Dunnett's multiple comparisons at $P < 0.05$ (*) or $P < 0.01$ (**).

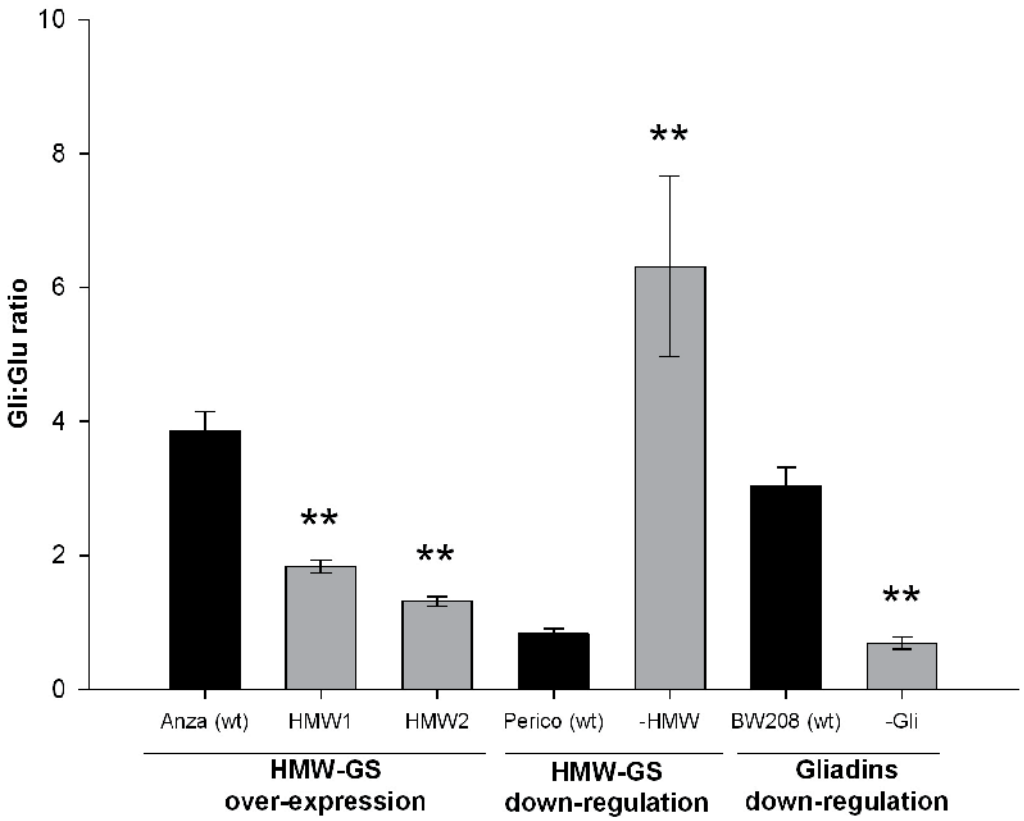

Fig. 4. Gliadins:glutenins ratio of untransformed wild types (black) and mean of transgenic lines (grey) for HMW-GS over-expression, HMW-GS down-regulation and gliadins down-regulation. Asterisks indicate significant differences between transgenics and the corresponding wild type line as determined by Dunnett's multiple comparisons at $P < 0.05$ (*) or $P < 0.01$ (**).

3.2.2 HMW-GS down-regulation

A bread wheat cultivar Perico was used to down-regulate all the HMW-GS by the transformation with a D Hordein gene from *H. chilense*. Thus, the transgenic line T619 showed a very low content of HMW-GS from the three genomes (Fig. 1 C). Grains composition analysis did not shown differences in total starch content, WSC and the simple carbohydrates between T619 and the wild type Perico. However, the total protein content was significantly lower in T619 in comparison with the wild type Perico (Table 2). This significant decrease in the protein content did not result in changes in the starch: protein ratio (Figure 3). There are no consistent data about the response of wheat grain components, and in particular the total protein content, when one or more HMW-GS genes are silenced (Alvarez et al., 2000, Yue et al., 2008). Consistently with the over-expression of HMW-GS genes, the down-regulation of HMW-GS results in a significant increase of the ratio Gli:Glu (Figure 4).

3.2.3 Gliadin down-regulation

Transgenic lines with reduction of all the gliadins had not differences in total protein, total starch, WSC and simple carbohydrates in comparison with non-transformed lines (Table 2). In addition, the ratio starch:protein did not present differences between transgenic and wild type lines (Figure 3). Gil-Humanes et al. (2010) reported no differences in total protein content between lines with reduction of gliadins and their wild types. This suggests a compensatory process that operates in the grain in response to gliadin silencing, to maintain a stable total protein content. However, the ratio Gli:Glu showed a significant decrease in the transgenic lines, overwhelmingly driven by the silencing of gliadins (Figure 4). Gil-Humanes et al. (2011) also reported increases in the amounts of glutenins, albumins and globulins in the transgenic lines with down-regulation of all the gliadins. Previously, compensatory effects were observed by Lange et al. (2007) who reported increases in the amounts of B hordeins and glutelins in transgenic lines of barley with reduced contents of C hordeins. Hansen et al. (2007) performed microarray analyses of the same lines and showed up-regulation of B and γ-hordein genes and of the gene encoding the barley prolamin-binding factor (BPBF), a transcription factor that regulates B hordein gene expression.

4. Conclusion

The genotype and quality of the explants used for transformation, and the reduction in the stress during particle bombardment with the application of the osmotic treatment, promotes an improvement in the transformation efficiency of wheat. The 4 h post-treatment produced the highest transformation efficiency and the lowest ratio of escapes, so it is highly recommended to subject the explants to this treatment in order to obtain high number of transgenic plants.

Over-expression and down-regulation of HMW-GS genes provide different changes in total protein content. It is increases with the over-expression of HMW-GS genes and decreases when they are silenced. The over-expression of HMW-GS genes causes the most severe changes in the composition of wheat grain, changing the total protein and starch contents, and the relationship between them. By contrast, the down-regulation of total gliadins does not cause major changes in the total content of protein and starch. Finally, the over-expression and down-regulation of HMW-GS and gliadin genes produce changes in the Gli:Glu ratio, which is closely related to wheat flour bread-making quality.

5. Acknowledgments

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6. References

Altpeter F., Vasil V., Srivastava V., Stöger E. & Vasil I.K. (1996). Accelerated producction of transgenic wheat (*Triticum aestivum* L.) plants. *Plant Cell Reports*, Vol 16, No. 12-17.

Alvarez M.L., Guelman S., Halford N.G., Lustig S., Reggiardo M.I., Ryabushkina N., Shewry P., Stein J. & Vallejos R.H. (2000). Silencing of HMW glutenins in transgenic wheat expressing extra HMW subunits. *Theoretical and Applied Genetics*, Vol 100, No. 2, (Jan), pp. 319-327.

Annison G. & Topping D.L. (1994). Nutrition role of resistant starch: Chemical structure vs. physiological function. *Annual Review of Nutrition*, Vol 14, No. 297-320.

Barro F., Rooke L., Bekes F., Gras P., Tatham A.S., Fido R., Lazzeri P.A., Shewry P.R. & Barcelo P. (1997). Transformation of wheat with high molecular weight subunit genes results in improved functional properties. *Nature Biotechnology*, Vol 15, No. 12, (Nov), pp. 1295-1299.

Barro F., Cannell M.E., Lazzeri P.A. & Barcelo P. (1998). The influence of auxins on transformation of wheat and tritordeum and analysis of transgene integration patterns in transformants. *Theoretical and Applied Genetics*, Vol 97, No. 5-6, 684-695.

Baulcombe D. (2004). RNA silencing in plants. *Nature*, Vol 431, No. 7006, 356-363.

Becker D., Brettschneider R. & Lorz H. (1994). Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. *Plant Journal*, Vol 5, No. 2, 299-307.

Blechl A.E. & Anderson O.D. (1996). Expression of a novel high molecular weight glutenin subunit gene in transgenic wheat. *Nature Biotechnology*, Vol 14, No. 7, 875-879.

Brinch-Pedersen H., Olesen A., Rasmussen S.K. & Holm P.B. (2000). Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an *Aspergillus* phytase. *Molecular Breeding*, Vol 6, No. 2, 195-206.

Choct M., Hughes R.J., Trimble R.P., Angkanaporn K. & Annison G. (1995). Non-starch polysaccharide-degrading enzymes increase the performance of broiler chickens fed wheat of low apparent metabolizable energy. *Journal of Nutrition*, Vol 125, No. 3, 485-492.

Christensen A.H. & Quail P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research*, Vol 5, No. 3, (MAY), pp. 213-218.

Finney K.F. & Barmore M.A. (1948). Loaf volume and protein content of hard winter and spring wheats. *Cereal Chemistry*, Vol 25, No. 5, 291-312.

Gil-Humanes J., Pistón F., Tollefsen S., Sollid L.M. & Barro F. (2010). Effective shutdown in the expression of celiac disease-related wheat gliadin T-cell epitopes by RNA

interference. *Proceedings of the National Academy of Sciences of the United States of America*, Vol 107, No. 39, 17023-17028.

Gil-Humanes J., Pistón F., Shewry P.R., Tosi P. & Barro F. (2011). Suppression of gliadins results in altered protein body morphology in wheat. *Journal of Experimental Botany*, *In press* .

Goesaert H., Brijs K., Veraverbeke W.S., Courtin C.M., Gebruers K. & Delcour J.A. (2005). Wheat flour constituents: how they impact bread quality, and how to impact their functionality. *Trends in Food Science & Technology*, Vol 16, No. 1-3, (Jan-Mar), pp. 12-30.

Hansen M., Lange M., Friis C., Dionisio G., Holm P.B. & Vincze E. (2007). Antisense-mediated suppression of C-hordein biosynthesis in the barley grain results in correlated changes in the transcriptome, protein profile, and amino acid composition. *Journal of Experimental Botany,* Vol 58, No. 14, pp. 3987-3995

Jordan M.C. (2000). Green fluorescent protein as a visual marker for wheat transformation. *Plant Cell Reports*, Vol 19, No. 11, 1069-1075.

Kim J.C., Mullan B.P., Simmins P.H. & Pluske J.R. (2003). Variation in the chemical composition of wheats grown in Western Australia as influenced by variety, growing region, season, and post-harvest storage. *Australian Journal of Agricultural Research*, Vol 54, No. 6, 541-550.

Lange M., Vincze E., Wieser H., Schjoerring J.K., Holm P.B. (2007). Suppression of C-hordein synthesis in barley by antisense constructs results in a more balanced amino acid composition. *Journal of Agricultural and Food Chemistry*, Vol 55, No. 15, pp. 6074-6081.

Lazzeri P.A. & Jones H.D. (2009). Transgenic wheat, barley and oats: Production and characterization. In: *Methods in Molecular Biology*, pp. 3-20.

León E., Marín S. & Barro F. (2006). Improvement of *in vitro* culture response of elite wheat cultivars by selecting the source spike, the scutellum size and the temperature for the induction of embryogenesis. *Plant Breeding*, Vol 125, No. 6, (Dec), pp. 580-583.

León E., Marin S., Gimenez M.J., Piston F., Rodriguez-Quijano M., Shewry P. & Barro F. (2009). Mixing properties and dough functionality of transgenic lines of a commercial wheat cultivar expressing the 1Ax1, 1Dx5 and 1Dy10 HMW glutenin subunit genes. *Journal of Cereal Science*, Vol 49, No. 1, 148-156.

León E., Aouni R., Pistón F., Rodriguez-Quijano M., Shewry P. & Martín A. (2010a). Stacking HMW-GS transgenes in bread wheat: Combining subunit 1Dy10 gives improved mixing properties and dough functionality. *Journal of Cereal Science*, Vol 51, No. 1, 13-20.

León E., Pistón F., Aouni R., Shewry P.R., Rosell C.M., Martín A. & Barro F. (2010b). Pasting properties of transgenic lines of a commercial bread wheat expressing combinations of HMW glutenin subunit genes. *Journal of Cereal Science*, Vol In press, No.

Li B.C., Caswell K., Leung N. & Chibbar R.N. (2003). Wheat (*Triticum aestivum* L.) somatic embryogenesis from isolated scutellum: Days post anthesis, days of spike storage, and sucrose concentration affect efficiency. *In Vitro Cellular & Developmental Biology-Plant*, Vol 39, No. 1, (Jan-Feb), pp. 20-23.

Lonsdale D.M., Lindup S., Moisan L.J. & Harvey A.J. (1998). Using firefly luciferase to identify the transition from transient to stable expression in bombarded wheat scutellar tissue. *Physiologia Plantarum*, Vol 102, No. 3, (Mar), pp. 447-453.

Meyer P. & Saedler H. (1996). Homology-dependent gene silencing in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, Vol 47, No. 23-48.

Miki D., Itoh R. & Shimamoto K. (2005). RNA silencing of single and multiple members in a gene family of rice. *Plant Physiology*, Vol 138, No. 4, 1903-1913.

Murashige T. & Skoog F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, Vol 15, No. 3, 473-497.

Ortiz J.P.A., Reggiardo M.I., Ravizzini R.A., Altabe S.G., Cervigni G.D.L., Spitteler M.A., Morata M.M., Elias F.E. & Vallejos R.H. (1996). Hygromycin resistance as an efficient selectable marker for wheat stable transformation. *Plant Cell Reports*, Vol 15, No. 12, 877-881.

Parsaie S., Shariatmadari F., Zamiri M.J. & Khajeh K. (2006). Evaluation of starch, soluble and insoluble non-starch polysaccharides and metabolizable energy of 15 cultivars of Iranian wheat. *Journal of agriculture & social sciences*, Vol 2, No. 4, 260-263.

Payne P.I. (1987). Genetics of wheat storage proteins and the effect of allelic variation on bread-making quality. *Annual Review of Plant Physiology and Plant Molecular Biology*, Vol 38, No. 141-153.

Pistón F., Shewry P. & Barro F. (2007). D hordeins of *Hordeum chilense*: a novel source of variation for improvement of wheat. *TAG Theoretical and Applied Genetics*, Vol 115, No. 1, 77-86.

Rakszegi M., Bekes F., Lang L., Tamas L., Shewry P.R. & Bedo Z. (2005). Technological quality of transgenic wheat expressing an increased amount of a HMW glutenin subunit. *Journal of Cereal Science*, Vol 42, No. 1, (Jul), pp. 15-23.

Rasco Gaunt S. & Barcelo P. (1998) Immature inflorescence culture of cereals: A highly responsive system for regeneration and transformation. In: *Methods in Molecular Biology* (ed R.D. Hall), pp. 71-81. Humana Press Inc., Totowa, NJ.

Rooke L., Barro F., Tatham A.S., Fido R., Steele S., Bekes F., Gras P., Martin A., Lazzeri P.A., Shewry P.R. & Barcelo P. (1999). Altered functional properties of Tritordeum by transformation with HMW glutenin subunit genes. *Theoretical and Applied Genetics*, Vol 99, No. 5, 851-858.

Shewry P. & Halford N. (2002). Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany*, Vol 53, No. 370, 947-958.

Shewry P.R., Halford N.G., Tatham A.S., Popineau Y., Lafiandra D. & Belton P.S. (2003) The high molecular weight subunits of wheat glutenin and their role in determining wheat processing properties. In: *Advances in Food and Nutrition Research*, pp. 219-302.

Shewry P.R. & Jones H.D. (2005). Transgenic wheat: where do we stand after the first 12 years? *Annals of Applied Biology*, Vol 147, No. 1, 1-14.

Stoger E., Williams S., Christou P., Down R.E. & Gatehouse J.A. (1999). Expression of the insecticidal lectin from snowdrop (*Galanthus nivalis* agglutinin; GNA) in transgenic wheat plants: effects on predation by the grain aphid *Sitobion avenae*. *Molecular Breeding*, Vol 5, No. 1, 65-73.

Vain P., McMullen M.D. & Finer J.J. (1993). Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Reports*, Vol 12, No. 2, 84-88.

Vasil V., Castillo A.M., From M.E. & Vasil I.K. (1992). Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. *BioTechnology*, Vol 10, No. 6, 667-674.

Vasil V., Srivastava V., Castillo A.M., Fromm M.E. & Vasil I.K. (1993). Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. *BioTechnology*, Vol 11, No. 13, 1553-1558.

Wiseman J. & Inborr J. (1990) The nutritive value of wheat and its effect on broiler performance. In: *Recent Advances in Animal Nutrition - 1990* (eds W. Haresign & D.J.A. Cole), pp. 79-102.

Witrzens B., Brettell R.I.S., Murray F.R., McElroy D., Li Z.Y. & Dennis E.S. (1998). Comparison of three selectable marker genes for transformation of wheat by microprojectile bombardment. *Australian Journal of Plant Physiology*, Vol 25, No. 1, 39-44.

Yue S.J., Li H., Li Y.W., Zhu Y.F., Guo J.K., Liu Y.J., Chen Y. & Jia X. (2008). Generation of transgenic wheat lines with altered expression levels of 1Dx5 high-molecular weight glutenin subunit by RNA interference. *Journal of Cereal Science*, Vol 47, No. 2, (Mar), pp. 153-161.

8

Maize Transformation to Obtain Plants Tolerant to Viruses by RNAi Technology

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1. Introduction

Plants represent the principal source of human foods and livestock feeds and efforts to improve them in many agronomic aspects have focused on plant breeding. The biotechnology revolution in the past decade made possible for plant breeders access new sources of genetic variability for the development of superior cultivars. It has been possible to define additional strategies for crop improvement through the introduction and stable integration of heterologous genes in plant cells with the knowledge of the regulation of the important agronomic characteristics. The genetic manipulation of plants allows their adaptation to different environmental stresses, whether biotic or abiotic. Currently, the production of genetically modified plants occupies a prominent place in both, basic and applied plant research. Genetically modified crops are generated through a process known as genetic engineering, in which genes of interest are transferred to plants without the need of natural crossing. The most widely used methods for introducing transgenes into the genome of plants are *Agrobacterium* mediated transformation and microprojectile bombardment. In the first case, scientists took advantage of the natural ability of *Agrobacterium* to transfer some of its wild genes to plant cells causing the diseases known as crown gall or hairy roots, and replace them by other genes expressing traits of agronomic interest. However, *Agrobacterium* is not able to infect all plants in a very efficient way, as a consequence, new systems for direct transfer of genes to plants emerged. The microprojectile bombardment system is a direct transfer of genes that involves an equipment known as gene gun. The DNA to be introduced into plant cells is physically attached to metal microparticles that are then propelled against the plant cells, using the gene gun. DNA that penetrates the plant cell can be integrated into the plant genome.

Maize is one of the most cultivated cereals in the world. The main maize producer's countries are the United States, China, and Brazil, followed by Mexico, France, Argentina and India. Among the big losses faced by agriculture are the attacks of pests and diseases. For maize, these problems have worsened since 1990 because of the increase of the cultivated areas in both the normal growing season and the off season, mainly due to intensive cultivation of maize in the irrigated areas, and lack of adoption of crop rotation in certain fields. In recent years, diseases that were not a problem, increased in importance such as the viruses. Among the strains of the virus complexes, potyviruses cause significant losses in grain and forage of maize susceptible genotypes. Plants have different mechanisms for protection against invasion by pathogens, and different genes directly related to

tolerance to viruses have been described in maize. Works have been published using methods of obtaining plants resistant to viruses by antisense, co-suppression and, more recently, RNA interference (RNAi).
This chapter reviews methodologies that have been used to introduce the RNAi construct in maize cells, such as *Agrobacterium* and microprojectile bombardment aiming to produce transgenic maize plants tolerant to SCMV. Topics covered in this chapter include maize regeneration in tissue culture, transformation mediated by *Agrobacterium* and microprojectile bombardment, isolation and cloning of the target DNA into RNAi based vectors, some results already obtained with this technology and its application to crop improvement.

2. RNA interference

RNA interference is a natural phenomenon of which double stranded RNA (dsRNA) activates a mechanism that degrades complementary RNA in the cell. This process has been described in many organisms such protozoa, flies, nematodes, insects, mouse and human cells (Napoli et al. 1990; Hammond et al., 2001; Agrawal et al., 2003; Baulcombe, 2004; Tang and Galili, 2004) and has been referred as cellular defense against viruses and post-transcriptional regulation of gene expression. In maize, there are extensive reviews done by McGinnis (2009) describing the application of this process as a reverse genetic tool.
Before the identification of the RNAi phenomenon, there were other methods such as T-DNA insertion, transposon elements and physical and chemical mutagens and antisense suppression to generate gene loss-of-function. These approaches, which have been used until today have allowed scientist study the function of many gene or gene families. The earliest version of gene silence was the process called antisense, which involves the introduction of the antisense strand of RNA to silence an internal RNA homologue (Knee and Murphy, 1997). The antisense strand once inside the cell binds to the target RNA by complementation preventing it to be translated. One possible explanation, on that time, was the inability of the ribosomes bind to the dsRNA. Another possible explanation that came up later, was that the dsRNA might also be a substrate for the DICER/RISC an enzymatic complex responsible for degradation of dsRNA in the RNAi process. The first description of the RNAi phenomenon was done by Fire et al. (1998). This group introduced sense and antisense RNA strands in *Caenorhabditis elegans* and obtained a gene silence ten times greater than with the sense or the antisense strand only. Injecting the sense and antisense strands together, in fact, created the double stranded RNA required for the RNA interference process. Later the same phenomenon was described in trypanosomes (Ngo et al., 1998) and flies (Kennerdell and Carthew, 1998). Due to the great interest in the RNAi technology and its applications, many other works were published trying to elucidate the mechanisms involved. Once the dsRNAs are formed in the cell, they are automatically recognized by an enzyme complex called DICER that cleaves them into small fragments known as small interference or siRNA. The DICER was discovered by Bernstein et al. (2001) in *Drosophila* and, it is an enzyme complex belonging to the RNase III family, which has four domains: a) an N-terminal helicase; b) an double RNase III domain; c) a binding domain to the C terminal dsRNA; d) a PAZ domain (Piwi / Argonaute / Zwill) (Agrawal et al., 2003). The siRNAs are composed of 21-25 base pairs (dsRNA) with a 3' end of an additional base. Wei et al. (2003) found that the hydroxyl group 3' was necessary to direct RNAi process *in vitro*. The PAZ domain of DICER seems

that physically interacts with the PAZ domain of the RISC complex. The RISC complex, also discovered in *Drosophila* by Hammond et al. (2001), is a system component that uses RNAi to trace siRNA and degrade complementary mRNAs. The dsRNA present in the tRNA are highly stable molecule that can not be degradated by DICER / RISC complex. A simplified form of the degradation of dsRNA by DICER / RISC complex is shown in Figure 1.

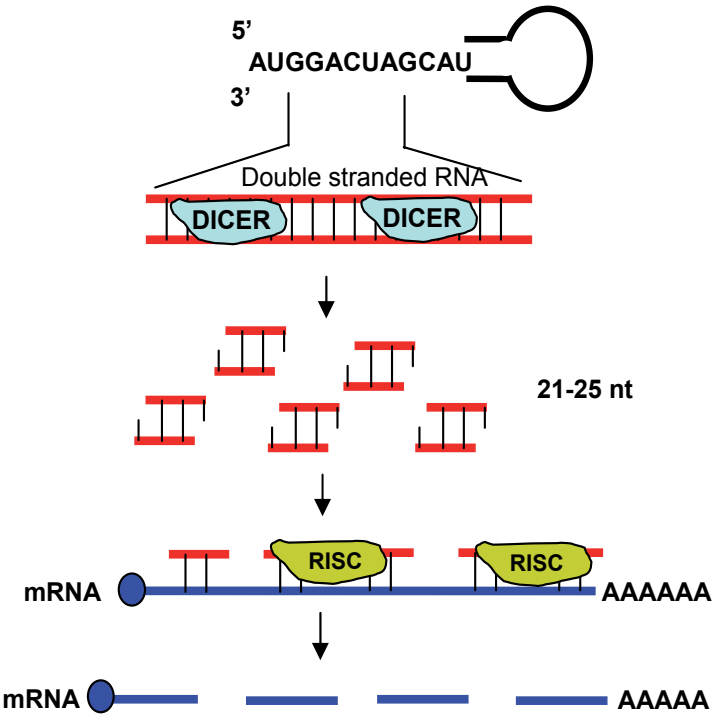

Fig. 1. A simplified diagram of the degradation of dsRNA by DICER / RISC enzyme complex. In the proposed mechanism the DICER RNAse III complex identifies the double stranded RNAs and cleaves them into 21 to 25 bp small pieces siRNA. These molecules are then recognized by the RISC complex, that unwound the siRNA, leaving the antisense strand in the RISC, resulting in the complex activation. The activated RISC then targets and cleaves mRNA that is complementary to the antisense strand of the siRNA (Zamore et al., 2000; Kim, 2003; Wall and Shi, 2003).

3. Application of RNAi to obtain transgenic maize lines tolerant to the SCMV

Fuchs and Grüntzig (1996) observed that Sugarcane Mosaic Virus (SCMV) and Maize Dwarf Mosaic Virus (MDMV) were the most important potyviruses, causing significant losses in grain and forage of susceptible maize genotypes. In Germany, the maize fields with mosaic symptoms were first found in the early 80's (Fuchs and Kozelska, 1984). Since then, MDMV and SCMV have been regularly observed in maize producing regions of Germany, where epidemiological studies have shown the prevalence of SCMV(Fuchs et al., 1996). For the tropical conditions observed in Brazil, it were described three viruses in maize: (i) mosaic

which can be caused by four distinct potyviruses transmitted mechanically and, by *Rhopalosiphum maidis*; (ii) MRFV (Maize Rayado Fino Virus) , transmitted in a persistent manner by the leafhopper *Dalbulus maidis* and, (iii) MMV (Maize Mosaic Virus), transmitted in a persistent manner by the leafhopper *Peregrinus maidis* (Waquil et al., 1996). The mosaic also attacks sorghum and sugarcane, crops of great economic importance. Besides, numerous species of wild Poaceae constitute reserves of virus inoculum for the cultivated species. Due to non persistent transmission of the potyviruses, control of aphid vectors by chemical is not effective. Therefore, due to ecological and economic reasons, the cultivation of resistant maize varieties is one of the most effective methods of controlling these diseases (Melching, 1998).

The particles of the potyvirus causing the mosaic disease are flexible and have a length of approximately 750 nm and width varying from 13 nm (MDMV and SCMV) to 12 nm for the Johnsongrass mosaic virus (JGMV) (Shukla et al., 1994). Like most plant viruses, the potyviruses have a genome consisting of sense strand RNA-positive, with a length of approximately 10,000 nucleotides and a protein (Vpg) connected to the terminal 5 'genome (Figure 2).

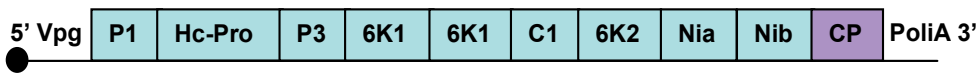

Fig. 2. Schematic representation of genomic organization of potyviruses indicating the proteins encoded by the virus and its possible functions. (P1): first protease; (HC-Pro): helper component - protease; (P3): third protease; (C1): protein with RNA helicase activity; (6K1 and 6K2): peptides; (Nia): nuclear inclusion protease; (Nib): a RNA dependent RNA polymerase; (CP): coat protein. By analogy with other viral systems, it is suggested that Vpg serve as a primer for synthesis of vRNA (Shukla et al., 1994) and stabilization of mRNA against attack by exonucleases.

Plants have different mechanisms for protection against invasion by pathogens such as physical barriers, secondary metabolites and antimicrobial proteins. Once established, elicited molecules produced and released by the pathogen induce new defenses such as cell wall strengthening, phytoalexin production, synthesis of proteins related to plant defense, among others. The identification and application of these mechanisms is one of the most effective manners to rapidly improve crop resistance to diseases. Microarray experiments have shown hundreds of genes regulated by plant-pathogen interactions, most of these are defense-related proteins (PRs) or system acquires resistance (SARs) (van Loon et al., 2006).

An alternative strategy for obtaining materials resistant to pathogens, specifically virus, was published by Grumete et al. (1987), when they over expressed part of the genome of the pathogen in a plant and showed a significant increase in resistance. The explanation given at the time was that the disfunction of the gene products derived from the pathogen could inhibit the pathogen. Similar work has also demonstrated the expression of the coat protein of TMV (Tobacco Mosaic Virus) in the generation of resistant tobacco plants (Abel et al., 1986). These plants in the presence of the virus showed no symptoms or showed a delayed onset of symptoms. Additional experiments showed immediately that the level of transgene expression was correlated with the level of expression of resistance (Fitchen and Beachy, 1993, Powell et al., 1990).

Many different types of viruses in plants have been shown to encode silencing suppressors. Suppressors of silencing of these viruses interfere with different steps of processing the RNA silencing present in plants and are important defense responses (Ratcliff et al., 1999). This process was one of the most evident in plants to identify viruses that have proteins that interfere with the system of the plant RNA silencing. In 1998, Anandalakshmi and collaborators and Brigneti and collaborators shown that HC-Pro protein of TEV and 2b of CMV could have this role. A classic paper demonstrated that the inhibition system of the 5' end corresponding to proteins P1 and Hc-Pro was efficient to obtain plants resistant to Plum pox virus (PPV) in tobacco (Di Nicola-Negri et al., 2005). In this same study were tested four regions of the virus genome: (i) nucleotide (nt) 1-733 of the protein corresponding to P1; (ii) nt 954-1603 corresponding to the end of the protein P1 and protein portion of Hc-Pro; (iii) nt 1680-2386 corresponding to the central part of Hc-Pro/P3 and, (iv) nt 1935 to 2613 corresponding to the end of Hc-Pro protein and part of P3. To access the efficiency of each construct in relation to the resistance of transgenic plants to PPV a large number of transgenics was analyzed by ELISA and, it was shown that 90% of transgenic plants were resistant to PPV. Despite all indicates that the target for this group of RNAi gene constructs are based on the 5 'end (mainly the Hc-Pro) there are works based on positive replication region (Guo and Garcia, 1997; Wittner et al., 1998) or in the 3 'end of the coat protein (Ravelonandro et al. 1992; Palkovics et al. 1995; Jacquet et al., 1998).

4. Maize transformation

The insertion of sRNAi in the plant can be accomplished by different ways such as eletroporation, *Agrobacterium*-mediated transfer, microparticle bombardment or viruses. Most of these methods use an RNA vector that produces stable dsRNA.

Significant progress has been achieved in developing technology for genetic transformation of maize in the last decade. Genetic transformation of maize became nowadays a routine procedure for various genotypes in most public and private laboratories working with this culture. For introducing a siRNA construct in maize is necessary (i) an *in vitro* regeneration protocol for transgenic maize cells and; (ii) methodologies to insert siRNA construct in the genome.

4.1 *In vitro* regeneration of transgenic maize cells

The establishment of maize regeneration systems from somatic cells constitutes a prerequisite of utmost importance within the process of transgenic maize plants production. Regeneration of maize plants in tissue culture can occur via organogenesis (Zhong et al. 1992) or somatic embryogenesis, and the last one is the most used method.

Grasses were considered recalcitrant species with regard to establishing of totipotent cultures *in vitro* (King et al. 1978). The intensification of research in this area enabled rapid progress, especially after 1980 with the discovery of somatic embryogenesis in several grass species (Prioli & Silva, 1989). In maize, plant regeneration from Type I callus cultures was first described in 1975 by Green & Phillips, using immature embryos as explants. For the induction of callus, imature embryos were collected 10-15 days after pollination, with approximately 1.0 to 2.0 mm long and grown with the embryonic axis in contact with the culture medium. This orientation induces better proliferation of the scutellum cells while reduce germination (Green & Phillips, 1975).

Armstrong & Green (1985) introduced the terms of Type I and II callus which are currently used for the classification of embryogenic cultures of maize. Type I callus is composed of hard, compact, yellow or white tissue and usually capable of regenerating plants (Vasil & Vasil, 1981). Type II is soft, friable and highly embryogenic (Armstrong & Green, 1985). Type II callus culture is fast-growing and can be kept for a long period of time without losing their totipotency (Vasil, 1987).
Although Type II calli are the most efficient in the production of transgenic maize, Type I calli can also be used. The occurrence of friable embryogenic Type II callus is not so common, only a limited number of maize genotypes are able to express this phenotype in tissue culture, notably the line A188 (Armstrong & Green, 1985) and the hybrid HiII (Armstrong et al. 1991). With the advancement of the *in vitro* culture methodologies, and particularly with changes in the composition of culture media including type and levels of plant growth regulators, it became possible to regenerate a growing number of genotypes (Rapela, 1985, Duncan et al., 1985, Prioli & Silva, 1989). However, most of these genotypes only form compact Type I callus.
It is known that, in maize, the initiation of regenerable callus as well as the frequency of regeneration of plants are affected by a genetic component and depend on the genotype used (Hodges et al. 1986; Prioli & Silva, 1989). Through a diallele involving eight cultivars of maize, Beckert & Qing (1984) found significant heritability for initiation of somatic embryogenesis and plant regeneration. The high heritability indicates that both the initiation of callus and plant regeneration can be improved by crossing genotypes recalcitrant to highly responsive genotypes (Hodges et al., 1986). The formation of somatic embryos and regenerative ability are under control of genes located in the genome of maize cells (Hodges et al. 1986; Vinh, 1989). However, the physiological and developmental stage at the time of explant excision, the time of the year and, the specific interactions between genotypes and growing conditions of the donor plant, may modify the expression of genes that control the induction of somatic embryogenesis and plant regeneration (Prioli & Silva, 1989).

4.2 Methods of genetic transformation of maize

The different methods of genetic transformation of maize can be divided into two major groups: direct and indirect methods. Indirect method of genetic transformation uses a bacterium, *Agrobacterium tumefaciens*, to introduce the gene of interest in the maize genome. In the transformation using direct methods, the gene of interest is introduced into the genome without the intervention of a bacterium. The most used method of direct genetic transformation of maize is the bombardment of cells with microparticles of metal physically covered with the DNA of interest.

4.2.1 Transformation of maize cells using microparticle bombardment

Since most of the monocots are not natural hosts for *Agrobacterium*, initially, the transformation of maize was performed using direct systems.
The particle bombardment of plant cells with DNA of interest is a direct method of transformation designed to introduce nucleic acids into the genome or plastome of cells (Taylor and Fauquet, 2002). It is a methodology commonly used by laboratories working with plant genetic transformation. It was developed in the late 80's to manipulate the genome of plants recalcitrant to *Agrobacterium*-mediated transformation, among which are included cereals (Klein et al. 1988; Taylor and Fauquet, 2002). In the transformation via

particle bombardment or biolistic, microparticles of metal physically coated with the gene of interest are accelerated toward the target cells, using equipment known as "gene gun" (Sanford et al. 1987), with sufficient acceleration to penetrate the cell wall and not cause cell death. Precipitated DNA on the microparticles is released gradually into the cell after the bombardment, and integrated into the genome (Taylor and Fauquet, 2002). The acceleration of microparticles is obtained by a high voltage electrical discharge, or a helium pulse. The particles used are non-toxic, non-reactive, and lower than the diameter of the target cell. Typically, the microparticles used are gold or tungsten and they are propelled toward the target cells by modern devices such as PDS 1000 (BioRad Laboratories, Hercules, CA, USA) or the Accell gene gun (Agracetus, Inc., Middleton, WI, USA).
Several physical parameters correlated with the biolistic equipment such as pressure, macrocarrier and microcarrier flight distance, and vacuum, must be optimized for successful transformation. Besides these parameters, the plant material and the gene of interest which will be used should also be tested in preliminary experiments (Sandford et al., 1993).
From the 90's the microparticle bombardment was used to transform a wide variety of plants, including maize. Gordon-Kamm et al. (1990) and Fromm et al. (1990) were the first groups to report the production of transgenic maize from the bombardment of embryogenic callus. Then, several reports of transformation of maize showed that the particle bombardment is a successful technique for inserting foreign genes into the genome of maize with high reproducibility of results (Brettschneider et al. 1997; Frame et al. 2000).
The main advantages of microparticle bombardment is related to the use of simple vectors and easy handling, plus the possibility of inserting more than one gene of interest into cells efficiently (Wu et al. 2002). Although considered a very efficient method of transforming maize, a possible disadvantage is the occurrence of multiple copies of the gene of interest and complex integration patterns, susceptible to silencing, of gene expression in future generations (Wang and Frame, 2004).

4.2.2 *Agrobacterium tumefaciens* mediated maize transformation

For several years the transformation of monocots by *Agrobacterium* had a very low efficiency, however, recently this is changing, and this method of gene transfer has become the method of choice for this group of plants. This transformation method uses a natural system of gene transfer developed by *Agrobacterium*. *Agrobacterium* is a soil bacterium capable of causing tumors in the region of plant infection. These tumors result from the presence of the Ti plasmid or plasmid tumor inductor in the bacterial cell. The Ti plasmid is a large circular molecule (200 to 800 kb), double stranded DNA that can replicate independently of the genome of *Agrobacterium tumefaciens* (Gelvin, 2003). Located in the Ti plasmid are two important regions for gene transfer from bacteria to the plant, the T-DNA region and the *Vir* region. The wild T-DNA contains genes that control the production of opines and hormones such as auxin and cytokinin, by the plant cell. Opines are amino acids used by *Agrobacterium* as a source of carbon and nitrogen, while the hormones are responsible for tumor induction in vegetables. The T-DNA is approximately 10 to 30 kb, and its ends are delimited by two 25 bp sequences highly homologous, called right and left ends. Wild *Agrobacterium* transfers its T-DNA across the membranes of plant cells and incorporates it into the plant genome. The T-DNA processing and transfer to the plant cells are largely due to the activity of virulence proteins encoded in the *Vir* region (Gelvin, 2003).
To enable the use of *Agrobacterium* in the biotechnology processes of gene transfer to plants is necessary that the endogenous tumor-causing genes of the T-DNA be inactivated, and

that the foreign genes, genes of interest and selection markers, be inserted between the right and left borders of the T-DNA. The resulting recombinant plasmid is again placed in the *Agrobacterium* to be transferred to plant cells (Gelvin, 2003). Transformed tissues or cells can be used for regeneration of transgenic plants (Hiei et al., 1994, Ishida et al., 1996).
Because it is very large, the Ti plasmid is difficult to manipulate, so binary vectors, which are smaller, able to grow both in *Agrobacterium* and *E. coli* and easy to manipulate in the laboratory were created. These vectors have an artificial T-DNA, in which different transgenes can be inserted and an origin of replication compatible with the *Agrobacterium* Ti. The binary vectors are introduced into an *Agrobacterium* that had the T-DNA region removed from its Ti plasmid, called disarmed *Agrobacterium*. The disarmed *Agrobacterium* Ti plasmid still possesses the virulence region (*Vir*) and its expressed proteins can act in trans to transfer the recombinant T-DNA of the binary vector (Gelvin, 2003).
Agrobacterium tumefaciens is an excellent system for introducing genes into plant cells because: (i) DNA can be introduced in different plant tissues, (ii) the integration of T-DNA is a relatively accurate process. The region of DNA to be transferred is defined by flanking sequences, right and left ends. Occasionally it produces rearrangements, but in most cases the T-DNA region is inserted into the plant genome intact and, (iii) usually the T-DNA integrated shows genetic maps consistent and adequate segregation. Furthermore, the characters introduced in this way have proven stable over many generations of crosses. This stability is critical for the generation of commercial transgenic plants (Hiei et al., 1994, Ishida et al., 1996).
The first maize transformation protocol mediated by *Agrobacterium* with high efficiency was reported in 1996 by a group of researchers from Japan Tobacco Inc. (Ishida et al., 1996). They were able to infect maize immature embryos of A188 using super-binary vectors (pSB131 or pTOK233) (Ishida et al. 1996). The super-binary plasmid developed by Komari (1990) contains an extra copy of the virulence genes *virB*, *virC* and *virG*. Subsequent work showed that the transformation of maize mediated by *Agrobacterium* was also possible with the use of standard vectors (Frame et al. 2002). For maize the technique of *Agrobacterium* mediated transformation has been reported to result in high efficiency of transgenic plants production, with high number of events with only one or a small number of copies of the transgene in the genome compared to biolistic (Ishida et al., 1996, Zhao et al. 2001; Gordon-Kamm et al 2002, Frame et al. 2002; Lupotto et al. 2004; Huang and Wei 2005, Ishida et al 2007).

5. Gene constructs for RNAi target genes

Transgenes or genes that are inserted via molecular biology techniques in plants such as maize, are basically composed of (i) regulatory sequences that control gene expression, (ii) the selection marker gene and, (iii) the gene of interest.
The main sequences controlling gene expression are promoters, enhancers, introns and terminators. Promoters are DNA sequences, normally present in the 5 'end of a coding region, used by RNA polymerase and transcription factors to initiate the process of gene transcription (Buchanan et al., 2000). Depending on the ability to control gene expression, the promoters are classified as weak or strong, according to the binding affinity of transcription factors with the promoter sequence (Browning & Busby, 2004). Strong or weak promoters can be further classified as constitutive, tissue and / or organ-specific and inducible. A constitutive promoter directs expression of a gene in all tissues of a plant during the various stages of development. The viral *35S mosaic virus* promoter isolated from

cauliflower (*CaMV35S*) is one of the most used to drive high constitutive expression in plants (Odell et al., 1985), however its function in monocots is not as efficient as in dicotyledons. The promoter used to drive the overexpression of a protein constitutively in maize is currently the promoter isolated from maize ubiquitin gene *Ubi1* (Christensen & Quail, 1996). A tissue-specific promoter directs gene expression only in certain tissue, which may or may not be activated during all stages of development. The use of this type of promoter may be advantageous to prevent an unnecessary waste of energy and nutrients by the transgenic plant when the protein of interest is not required throughout the plant. For example, the expression of genes related to absorption of nutrients is required only at the root. An inducible promoter initiates gene expression in response to chemical, physical, or biotic and abiotic stresses (Liu, 2009). Similar to specific promoters, inducible ones avoid the unnecessary consumption of energy and nutrients, since the protein is only produced in response to right stimulus. An example of an inducible promoter is the one isolated from the *AtPHT1; 4* phosphate transporter gene from *Arabidopsis thaliana*, which was shown to direct expression of the *uidA* reporter gene only in roots of maize subjected to phosphorus stress (Coelho et al. 2010). These features of promoters allow the expression of the transgenic protein be controlled according to the project objectives.

Enhancers are regions of DNA that bind transcription factors responsible for an increase in transcription of a gene, and consequently by an increase in protein expression. Enhancers can be located before or after the coding region. In the genome, sequences of plant enhancers can be located physically distant from the gene which they are controlling, however because of the packaging of DNA in the nucleus, these sequences are geometrically positioned near the promoter. This position allows for an interaction between transcription factors and RNA polymerase II (Arnosti & Kulkarni, 2005).

Introns are non-coding sequences within a gene that are removed during transcription. Although the mechanisms underlying the phenomenon are not completely clear, the incorporation of introns in genes can increase or decrease promoter activity and the levels of transcription (Chaubet-Gigot et al., 2001). Typically, the intron is inserted between the 3 'end of the promoter and the initial codon of the protein of interest (Liu, 2009). Introns such as the rice actin *Act1* (McElroy et al., 1991), *Ubi1* of ubiquitin from maize (Christensen & Quail, 1996), *SH1* sucrose synthase from maize (Vasil et al., 1989), and *Adh1* corn alcohol dehydrogenase (Rathus et al., 1993) has been used in gene constructs in order to increase the expression of transgenes.

The regions 3 'UTRs also known as terminator regions are used to confer greater stability to the mRNA, and to signal the end of the transcript preventing the occurrence of the production of chimeric RNA molecules and consequently the formation of new proteins, if the polymerase complex continues transcribing beyond the end of the gene (Lessard et al. 2002). 3' UTRs sequences used in most gene constructs for transformation of maize include the nopaline synthase gene from *Agrobacterium* (Depicker et al., 1982), the 3 'region of *CaMV35S* (Frame et al., 2002), and inhibitor gene proteinase *pinII* from potato (An et al., 1989).

The selection gene is a sequence encoding a protein that when expressed in transgenic cells confer an adaptive advantage. The selection gene is used to identify and select cells that have the heterologous DNA integrated into their genome. Selection genes are fundamental to the development of technologies for plant transformation because the process of transferring a transgene to a recipient cell and its integration into the genome is very inefficient in most experiments, and the chances of recovery transgenic lines without selection are generally very low (Liu, 2009).

Currently, the most used selection markers for the production of transgenic maize are those that confer tolerance to herbicides. Among these, the *bar* gene, isolated from *Streptomyces hygroscopicus* and the *pat* gene, isolated from *Streptomyces viridochromogenes*, both encoding the enzyme phosphinothricin acetyltransferase (PAT) (De Block et al., 1989) are often mentioned.

In majority the gene of interest is a coding sequence or ORF (Open Reading Frame) of a certain protein that when expressed define a characteristic or phenotype of interest. In other cases, is a gene sequence used to silence gene expression, such as the RNAi technology.

An important aspect regarding the use of RNAi for plant biologists is the ability to decide the target region of the gene that should be used to efficiently produce the dsRNA. In 2002 the company Dharmacon (www.dharmacom.com) was the first to develop an algorithm as a tool for rational design of a potent silencing, based on data by Reynolds et al., (2004). Today, there are several companies that have developed algorithms for analysis of gene sequence based on a number of parameters that predispose to more effective use of this technology. Many of these softwares are freely accessible on the Internet:

1. http://www.ambion.com/techlib/misc/sRNAi_finder.html;
2. http://biotools.idtdna.com/rnai/;
3. http://hydra1.wistar.upenn.edu/Projects/sRNAi/sRNAiindex.htm;
4. https://rnaidesigner.invitrogen.com/sirna/;
5. http://jura.wi.mit.edu/sRNAiext/register.php;
6. http://www.protocol-online.org/prot/Molecular_Biology/RNA/RNA_Interference/sRNAi_Design_Rules/;
7. http://www.ambion.com/techlib/resources/RNAi/.

The new synthesized siRNA can target other RNAs on the basis of sequence similarity. Any RNA that possesses sequence similarity with the original trigger dsRNA may be silenced. This fact may limit the use of RNA silencing in plants due to gene family with high sequence similarity (Miki et al., 2005).

One alternative way to express dsRNA in maize is described as followed. The interested cDNA fragment is initially amplified with primers forward containing the *Xba*I-*Xho*I-*Bam*HI and primer reverse containing *Hind*III – *Kpn*I sites on the 5′ of each primer. The cDNA fragment (around 450 bp) is cloned in two steps in the multiple cloning site of an RNAi induced transgene in the pKANNIBAL vector (Wesley et al., 2001). In the first step, the cDNA is cloned into the pKANNIBAL *Xho*I –*Kpn*I. In the second step, the original cDNA fragment is cloned again in the inverted direction in the pKANNIBAL *Xba*I – *Hind*III already containing the first copy of the fragment. After the double cloning into the pKANNIBAL, the cassette is excised and cloned into pCAMBIA3301 *Bam*HI site already containing the Ubiquitin promoter and NOS terminator. The transgene expression results in a transcript that terminates within the 3′ sequence of the NOS terminator and folds back on itself by virtue of the inverted repeats, thus generating the dsRNA (Fig. 4). The dsRNA is then substrate for the DICER and RISC enzyme complex that cleaves it into siRNA as already explain in the Figure 1. The confirmation of the cloning in an appropriate direction might be either done with restriction mapping or sequencing analysis. However, with the advantage of DNA synthesis today none of the first steps might be required for final cloning. The RNAi cassette in the binary vector pCAMBIA 3301 is then transformed in Hi-II maize genotype by microprojectile bombardment.

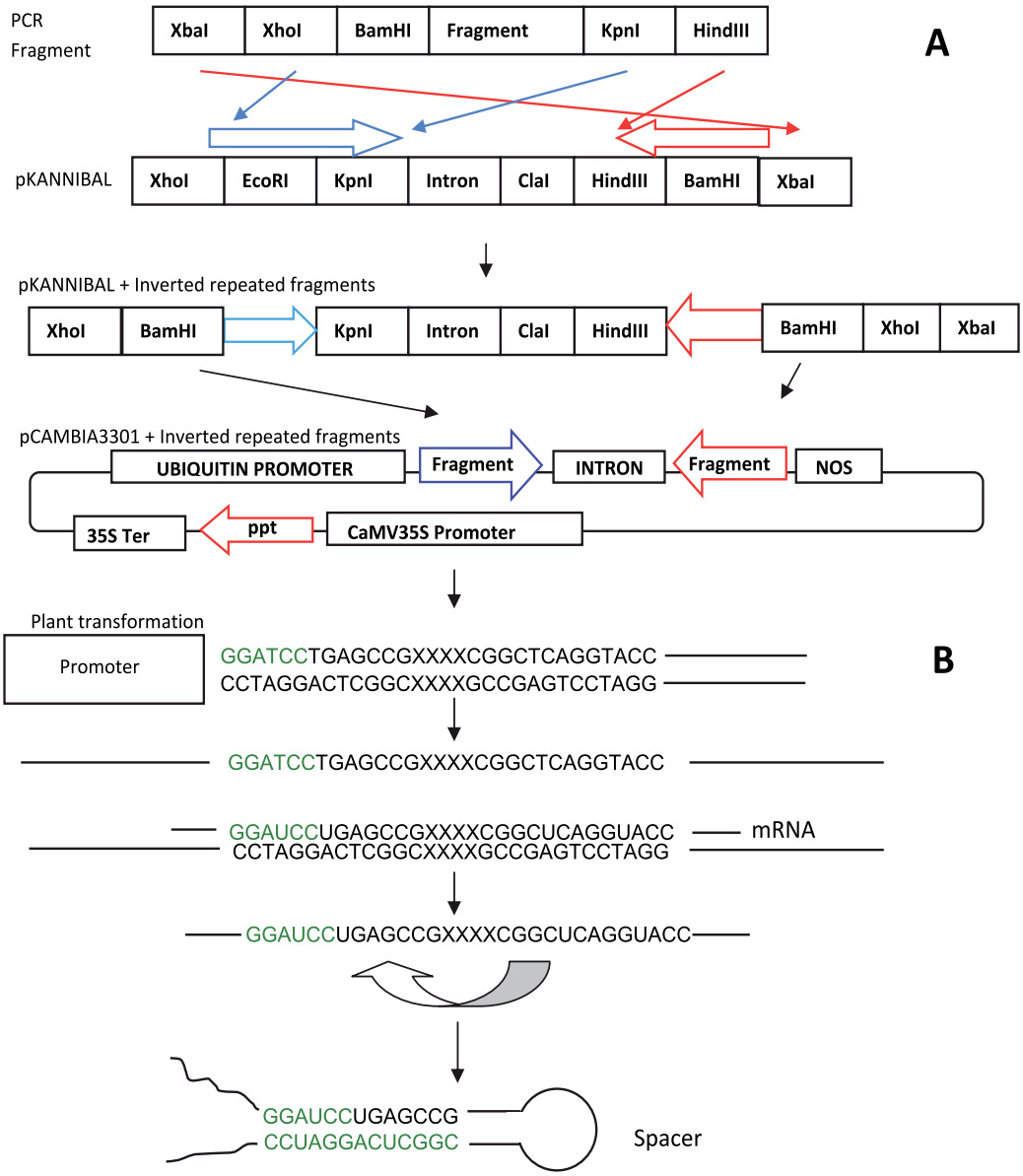

Fig. 4. (A) Diagram of the cloning of a gene fragment in an inverted repeat direction into de pKANNIBAL vector and the transfer of the cassette to the pCAMBIA3301 which carries the selection marker phosphinothricin acetyl transferase (*bar*) (B) Diagram of hpRNA prior to folding into the characteristic hairpin structure. It has two inverted oriented repeated sequences between a spacer.

The transgenic T1 plants arise in the frequency around 1% relative to the original number of explants. The first confirmation of the transgenic is done by spraying leaves with 3 mg/L Finale herbicide (ammonium glyfosinate - AgrEvo Environmental Health, Montvale, NJ). The *bar* gene present in the pCAMBIA3301 plasmid confers resistance to this herbicide.

Transgenic plants that express this selectable marker gene survive herbicide spraying whereas the nontransgenic plants die (Figure 5).

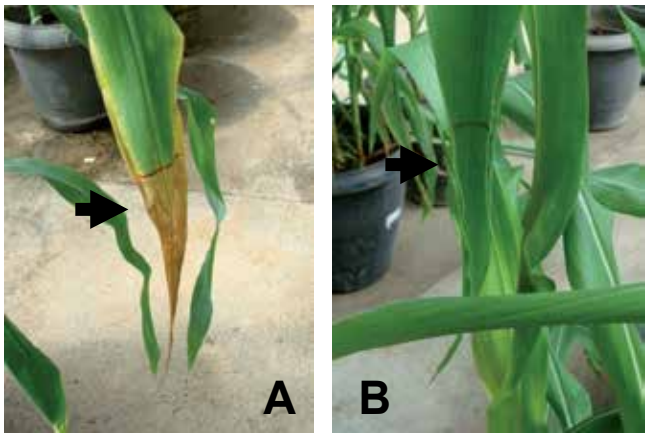

Fig. 5. Test with the Finale herbicide (ammonium glyfosinate) in maize leaves. (A) Sample sensitive and (B) insensitive to the herbicide.

The second confirmation of the transgenic is done by PCR using primers specific to the gene construct. To produce high-quality, stable transgenic lines it is necessary to define individuals with a single copy insertion and in homozygosity. This decision is based on the premise that expression of one copy is more stable and reliable than multicopy in the following generations. DNA purified from a single leaves (~100 mg of tissue) of T1 transformed plants is screening in a Southern blot analysis to identify events that possess single copy insertion. DNA is digested with restriction enzyme and subjected to gel electrophoresis. After the transfer of the DNA to the nylon membrane it is hybridized either with the *bar* gene or any other fragment present on the genetic cassette. The choice of the enzyme depends on the way the cassette was prepared. If there is no site in the cassette of the restriction enzyme used for the initial digestion of the DNA, the number of bands reflects the number of copies of the fragment integrated into the genome. Even for the self pollinated T0 plants many of the T1 generation are still heterozygous specially if there is more than one insertion. In this case, the test of herbicide and PCR in a sample of the following generation will help identify the one that are homozygous. If 100% of the T2 progeny of a single T1 plant are resistance to the herbicide (or show positive for the PCR) it indicates that the T1 parent (as well as all the T2 sibs) is homozygous for the transgene.

Recent works at Embrapa Maize and Sorghum (Brazil) obtained SCMV resistant transgenic maize plants by transforming friable callus of maize HiII using a construction based on the RNAi technology (data not published). Previous study on the SCMV gene family identified the region of the coat protein as a conserved region that might be used to produce the cassette to silence the expression of the SCMV virus in maize. Once this fragment from the SCMV genome was choose and isolated, it was cloned twice, in inverted position, into the vector pKANNIBAL containing a spacer, transferred to a binary vector pCAMBIA 3301

containing the ubiquitin promoter and NOS terminator and used to transform maize by particle bombardment as explained above.
The phenotypic evaluation of the transgenic plants was done by inoculation of the SCMV virus complex every week for three consecutive weeks starting in a maize V5 stage. The inoculation was confirmed by PCR and microscopy. From the 20 events obtained 30% of the plants did not show any viruses symptoms and in approximately 46% the symptoms reduces along the plant life cycle. These results indicated that the technique of RNAi based on the Coat protein sequence was capable of generating transgenic maize resistant to the SCMV virus (Figure 6).

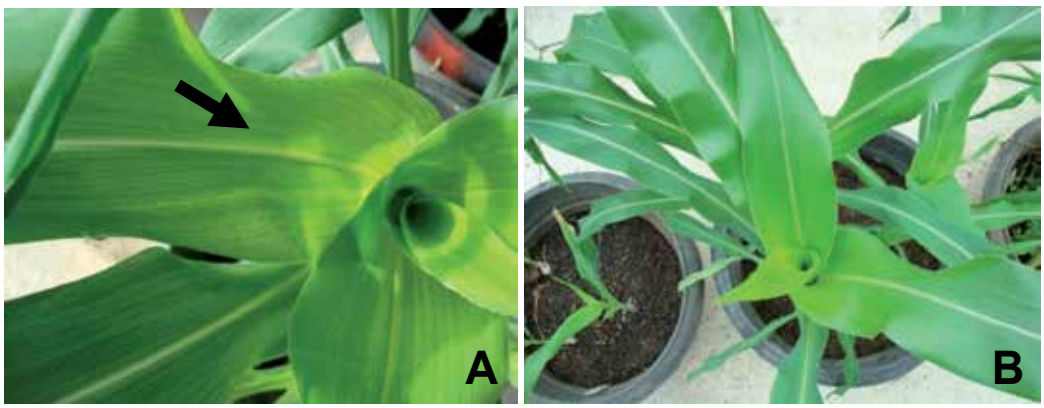

Fig. 6. Transgenic and non-transgenic plants inoculated with SCMV in the greenhouse. (A) Plant with symptoms and (B) transgenic plants with no symptoms; Black arrow indicates the symptoms.

Other groups also got similar results, in maize, by induced RNAi-mediated transgenic virus resistance. Bai et al. (2008) transformed maize with an hpRNA expression vector p3301 containing the inverted-repeat sequence of the SCMV Nib gene, and obtained transgenic resistant lines. Also, Zhang et al. (2001) constructed an hpRNA expression vector containing reverted-repeat sense and antisense arms to target the MDMV gene encoding the P1 protein (protease) and used this cassette to transform maize embryonic calli and obtain plants tolerant to MDMV viruses.

6. Conclusions

In the 60's and 70's the world experienced a vast increase in the agricultural productivity based on conventional breeding techniques, intensive use of industrial inputs (fertilizers and pesticides), mechanization and cost reduction of management. In the 21st century, molecular biology techniques have been coupled with the conventional breeding techniques to boost up crops productivity. In the mid 90's the discovery of the RNAi added a new perspective to the gene regulation. This technology became a powerful tool to understand gene function and to the breeders improve crop varieties such as the development of barley varieties resistant to BYDV (Barley Yellow Dwarf Virus) (Wang et al., 2000), reduce the level

of glutenin in rice which is important for patients that are incapable to digest it (Kusaba et al., 2003) and among others, to obtain varieties of banana resistant to BBrMV (Banana Bract Mosaic Virus), a virus that has devastated the Southeast of Asia and Indian .

Some applications of RNAi in plants have relied in non *Agrobacterium* mediated methods to induce dsRNA into the cells. This chapter described the potential use of RNAi to knock out gene in plants and obtain tolerant transgenic maize lines using a vector capable to form dsRNA. The results implicate in the creation of an improved maize cultivar resistance to SCMV. This approach might be a very interesting alternative and innovation to narrow the gap between productivity and disease, insects and virus resistance, nutritionally rich and toxic-free crops and abiotic stresses.

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8. References

Abel, P.P.; Nelson, R.S.; De, B.; Hoffmann, N.; Rogers, S.G.; Fraley, R.T. & Beachy, R.N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science. Vol.232, No.4751, pp. 738-43.

Agrawal, N.; Dasaradhi, P.V.; Mohmmed, A.; Malhotra, P.; Bhatnagar, R.K. & Mukherjee, S.K. (2003). RNA interference: biology, mechanism, and applications Microbiol Mol Biol Rev. Vol.67, No.4, pp. 657-685

An, G.; Mitra, A.; Hong, K.C.; Costa, M.A.; Na, K.; Thornburg, R.W. (1989). Functional analysis of the 3' control region of the potato wound-inducible proteinase inhibitor II gene. Plant Cell Vol.1, pp. 115-122

Anandalakshmi, R.; Pruss, G.J.; Ge, X.; Marathe, R.; Mallory, A.C.; Smith, T.H. & Vance, V.B. (1998). A viral suppressor of gene silencing in plants Proc Natl Acad Sci U S A. Vol.95, No.22, pp. 13079-13084.

Armstrong, C.L. & Green, C.E. (1985). Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta. Vol. 164, No.2, pp. 207-214

Armstrong, C.L.; Green, C.E. & Phillips, R.L. (1991). Development and availability of germplasm with high type II culture formation response. Maize Genet. Coop. Newsl. Vol.65, pp. 92-93

Arnosti, D.N. & Kulkarni, M.M. (2005) Transcriptional enhancers: intelligent enhanceosomes or flexible billboards? J. Cell.Biochem. 94:890–898.

Baulcombe, D. (2004) RNA silencing in plants, Nature. Vol.431, pp. 356-363

Bai, Y.; Yang, H.; Qu, L.; Zheng, J.; Zhang, J.; Wang, M.; Xie, W.; Zhou, X.; Wang, G. (2008). Inverted-repeat transgenic maize plants resistant to sugarcane mosaic virus. Front. Agric. China Vol. 2, No.2, 125–130

Beckert, M. & Qing, C.M. (1984). Results of a diallel trial and a breeding experiment for *in vitro* aptitude in maize. Theor. Appl. Genet. Vol.68, pp.247-251

Bernstein, E.; Caudy, A.A.; Hammond, S.M. & Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. Vol.409, No.6818, pp. 363-366.

Brettschneider, R.; Becker, D. & Lorz, H. (1997). Efficient transformation of scutellar tissue of immature maize embryos. Theor. Appl. Genet. Vol.94, pp. 737-748

Brigneti, G.; Voinnet, O.; Li, W.X.; Ji, L.H.; Ding, S.W. & Baulcombe , D.C. (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. EMBO J. Vol.17, No.22, pp.6739-6746.

Browning, D.F. & Busby, S. J. (2004). The regulation of bacterial transcription initiation. Nat. Rev. Microbiol. Vol.2, pp. 57-65

Buchanan, B.B.; Gruissem, W.; , Jones RL eds. Biochemistry and molecular biology of plants. Rockville, MD: American Society of Plant Physiologists; 2000:340–342.

Chaubet-Gigot, N.; Kapros, T.; Flenet, M.; Kahn, K.; Gigot, C. & Waterborg, J.H. (2001). Tissue-dependent enhancement of transgene expression by introns of replacement histone H3 genes of Arabidopsis. Plant Mol. Biol.Vol.45, pp. 17–30

Christensen, A.H. & Quail, P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res. 5213–5218.

Coelho, G.T.C.P.; Carneiro, A.A., Karthikeyan, A.S.; Carneiro, N.P.; Brandão, R.L.; Paiva, L.V.; Souza, I.R.P.; Alves, V.M.; Imolesi, A.; Carvalho, C.H.S.; Raghothama, K.G. & Schaffert, R.E. (2010). Phosphate transporter promoter from *Arabidopsis thaliana AtPHT1;4* gene drives preferential gene expression in transgenic maize root under phosphorus starvation. Plant Mol Biol Rep. Vol.28, pp. 717–723

DeBlock, M.; De Brower, D. & Tenning, P. (1989). Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol. Vol.91, pp. 694–701

Depicker, A.; Stachel, S.; Dhaese, P.; Zambryshi, P. &, Goodman, H.M. (1982). Nopaline synthase, transcript mapping and DNA sequence. J. Mol. Appl. Genet.. Vol1, pp. 561-573

Di Nicola-Negri, E.; Brunetti, A.; Tavazza, M. & Ilardi, V. (2005). Hairpin RNA-mediated silencing of Plum pox virus P1 and HC-Pro genes for efficient and predictable resistance to the virus. Transgenic Res. Vol.14, No.6, pp. 989-94

Duncan, D.R.; Willians, M.E.; Zehr, B.E. & Widholm, J.M. (1985). The production of callus capable of plant regeneration from immature embryos of numerous *Zea mays* plants. Bio/Technol. Vol.8, pp. 833-839

Fire, A.; Xu, S.; Montgomery, M.K.; Kostas, S.A.; Driver, S.E. & Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. Vol.391, No.6669, pp. 744-745

Fitchen, J.H. & Beachy, R.N. (1993). Genetically engineered protection against viruses in transgenic plants. Annu Rev Microbiol. Vol.47, pp. 739-63

Frame, B.R.; Shou, H.; Chikwamba , R.K.; Zhang, Z.; Xiang, C.; Fonger, T.M.; Pegg, E.K.; Li, B.; Nettleton, D.S.; Pei, D. & Wang, K. (2002). *Agrobacterium tumefaciens*-Mediated

transformation of maize embryos using a standard binary vector system. Plant Physiol. Vol.129, pp. 13-22

Frame, B.; Zhang, H.; Cocciolone, S.; Sidorenko, L.; Dietrich, C.; Pegg, S.; Zhen, S.; Schnable, P. & Wang, K. (2000). Production of transgenic maize from bombarded Type II callus: effect of gold particle size and callus morphology on transformation efficiency. In Vitro Cell Dev. Biol-Plant. Vol.36, pp.21-29

Fromm, M.E.; Morrish, F.; Armstrong, A.; Willians, R.; Thomas, J. & Klein, T.M. (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio/Technol. Vol.8, No.9, pp. 833-839

Fuchs, E. & Grüntzig, M. (1995). Influence of sugarcane mosaic virus (SCMV) and maize dwarf mosaic virus (MDMV) on the growth and yield of two maize varieties. J. Plant Dis. Protect. Vol. 102, pp. 44-50

Fuchs, E.; Grüntzig, M.; kuntze, L.; Oertel, U. & Hohmann, F. (1996) Zur Epidemiologie der Potyviren des Maises in Deutschland. Bericht 46. Arbeistag. AG Saatzuchleiter der "Vereinigung österreichischer Pflanzenzüchter". Gumpenstein. Vol. 21-23, pp. 43-49

Fuchs, E. & Kozelska, S. (1984). Zum Auftreten und zur Schadwirkung des Europäischen Mais Mosaik Virus (EMMV) in der DDR, sowie Ergebnisse der Prüfung der Anfälligkeit von Inzuchtlinien und Hybriden. Nachrichtenbl. Pflanzenschutz DDR. Vol.38, pp. 162-164

Gelvin, S.B. (2003) *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol. Mol. Biol. Rev. Vol.67, No.1, pp. 16-37

Gordon-Kamm, W.J.; Spencer, T.M.; Mangano, M.L.; Adams, T.R.; Daines, R.J.; Start, W.G.; O'Brien, J.V.; Chambers, S.A.; Adams Jr., W.R.; Willetts, N.G.; Riche, T.B.; Mackey, C.J.; Krueger, R.W.; Kausch, A.P. & Lemaux, P.G. (1990). Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell Vol.2, pp.603–618

Green, C.E. & Phillips, R.L. (1975). Plant regeneration from tissue cultures of maize. Crop Sci. Vol.15, pp. 417-421

Grumet, R.; Sanford, J.C. & Johnston, S.A. (1987). Pathogen-derived resistance to viral infection using a negative regulatory molecule. Virology. Vol.161, No.2, pp. 561-569

Guo, H..S. & Garcia, J.A. (1997). Delayed resistance to Plum pox potyvirus mediated by a mutated RNA replicase gene: involvement of a gene-silencing mechanism. MPMI. Vol.10, pp. 160-170

Hammond, S.M.; Boettcher, S.; Caudy, A.A.; Kobayashi, R. & Hannon, G.J. (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. Science. Vol.293, No.5532, pp.1146-11450

Hiei, Y.; Ohta, S.; Komari, T. & Kumasho, T. (1994). Efficient transformation of rice mediated by *Agrobacterium* and sequence analysis of boundaries of the T-DNA. Plant J.Vol.6, pp.271-282

Hodges, T.K.; Kamo, K.K.; Imbrie, C.W. & Becwar (1986). Genotype specificity of somatic embryogenesis and regeneration in maize. Bio/Technol. Vol4, pp.199-223.

Huang, X. & Wei, Z. (2005). Successful *Agrobacterium*-mediated genetic transformation of maize elite inbred lines. J. Plant Biotechnol. Vol.83, No.2, pp. 187-200

Ishida, Y.; Hiei, Y. & Komari, T. (2007). *Agrobacterium*-mediated transformation of maize. Nature Protocols Vol.2, pp. 1614-1621

Ishida, V.; Saito, H.; Ohta, S.; Hiei, Y.; Komari, T. & Kumashiro, T. (1996). High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnol. Vol.6, pp. 745-750

Jacquet, C.; Ravelonandro, M.; Bachelier, JC. & Dunez, J. (1998). High resistance to plum pox vírus (PPV) in transgenic plantas containing modified and truncated formo f PPV coat protein gene. Transgenic Res Vol.7, pp. 29-39

Knee, R.; Murphy, PR,. (1997). Regulation of gene expression by natural antisense RNA transcripts. Neurochem Int. Sep;31(3):379-92

Kennerdell, J.R. & Carthew, R.W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell. Vol.95, pp. 1017-1026

Kim, V.N. (2003). RNA interference in functional genomics and medicine. J. Korean Med. Sci. Vol.18, pp. 309-318

King, P.J.; Potrykus, I. & Thomas, E. (1978). *In vitro* genetics of cereal: Problems and perspectives. Physiol. Veg. Vol.16, pp. 381-399

Klein, T.; Gradiziel, T.; Fromm, M. & Sandford, J. (1988). Factors influencing gene delivery into *Zea mays* cells by high-velocity microprojectiles. Bio/Technol. Vol6, pp. 559-563

Komari, T. (1990). Transformation of callus cells of *Chenopodium quinoa* by binary vectors that carry a fragment of DNA from the virulence region of pTiBo542. Plant Cell Rep. Vol.9, pp.303-306

Kusaba, M.; Miyahara, K.; Iida, S.; Fukuoka, H.; Takano, T.; Sassa, H.; Nishimura, M.; Nishio, T. (2003) Low glutelin content1: a dominant mutation that suppresses the glutelin multigene family via RNA silencing in rice. Plant Cell. Vol.15, No.6, pp. 1455-67

Lessard, P.A.; Kulaveerasingam, H.; York, G.M.; Strong, A. & Sinskey, A.J. (2002). Manipulating gene expression for the metabolic engineering of plants. Metab. Eng. Vol.4, pp. 67-79

Liu, D. (2009). Design of gene constructs for transgenic maize. In: Methods in Molecular Biology: Transgenic Maize. M. Paul Scott (ed.). Humana Press. USA. 526:3-20.

Lupotto, E.; Conti, E.; Reali, A.; Lanzanova, C.; Baldoni, E. & Allegri, L. (2004). Improving *in vitro* culture and regeneration conditions for *Agrobacterium*-mediated maize transformation. Maydica. Vol.49, pp. 221-229

McElroy, D.; Blowers, A.D.; Jenes, B. & Wu, R. (1991). Construction of expression vectors based on the rice actin 1 (*Act1*) region for use in monocot transformation. Mol. Gen. Genet. Vol.231, pp. 150–160

McGinnis K. Use of transgene-induced RNAi to regulate endogenous gene expression. (2009). Methods Mol Biol.;526:91-9.

Melchinger, A.E.; Utz, H.F. & Schon, C.C. (1998). Quantitative trait locus (QTL) mapping using different testers and independent population samples in maize reveals low power of QTL detection and large bias in estimates of QTL effects. Genetics. Vol.149, No.1, pp. 383-403

Miki. D.; Itoh, R.; Shimamoto, K. (2005)RNA silencing of single and multiple members in a gene family of rice. Plant Physiol. Vol.138, No.4, pp. 1903-1913

Napoli, C.; Lemieux, C. & Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell. Vol.2, No.4, pp. 279-289

Ngo, H.; Tschudi, C.; Gull, K. & Ullu, E. (1998). Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. Proc. Natl. Acad. Sci. USA. Vol.95, pp. 14687-14692

Odell, J.T.; Nagy, F. & Chua, N-H, (1985), Identification of DNA sequences required for activity of the cauliflower mosaic virus *35S* promoter. Nature. Vol.313, pp. 810–812.

Palkovics, L.; Wittner, A. & Balazs, E. (1995). Pathogen-derived resistance induced by integrating the plum po virus coat protein gene into plantas of *Nicotiana benthamiana*. Acta Hort. Vol.386, pp. 311-317

Powell, P.A.; Sanders, P.R.; Tumer, N.& Fraley. (1990). Protection against tobacco mosaic virus infection in transgenic plants requires accumulation of coat protein rather than coat protein RNA sequences.RT. Virology. Vol.175, No.1, pp. :124-30

Priolli, L.M. & Silva, W.J. (1989). Somatic embryogenesis and plant regeneration capacity in tropical maize inbreds. Rev. Brasil. Genet.Vol.12, pp. :553-566

Rapela, M.A. (1985). Organogenesis and somatic embryogenesis in tissue cultures of Argentina maize (*Zea mays* L.) J. Plant Physiol. Vol.121, pp.119-122

Ratcliff, F.G.; MacFarlane, S.A. & Baulcombe, D.C. (1999) Gene silencing without DNA. RNA-mediated cross-protection between viruses. Plant Cell. Vol.11, No7, pp. 1207-16

Rathus, C.; Bower, R. & Birch, R.G. (1993). Effects of promoter, intron and enhancer elements on transient gene expression in sugar-cane and carrot protoplasts. Plant Mol. Biol. Vol.23, pp. 613–618

Ravelonandro, M.; Monsion, M.; Teycheney, P.Y.; Delbos, R. & Dunez J. (1992). Construction of a chimeric viral gene expressing plum pox virus coat protein. Gene. Vol.120, No.2, pp. 167-73

Reynolds, A.; Leake, D.; Boese, Q.; Scaringe, S.; Marshall, W.S. & Khvorova A. 2004. Rational sRNAi design for RNA interference. Nat Biotechnol. Vol.22, No.3, pp. 326-330

Sanford, J.C.; Smith, F.D. & Russel, J.A. (1993). Optimizing the biolistic process for different biological applications. In: WU, R. Ed. Recombinant DNA – Part H. San Diego: Academic Press. p. 483-510 (Methods in Enzymology, 217).

Sanford, J. (1988). The biolistic process. Trends Biotech. Vol.6, pp. 299-302

Sandford, J.; Klein, T.; Wolf, E. & Allen, N. (1987). Delivery of substances into cells and tissues using particle bombardment process. J. Part. Sci. Tech. Vol.5, pp. 27-37

Shukla, D.D.; Ward, C.W.; Brunt, A.A. (1994). The Potyviridae. Cambridge. CAB International. 516p

Tang, G. & Galili, G. (2004). Using RNAi to improve plant nutritional value: from mechanisms to application. Trends in Biotechnology. Vol.22, No.9, pp. 463:469

Taylor, N.J. & Fauquet, C.M. (2002). Particle bombardment as a tool in plant science and agricultural biotechnology. DNA Cell Biol. Vol.21, No.12, pp. 963-977.

van Loon, L.C., Rep, M., Pieterse, C.M. (2006). Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol.; Vol. 44, pp. 135-162

Vasil, V.; Clancy, M.; Ferl, R..; Vasil, I.K. & Hannah, L.C. (1989). Increased gene expression by the first intron of Maize *Shrunken-1* locus in grass species. Plant Physiol. Vol.91, pp. 1575-1579

Vasil, I.K. (1987). Developing cell and tissue culture systems of the improvement of cereal and grass crops. J. Plant. Physiol. Vol.128, pp. 193-218

Vasil, V. & Vasil, I.K. (1981). Somatic embryogenesis and plant regeneration from tissue cultures of *Pennisetum americanum* and *P. americanum* X *P. purpureum* hybrid. Amer. J. Bot. Vol.68, pp. 864-872

Vinh, D.N. (1989). Factors affecting the formation and differentiation of embryogenic callus in cultured in vitro of immature maize embryos. Genet Breed. Vol.22, pp. 45-51

Wall, N.R. & Shi, Y. (2003). Small RNA: can RNA interference be exploited for therapy? Lancet. Vol.362, pp. 1401-1403

Wang, K. & Frame, B. (2004). Maize transformation. In: Transgenic crops of world – Essential Protocols. Ian S. Curtis (ed) Kluwer Acad. Publish. Netherlands pp 45-62.

Wang, M.B.; Abbott, D.C.; Waterhouse, P.M. (2000). A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus. Mol Plant Pathol. Vol.1, No.6, pp. 347-356

Waquil, J.M.; Oliveira, E.; Pinto, N.F.J.A.; Fernandes, F.T.; Correia, L.A. (1996). Efeito na produção e incidência de viroses em híbridos comerciais de milho. Fitopatol. Bras. Vol.21, No.4, pp. 460-463

Wei, Q.; Lipardi, C. & Paterson, B.M. (2003). Analysis of the 3(')-hydroxyl group in *Drosophila* sRNAi function. Methods. Vol.30, No.4, pp. 337-347

Wesley, S.V.; Helliwell, C.A.; Smith, N.A.; Wang, M.B.; Rouse, D.T.; Liu, Q.; Gooding, P.S.; Singh, S.P.; Abbott, D.; Stoutjesdijk, P.A.; Robinson, S.P.; Gleave, A.P.; Green, A.G. & Waterhouse PM. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. Vol.27, No.6, pp. 581-590

Wittner, A.; Palkovics, L. & Balazs, E. (1998). *Nicotiana benthamiana* plants transformed with the plum pox virus helicase gene are resistant to virus infection. Virus Res. Vol.53, No.1, pp. 97-103

Wu, L.; Nandi, S.; Chen, L.; Rodriguez, R.L. & Huang, N. (2002). Expression and inheritance of nine transgenes in rice. Transgenic Res.Vol.11, pp. 533-541

Zamore, P.D.; Tuschl, T.; Sharp, P.A. & Bartel, D.P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21-23 nucleotide intervals. Cell. Vol.101, pp. 25-33

Zhang, X., Lyle, C., Wang, Y., Zeng, L. (2001). Role of hnRNP A1 in coronavirus RNA synthesis. Adv Exp Med Biol.; Vol. 494 pp437-46

Zhao, Z-Y.; Gu, W.; Cai, T.; Tagliani, L.; Hondred, D.; Bond, D.; Schroeder, S.; Rudert, M. & Pierce, D. (2001). High throughput genetic transformation of maize mediated by *Agrobacterium tumefaciens*. Mol Breed. Vol.8, pp. 323-333

Zhong, H.; Srinivasan, C. & Sticklen, M.B. (1992). *In-vitro* morphogenesis of corn *Zea mays* L. I. Differentiation of multiple shoots clumps and somatic embryos from shoot tips. Planta. Vol.187, pp. 483-489

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Genetic Transformation of *Triticeae* Cereals for Molecular Farming

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1. Introduction

The *in planta* production of recombinant proteins is a newly emerging area. The use of transgenic crops enjoys several comparative advantages over established heterologous protein production systems based on bacteria, yeasts, mammalian or plant cells, particularly in terms of cost and practicality. Thanks to the development of effective transformation protocols, the generation of recombinant vaccines, antibodies and enzymes in the grains of the *Triticeae* cereals has become a feasible proposition in recent years. A further advantage of *in planta* synthesized recombinant proteins over bacterial and yeast-derived ones relates to post-translational modifications, in particular glycosylation. Since the majority of pharmaceutically active proteins are glycoproteins, their synthesis in bacteria and yeast is not possible. Therefore most of these proteins are currently synthesized in mammalian cell cultures. Since such cultures need complex (and therefore expensive) media, they also bear the risks of contamination by human pathogens. At present, about a dozen plant-derived pharmaceuticals are in the clinical phase of testing. Beside that a secretory IgA targeting tooth decay (CaroRx™-from Planet Biotechnology Inc, Ma et al., 1998, 2005) and a human intrinsic factor targeted as a dietary supplement to alleviate vitamin B-12 deficiency (Cobento Biotech AS) are already approved for human use (Faye & Gomord, 2010). A number of field trials are currently underway to investigate and validate additional products (Dunwell, 2009; APHIS, 2011).

The *Triticeae* family includes the major temperate crop species barley and wheat, which have been intensively bred over many decades to become well adapted to a wide range of growing environments. Although the major end-use of the temperate cereal grain is for food and feed, a significant focus of certain improvement programmes is aimed at the bioenergy market. Barley is seen as a more suitable host than wheat for transgenic applications because it is more easily transformed. An important advantage of barley and wheat in the context of biosafety is that they are largely self-pollinating, and so have been accorded G.R.A.S. (generally recognized as safe) status by the European regulatory agency EFSA. The infrastructure associated with cereal grain production, harvest and post-harvest storage is well established, and production volume is readily scalable by simply adjusting acreage. A number of transgene expression systems are available, some designed to restrict expression to the grain, but others allowing ubiquitous expression (for review, see Hensel et al., 2011).

The purification of heterologous products can be a costly process, although in some situations this step is not needed; a good example is provided by the feeding to poultry of

transgenic pea expressing an scFv antibody directed against the *Eimeria* parasite (Zimmermann et al., 2009). In dicotyledonous species such as *Nicotiana benthamiana*, pharmaceutical proteins have been produced primarily using virus-based magnICON system (ICON Genetics, Germany) in combination with agroinfiltration of the leaf: this approach has been exploited by Bayer Innovation GmbH to develop a patient-specific tumour-vaccine against non-Hodgkin's lymphoma (NHL) which is at present in the clinical phase of testing (Bayer Innovation GmbH, Germany). So far, however, this technology has not been usable in *Triticeae* species. At present either transient expression based on particle bombardment or virus vectors, or via stable expression by integration within the nuclear genome or the plastome, using *Agrobacterium*-mediated transformation or particle bombardment are used, respectively.

To date, the main focus of pharma transgenic research in the *Triticeae* cereals has been concerned with the expression of human growth factors in the barley endosperm (Maltagen Forschung GmbH, Germany; ORF Genetics, Iceland; Ventria Bioscience, CO). The transgenic production of antigens, vaccines and antibodies must abide by GMP (Good Manufacturer Practice), which is intended to guarantee the quality and uniformity of the approved product. The major challenge that this creates is to provide a uniform product given that transgene expression and the accumulation of the transgene product can be affected by uncontrollable variation in a field environment. Although it is possible to grow material in a closely controlled environment, such as in a sophisticated glasshouse, this forfeits much of the advantage of plant production systems, as such facilities are expensive to operate, and thus are only appropriate for high value products which require only small production volumes.

Uniform planting material is a necessity, and one means of obtaining this in the cereals is to generate doubled haploid plants from immature pollen. In barley, Kumlehn et al. (2006) were able by using *Agrobacterium*-mediated gene transfer into embryogenic pollen cultures to produce haploid primary transformants, which were subsequently treated with colchicine to diploidize the material, thereby avoiding segregation of the transgene in later progeny. This immediate fixation of the transgene is particularly attractive in terms of time-saving in winter varieties of wheat and barley.

This review aims to summarize the current state of the art regarding strategies, targets and future challenges in order to achieve high expression levels of *Triticeae* species-based recombinant proteins.

2. The generation of transgenic plants

The progress achieved over the past 20 years towards *Triticeae* cereal transformation has been reviewed recently by Kumlehn & Hensel (2009). The various approaches differ from one another with respect to the means employed to transfer the alien DNA, and/or in the choice of recipient host tissue. Methods include the use of PEG to transfer the DNA into isolated protoplasts, the exploitation of a virus as a vector, the biolistic introduction of DNA-coated particles and *Agrobacterium*-mediated gene transfer. The two latter methods will be described here in some detail, since they have been used intensively in the temperate cereals. Most transformation events involve the integration with nuclear DNA, but transplastomic *Triticeae* plants have also been reported (Cui et al., 2011). The commonest target tissue has been immature embryos, although isolated ovules have also shown some potential (Holme et al., 2008), and embryogenic barley pollen has distinct advantages

(Kumlehn et al., 2006). In wheat, Chauhan et al. (2010) have demonstrated that *Agrobacterium*-mediated gene transfer is also feasible for anther-culture derived haploid embryos.

2.1 Biolistic gene transfer

The biolistic technique involves the bombardment of the recipient tissue with gold or tungsten particles coated with the transgene DNA. It has been widely used to achieve transient expression, particularly where the purpose has been to assess the functionality of gene candidates, the effectiveness of RNAi constructs or the activity of promoter/reporter fusions (Onate et al., 1999; Rubio-Somoza et al., 2006). The major advantage of the technique is that it can rapidly characterize a large number of sequences (Ihlow et al., 2008). Most biolistic protocols seek to effect transfer into either leaf epidermal cells (Douchkov et al., 2005) or into the scutellar tissue of an immature embryo (Knudsen & Müller, 1991). The first stable transgenic wheat plants generated by this means involved the introduction of a gene determining herbicide resistance into embryogenic callus (Vasil et al., 1992). Thereafter, the method was improved and applied successfully to barley (Wan & Lemaux, 1994), cereal rye (Castillo et al., 1994), triticale (Zimny et al., 1995) and macaroni wheat (Bommineni et al., 1997).

2.2 *Agrobacterium*-mediated gene transfer

Although *Agrobacterium*-mediated gene transfer is based on a natural process, the *Triticeae* cereals were not originally considered as being amenable to the technique, as they are not infected by *Agrobacterium* spp. in nature. After the first reports of its successful use to transform wheat (Cheng et al., 1997) and barley (Tingay et al., 1997), the range of transformable species was extended to cereal rye (Popelka & Altpeter, 2003) and triticale (Hensel et al., 2009; Nadolska-Orczyk et al., 2005). However, transformation efficiency remains still variable and rather genotype dependent. In barley, the most readily transformed cultivar is 'Golden Promise', which allows an average of >10 independent transformation events per immature embryo (Bartlett et al., 2008; Hensel et al., 2009; Murray et al., 2004); other cultivars, while being amenable to transformation, show a lower level of efficiency (Hensel et al., 2008; Murray et al., 2004). One suggested means of overcoming this genotype dependency was to replace immature embryos with isolated ovules as the recipient tissue. Holme and colleagues (2008) showed that genotypes with a poor regeneration capacity can be transformed by this method, although the efficiency was lower but not statistically different from that of 'Golden Promise'. Kumlehn et al. (2006) preferred to target embryogenic cultures of pollen as the target plant tissue for transformation in barley.

3. Expression systems

A comprehensive summary of the expression systems developed to date has been given by Hensel et al. (2011). In the context of the cereal grain, a prime target has been to exploit the regulatory system responsible for the expression of the endosperm storage proteins, which represent a major proportion of the protein synthesized within the grain. A particularly frequently exploited sequence for barley is the *HORDEIN D* promoter, and for wheat the various *GLIADIN* and *GLUTENIN* promoters. Vickers et al. (2006) suggested that even

higher levels of transgene expression in barley and wheat endosperms could be obtained by using the oat *GLOBULIN 1* promoter. But till now there is no published study using this expression system. One strategy to maximize transgene expression involves the directed targeting to a particular cellular compartment, by attaching a signal peptide to the 5'- or 3'- terminus of the transgene; a second approach exploits promoter sequences that are only active during a distinct developmental stage or within a specific tissue. Further possibilities involve the use of viral transcriptional enhancer elements or the suppression of the recipient's endogenous protein degradation machinery. When transgenes encoding either antibodies or vaccines have been expressed in both tobacco and maize, only weak accumulation of the recombinant protein occurred in the cytosol, but targeting to the endoplasmic reticulum (ER) by attaching a H/KDEL tag led to a dramatically improved level of heterologous product accumulation (Schillberg et al., 1999; Streatfield et al., 2003). Unfortunately, the choice of the (a) signal peptide remains somewhat empirical, and to a large extent varies from one recombinant protein to other. Where glycosylation is required, targeting to the ER is essential, but nevertheless it remains necessary to evaluate the glycosylation pattern, since this property can itself be polymorphic (Floss et al., 2009).

4. Targets

Three major groups of products have been targeted to date for molecular farming. The first two consist of human or animal antigens and antibodies, which have applications in disease diagnosis, prophylaxis and recovery. The third, which has reached a more advanced stage thanks to a lesser regulatory load, is a range of technical enzymes. The first plant-made protein to be marketed was chicken avidin, produced in maize by ProdiGene (Hood et al., 1997). A number of companies have been active in making recombinant proteins in *Triticeae* plants - these include Ventria Bioscience, ORF Genetics and Maltagen Forschung GmbH. The full set of published outcomes in this area has been summarized in Table 1, and each is described in more detail below.

Promoter, specificity	Coding sequence	Effect	Species	References
Vaccines, Antigenes				
Barley *TRYPSIN INHIBITOR* (*TI*), endosperm	Enterotoxigenic *Escherichia coli FIMBRIAL ADHESIN FaeG* F4 (K88)	Edible vaccine for pigs partialy effective against ETEC-induced diarrhea	Barley	Joensuu et al., 2006
Antibodies				
Maize *UBIQUITIN-1* (*UBI-1*), ubiquitous	ScFvT84.66	Antibody against carcinoembryonic antigen (CEA), tumor-associated diagnostic reagent	Wheat	Stoeger et al., 2000
Wheat High-molecular-weight *GLUTENIN 1Bx17* (*HMW 1Bx17*), endosperm	Synthetic anti glycophorin scFv-HIV epitope fusion	HIV diagnostic reagent	Barley	Schuenmann et al., 2002

Promoter, specificity	Coding sequence	Effect	Species	References
Human Proteins and Growth Factors				
Barley *a-AMYLASE*, aleurone	*ANTITHROMBIN III*	Molecular farming of pharmaceutical proteins	Barley	Stahl et al., 2002
Barley *HORDEIN D* (*HOR-D*), endosperm				
Barley *a-AMYLASE*, aleurone	*a1-ANTITRYPSIN*	Molecular farming of pharmaceutical proteins	Barley	Stahl et al., 2002
Barley *a-AMYLASE*, aleurone	*SERUM ALBUMIN*	Molecular farming of pharmaceutical proteins	Barley	Stahl et al., 2002
Maize *UBIQUITIN-1* (*UBI-1*), ubiquitous	*COLLAGEN Ia*	Molecular farming of pharmaceutical proteins	Barley	Ritala et al., 2008; Eskelin et al., 2009
Rice *GLUTENIN B1* (*GLUB-1*), endosperm				
Barley *HORDEIN D* (*HOR-D*), endosperm	*FLT3-LIGAND*	Molecular farming of pharmaceutical proteins	Barley	Erlendsson et al., 2010
Barley *HORDEIN D* (*HOR-D*), endosperm	*LACTOFERRIN*	Molecular farming of pharmaceutical proteins	Barley	Stahl et al., 2002
Maize *UBIQUITIN-1* (*UBI-1*), ubiquitous				Kamenarova et al., 2007
Rice *GLUTENIN B1* (*GLUB-1*), endosperm				
Barley *a-AMYLASE*, aleurone	*LYSOZYME*	Molecular farming of pharmaceutical proteins	Barley	Stahl et al., 2002
Rice *GLUTENIN B1* (*GLUB-1*), endosperm				Huang et al., 2006
Wheat High-molecular-weight *GLUTENIN 1Bx17* (*HMW 1Bx17*), endosperm			Wheat	Huang et al., 2010
Barley *HORDEIN D* (*HOR-D*), endosperm	*ISOkine™, DERMOkine™*	Molecular farming of pharmaceutical proteins	Barley	ORF Genetics
Technical Enzymes and Recombinant Proteins				
Wheat High-molecular-weight *GLUTENIN 1-D1* (*HMW GLU-1 D1*), endosperm	An-*FERULIC ACID ESTERASE*	Molecular farming of second generation biofuels	Wheat	Harholt et al., 2010

Promoter, specificity	Coding sequence	Effect	Species	References
Barley *HORDEIN-D* (*HOR-D*), endosperm	Heat stable *(1,3-1,4)-β-GLUCANASE*	Grains containing thermostable 1,3-1,4-β-glucanase for better malting	Barley	Horvath et al., 2000
Maize *UBIQUITIN-1* (*UBI-1*), ubiquitous	*Vitreoscilla HAEMOGLOBIN* (*VHb*)	Grains with altered oxygen availability	Barley	Wilhelmson et al., 2007
Wheat Low-molecular-weight *GLUTENIN G1D1* (LMWG1D1), endosperm	Ps-*LEGUMIN A*	Grains with altered protein composition	Wheat	Stoeger et al., 2001
Cauliflower Mosaic Virus 35S (*35S*), ubiquitous	Hv-*LIPOXYGENASE2* (*LOX2*)	Plants with modified oxylipin signature	Barley	Sharma et al., 2006
Maize *UBIQUITIN-1* (*UBI-1*), ubiquitous	Heat-stable An-*PHYTASE*	Grains with improved digestibility for non-ruminant animal feed	Wheat	Brinch-Pederson et al., 2000
Barley *HORDEIN D* (*HOR-D*), endosperm	Td-*THAUMATIN*	Grains containing a natural sweetener for brewing industry	Barley	Stahl et al., 2009
Wheat High-molecular-weight *GLUTENIN 1-D1* (*HMW GLU-1 D1*), endosperm	Bs-*ENDO-XYLANASE*	Grains with improved baking quality	Wheat	Harholt et al., 2010

Table 1. Bio-pharmaceuticals and technical enzymes expressed in *Triticeae* species.

4.1 Vaccines and antigens

Epidemics of the major infectious human diseases are becoming rare in the developed world thanks to the widespread use of vaccination. In less developed countries, the high cost of vaccine and a poorer level of social infrastructure exposes the population to such diseases. The production of a cheap prophylactic product, such as a plant-made vaccine, would make a material contribution to development. The ideal expression system for producing such vaccines needs to be readily transformable, inherently safe and economical, and therapeutically effective (Fischer and Schillberg, 2004). Current systems capable of producing antigens and antibodies in transgenic plants have recently been described (Daniell et al., 2009; Floss et al., 2009; Joensuu et al., 2008). While vaccines can be administered either orally or by injection, the former method is preferably from an organizational point of view and the use of grains (or other plant parts) is particularly attractive for the vaccination of domesticated animals. A disadvantage of the oral delivery route is the relatively large quantity of antigen required (Streatfield & Howard, 2003). The only published report which describes the use of *Triticeae* plants as a vehicle for producing/ expressing antigens is concerned with the control of infection of enterotoxigenic *E. coli* in pigs, chickens and cows (Joensuu et al., 2006). Here, the major subunit of the F4 fimbriae (FaeG) protein was expressed in barley grains, where it comprised up to 1% of total soluble protein. The recombinant protein was able to evoke F4 fimbria-specific antibodies in mice. In a second approach, a company (Novoplant, Germany) expressed a gene responsible for

the production of an FaeG-specific antibody in transgenic pea, and were able to demonstrate a level of antibody expression in the seed of up to 1-2 g scFv/kg.

4.2 Antibodies

Following the first discovery of immunity-conferring substances in the blood (Behring & Kitasato, 1890), antibodies have been exploited in the fight against several diseases. Most antibodies are large Y-shaped proteins that include an antigen-binding site formed by the two variable segments of their heavy and light chain. The five major classes of antibody (IgA, IgD, IgE, IgG and IgM) are recognized by their conserved region structure and their immunological function (Woof & Burton, 2004). Hiatt et al. (1989) pioneered the expression of immunoglobulin chains in tobacco, since then, various portions of these chains have been expressed heterologously, including single chain molecules (scFvs), Fab fragments, small immune proteins (SIPs), IgGs and chimeric secretory IgAs (for a review, see De Muynck et al., 2010). The commonest plant host to date has been tobacco, with only a small number of examples among the *Triticeae* species. In wheat, the earliest success was achieved with the single chain Fv antibody ScFvT84.66, active against carcinoembryonic antigen (CEA), a well characterized tumour-associated marker (Stoeger et al., 2000). The production level was around 1 μg antibody/g grain, which compared unfavourably with what was possible at the time in rice. Storage of the dry grain at room temperature produced no discernible alteration in the antibody's biological activity, demonstrating the attractiveness of the *in planta* transgene expression of therapeutic molecules. A second example concerned a diagnostic antibody for HIV (Schuenmann et al., 2002), where an anti-glycophorin single-chain antibody was fused to an HIV epitope and expressed in tobacco leaves and stems, in potato tubers and in barley grains. In each case, the production level of the fusion protein was adequate, allowing the *in planta* method to replace the more conventional one based on bacterial and murine cells. The yield of heterologous protein in the barley grain reached as much as 150 μg/g, suggesting that transgenic barley could represent a highly suitable means of producing this particular antibody. The rather strict regulatory framework associated with GM plants in Europe has meant that no other example of *in planta* vaccine or antigen production in *Triticeae* has been published in the last ten years.

4.3 Human proteins and growth factors

The earliest published account of the use of cereal grain to express human genes concerned the five proteins antithrombin III, α1-antitrypsin, lysozyme, serum albumin and lactoferrin (Stahl et al., 2002). Here, the concern was not the quantity or quality of the recombinant proteins, but rather the detection of the T-DNA integration sites in the barley genome. However, these targets remain in the portfolio of Maltagen Forschung GmbH, whose website provides detailed information concerning the company's interest in these genes (Maltagen, Germany). Similar products are also offered by ORF Genetics, which exploits an endosperm-specific expression system. They produce a number of hormones and cytokinines like endothelial monocyte activating polypeptide-2 (EMAP2), various fibroblast growth factors, interferons and interleukins. A recent product from this company was human FLT3-ligand, with the gene under the control of the barley *HORDEIN D* promoter (Erlendsson et al., 2010). Ritala et al. (2008) were able to express a codon-optimized version of *COLLAGEN Ia* in barley endosperm-derived suspension cells, and showed that the recombinant protein was equivalent to a version produced in *Pichia pastoris* yeast. The gene

was driven by the maize *UBIQUITIN-1* promoter and the resulting protein yield was rather low (2-9 µg/l). However, the yield was improved by substituting the endosperm-specific rice *GLUTENIN B1* promoter and expressing the construct in the barley grain. The collagen content in the transgenic grain reached ~45 mg/kg dry weight in the best-performing transgenic derivatives. By way of comparison, the heterologous protein content of grain carrying the transgene driven by the same *UBIQUITIN-1* promoter was just ~13 mg/kg (Eskelin et al., 2009). This level was calculated to be sufficient to produce some 5 t of product were ~10% of Finland's barley production to be used for this purpose. Since the annual demand of the pharmaceutical sector is for at least ten times this amount, there is clearly a need to improve the efficiency to compete with existing production systems.

4.4 Technical enzymes and recombinant proteins

Here, the focus was on transgenes whose products are designed to either improve the technical quality of wheat (baking) or barley (brewing), to alter feed quality, or to improve biofuel properties. The earliest report of this sort of manipulation dates back about a decade, when Horvath et al. (2000) described the heterologous expression of a gene encoding a heat-stable *(1,3-1,4)-β-GLUCANASE*, designed to improve the digestibility of barley-based feed pellets used as chicken feed. The chicken gut is unable to break down complex glycans, and this failure can lead to the formation of excessive viscosity in the intestine. In commercial practice, this problem is commonly resolved by the addition to the diet of purified (1,3-1,4)-β-glucanase extracted from *Bacillus amyloliquefaciens*. A fully active and heat non-labile enzyme is present in the transgenic barley grain, which therefore represents an improvement in the nutritional value of the feed containing it. In a related approach, Brinch-Pederson et al. (2000) expressed in the wheat grain a heat-stable *PHYTASE* driven by the *UBIQUITIN-1* promoter in an attempt to encourage the release of phosphate, iron and zinc from the feed. Note that up to 85% of the phosphate present in the cereal grains is bound to phytic acid (Lott, 1984), which is deposited in the grain as phytin, a mixed salt containing potassium, magnesium, iron, calcium and zinc (Raboy, 1990). In the dry grain (as well as in the digestive tract of non-ruminant animals), no phytase activity is detectable (Lantzsch et al., 1992; Usayran & Balnave, 1995), so chicken diets are commonly supplemented by *Aspergillus niger* derived phytase (Nelson et al., 1968, 1971). The presence of the transgenic wheat increased grain phytase activity by a factor of four (from 0.7 to 3 kFTU/kg), whereas even an increase of 10% would have been sufficient to significantly improve the quality of wheat-based feed.

Barley malt and wheat flour are common ingredients of processed food and beverages, so the improvement of their technical quality is of commercial interest. The protein thaumatin is a low-calorie sweetener and flavour modifier (Gibbs et al., 1996; Green, 1999), initially isolated from the West African katemfe fruit (*Thaumatococcus daniellii* Bennett). It is heat stable up to 70°C and is 2,000-3,000 times sweeter than sugar. It has been produced heterologously in bacteria, yeast and various dicotyledonous plants, with an *in planta* yield reaching 1 g/kg leaf in tobacco (Icon Genetics). It has also been successfully synthesized in the barley grain, yielding 2-3 g/kg on a dry matter basis (Stahl et al., 2009).

The germinating seed frequently suffers from oxygen deficiency (Bewley & Black, 1994). This presents a problem during the malting process, and is not readily counteracted by continuous aeration (Wilhelmson et al., 2006). The hypoxia inhibits the *de novo* production of

starch-hydrolyzing enzymes (Guglielminetti et al., 1995), but the heterologous expression of *Vitreoscilla HAEMOGLOBIN* (VHb) in the barley grain reduces the level of hypoxia, and thus increases the availability of starch-hydrolysing enzymes during malting (Wilhelmson et al., 2007). However, the constitutive expression of *VHb* did not improve the germination rate of barley.

Several studies have highlighted the role of oxylipins in the regulation of environmentally induced or developmental-specific processes (Weber, 2002). Oxylipins are a product of the lipoxygenase pathway. When barley *LIPOXYGENASE2* was over-expressed as a means of determining the effect of altering the oxylipin status, Sharma et al. (2006) were able to show that they act as regulators, possibly by enhancing the level of endogenous jasmonic acid.

The baking property of wheat flour is influenced largely by the quantity and quality of the endosperm storage proteins, but arabinoxylan, the major non-starch polysaccharide present in the flour, also has some influence. When Harholt et al. (2010) created transgenic wheat plants expressing an *A. niger* gene responsible for the synthesis of ferulic acid esterase, the resulting grains were shrivelled and their test weight was reduced by up to 50 per cent. The increased ferulic acid esterase activity in the transgenic grain produced a higher than wild type level of water non-extractable arabinoxylan in the cell wall, but the effect of this alteration on the baking property of the flour has yet to be determined. The same authors performed similar experiments using a *B. subtilis ENDO-XYLANASE* gene, the product of which is used as an additive in some commercial baked wheat products. Just as for the ferulic acid esterase grain, the transgenic grains were shrivelled and of smaller test weight than the wild type. In the cell walls of these transgenic materials, the arabinose to xylose ratio was increased by 10-15%, and the proportion of water-extractable arabinoxylan was increased by 50%; the molecular weight range of this water-extractable arabinoxylan was reduced from >85 kDa to 2-85 kDa. There may be some potential for this transgene in the use of wheat as a bioenergy crop.

The major classes of endosperm storage proteins in the *Triticeae* species grain are the albumins, the globulins and particularly the prolamins. Transgenic wheat expressing a pea *LEGUMIN A* gene under the control of an endosperm-specific promoter were studied by Stoeger et al. (2001) to determine whether this globulin protein would be correctly processed and form the hexameric structure which it adopts in the pea seed. An unexpected result was that the legumin was condensed within endosperm inclusion bodies, and eventually formed crystals. This led the authors to suggest this transgenic material as a suitable means of producing large quantities of pure 11S globulin protein.

5. Protein modifications

Several modifications occur during the processing of proteins; these include cleavage of signal peptides after entry into the ER, formation of disulphide bonds in the lumen of the rough ER, phosphorylation by protein kinases, and the attachment of sugar side chains (glycosylation) initiated in the ER but occurring primarily in the Golgi apparatus. These modifications can be an important determinant of a protein's stability and activity.

5.1 Disulfid bridges

The conformation of a protein is sequence-dependent. One of the primary determinants of folding is the formation of a disulphide bridge between pairs of thiol groups. Most prolamins contain a number of cysteine residues capable of forming such disulphide bonds.

The retention of a phaseolin γ-zein fusion protein in the ER of tobacco protoplasts was shown to be dependent on disulphide bonding (Pompa & Vitale, 2006). Prolamins are synthesized in the ER of the wheat and barley endosperm, and are then transported to protein storage vacuoles (PSVs) in a process thought to involve both Golgi-dependent and independent pathways (Galili et al., 1993; Levanony et al., 1992; Rechinger et al., 1993). Autophagy and the *de novo* formation of PSVs has also been reported to mediate the transport of prolamins to the PSVs in wheat (Levanony et al., 1992), but the molecular and cellular mechanisms underlying these routes remain unknown.

5.2 Glycosylation

More than 50% of eukaryotic proteins are glycosylated (Apweiler et al., 1999), with the sugar linked either to an asparagine (*N*-glycosylation) or to a serine or threonine (*O*-glycosylation) residue. The synthetic pathway of N-glycans is conserved among animals, plants and fungi (for a review, see Kukuruzinska & Lennon, 1998). The majority of mammalian N-glycans are terminated by Neu5Ac and other sialic acids linked to terminal β1,4- or β1,3-Gal residues. These negatively charged sugars affect the biological activity and half-life of many therapeutic glycoproteins (Erbayraktar et al., 2003; Schauer, 2000; Varki, 2007). The synthesis of complex N-glycans takes place in various compartments of the plant cell and has been recently reviewed in the context of therapeutic protein production by Gomord et al. (2010). Retention in the ER prevents the addition of xylose and fucose residues to a recombinant antibody (Sriraman et al., 2004) that limites its applications to some human antibodies or antigenes. In tobacco, the pattern of glycosylation depends on whether the antibody is expressed in the leaf or in the seed, a phenomenon explained by proposing that the transport pathways from the ER to the protein storage vacuole differ in these organs (Floss et al., 2009), as suggested by Vitale and Hinz (2005). In monocotyledonous species, as in dicotyledonous ones, leaves (Fitchette et al., 1999; Wilson et al., 1998) and roots (Mega, 2004; Wilson et al., 2001) produce both high-Mannose-type N-glycans and complex N-glycans containing β1,2-xylose, α1,3-fucose and terminal GlcNAc or Lea antennae. A similar structural glycoprotein diversity has also been described for the fruits of both monocotyledonous (Leonard et al., 2004) and dicotyledonous (Wilson et al., 2001) species. The N-glycosylation patterns of seed glycoproteins differ significantly between monocotyledonous and dicotyledonous species. In the former, there is a little, if any presence of terminal Lea antennae (Bardor et al., 2003; Leonard et al., 2004), whereas this structural element is common in the seed of buckwheat, walnut, hazelnut, peanut, pea and mung bean (Wilson et al., 2001).

6. Concluding remarks

This review has set out to summarize the information in the public domain regarding the use of *Triticeae* species for the heterologous production of valuable products. A number of plant species have been suggested as vehicles for molecular farming, but relatively little attention has been paid to this important group of crop species, perhaps because they have been regarded as rather difficult to transform and/or because expression systems are less developed than in more commonly used plants such as tobacco.

A number of challenges remain before plant-made pharmeceuticals (PMPs) can reach the market. A major one is the expense and low efficiency of target purification. The attachment of fungal hydrophobins, elastin-like polypeptides (ELPs) or the use of a domain of the maize

storage protein zein as a purification tag represents promising strategies. The principle behind these purification tagging approaches can be based on either a temperature dependent change in solubility (ELP) termed inverse transition cycling (Meyer & Chilkoti, 1999), on a change in hydrophobicity in the case of the hydrophobins (Linder et al., 2001), or on the assembly of the proteins into so-called protein bodies by the use of γ-zein (Coleman et al., 1996; Geli et al., 1994). Although inverse transition cycling has been used to purify cytokines (Lin et al., 2006), antibodies (Floss et al., 2009; Joensuu et al., 2009) and spider silk proteins (Scheller et al., 2004) from transgenic plants, no application has yet been reported in *Triticeae* species. The same applies also for hydrophobins. Recently Joensuu et al. (2010) showed that the transient expression of a hydrophobin-GFP fusion transgene increased the accumulation in the leaves of *N. benthamiana* and eased the purification of the product. The γ-zein protein induces the formation of ER-derived protein bodies (PBs) in the seed and some vegetative tissues in dicotyledonous transformants in the absence of other zein subunits (Coleman et al., 1996; Geli et al., 1994). This observation has been exploited in the development of the Zera® expression system by ERA Biotech (Barcelona, Spain), which is effective in a number of plant species (Ludevid Mugica et al., 2007, 2009; Saito et al., 2009; Torrent et al., 2009a, 2009b). A rather different system has been pioneered by ORF Genetics, in which a carbohydrate-binding domain is used to purify the target protein (Mantyla & Orvar, 2007).

A more inexpensive approach is possible where the whole seed (or grain) is a component of feed, since in this case no purification is necessary. Nevertheless it remains important that the PMP is stable under ambient temperature conditions for several weeks. The stability of an antibody in the wheat grain was already demonstrated a decade ago (Stoeger et al., 2001). Where the PMP is heat stable, then heat treatment during feed processing is possible (Horvath et al., 2000). Achieving an adequate level of expression is essential, one approach would be to lower the amount of endogenous storage proteins competing with the transgene. Such a strategy has been followed by ORF Genetics by the down regulation of an transcription factor (*Hv-HoxB4*) which specifically affects the expression of the barley *HorB* and *HorC* genes (Orvar, 2005).

Public acceptance of GM products and a straightforward means of their detection require the availability of clear markers. In barley it is possible to use testa colour for this purpose by conventionally transferring an exotic testa colour into a readily transformable cultivar, which then becomes suitable for the production of PMPs (Orvar, 2006). With the imminent acquisition of the genomic sequences of barley and wheat, it can be expected that the key genes for the synthesis and processing underlying the pattern of glycosylation of *Triticeae* proteins will soon be known. Progress towards establishing plants as a vehicle for the production of PMPs is likely to accelerate in the coming years.

7. References

Aphis. (March 2011). Release Permits for Pharmaceuticals, Industrials, Value Added Proteins for Human Consumption, or for Phytoremediation Granted or Pending by APHIS. In: USDA Animal and Plant Health Inspection Service, 28.03.2011, Available from: http://www.aphis.usda.gov/brs/ph_permits.html

Apweiler, R., Hermjakob, H. & Sharon, N. (1999). On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochimica et Biophysica Acta*, Vol.1473, No.1, (December 1999), pp. 4–8, ISSN 0304-4165

Bardor, M., Faveeuw, C., Fitchette, A.-C., Gilbert, D., Galas, L., Trottein, F., Faye, L. & Lerouge, P. (2003). Immunoreactivity in mammals of two typical plant glyco-epitopes, core alpha(1,3)-fucose and core xylose. *Glycobiology*, Vol.13, No.6, (June 2003), pp. 427–434, ISSN 0959-6658

Bartlett, J.G., Alves, S.C., Smedley, M., Snape, J.W. & Harwood, W.A. (2008). High-throughput *Agrobacterium*-mediated barley transformation. *Plant Methods*, 4:22 (26 September 2008), ISSN 1746-4811

Bayer Innovation GmbH. (September 2010). Plant made pharmaceuticals In: Bayer: Science for a better live, 06.04.2011, Available from: http://www.bayer-innovation.com/en/plant-made-pharmaceuticals.aspx

Behring, E. & Kitasato, S. (1890). Ueber das Zustandekommen der Diphtherieimmunität und der Tetanusimmunität bei Tieren. *Deutsche Medizinische Wochenschrift*, Vol.16, No.49, (December 1890), pp. 1113–1114, ISSN 0012-0472

Bewley, J.D. & Black, M. (1994). *Seeds – physiology of development and germination* (2nd edn.), Plenum Press, ISBN 0-306-44748-7, New York

Bommineni, V.R., Jauhar, P.P. & Peterson, T.S. (1997). Transgenic durum wheat by microprojectile bombardment of isolated scutella. *Journal of Heredity*, Vol.88, No.6, (November 1997), pp. 475–481, ISSN 0022-1503

Brinch-Pedersen, H., Olesen, A., Rasmussen, S.K. & Holm, P.B. (2000). Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an Aspergillus phytase. *Molecular Breeding*, Vol.6, No.2, (April 2000), pp. 195–206, ISSN 1380-3743

Brinch-Pedersen, H., Hatzack, F., Stoger, E., Arcalis, E., Pontopidan, K. & Holm, P.B. (2006). Heat-stable phytases in transgenic wheat (*Triticum aestivum* L.): deposition pattern, thermostability, and phytate hydrolysis. *Journal of Agricultural Food Chemistry*, Vol.54, No.13, (June 2006), pp. 4624–4632, ISSN 0021-8561

Castillo, A.M., Vasil, V. & Vasil, I.K. (1994). Rapid production of fertile transgenic plants of rye (*Secale cereale* L.). *Nature Biotechnology*, Vol.12, No.12, (December 1994), pp. 1366–1371, ISSN 1087-0156

Chauhan, H. & Khurana, P. (2010). Use of double-haploid technology for development of stable drought tolerant bread wheat (*Triticum aestivum* L.) transgenics. *Plant Biotechnology Journal*, Vol.9, No.3, (April 2011), pp. 408–417, ISSN 1467-7652

Cheng, M., Fry, J.E., Pang, S., Zhou, H., Hironaka, C., Duncan, D.R., Conner, T.W. & Wan, Y. (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiology*, Vol.115, No.3, (November 1997), pp. 971-980, ISSN 0032-0889

Coleman, C.E., Herman, E.M., Takasaki, K. & Larkins, B.A. (1996). The maize gamma-zein sequesters alpha-zein and stabilizes its accumulation in protein bodies of transgenic tobacco endosperm. *Plant Cell*, Vol.8, No.12, (December 1996), pp. 2335–2345, ISSN 1040-4651

Cui, C., Song, F., Tan, Y., Zhou, X., Zhao, W., Ma, F., Liu, Y., Hussain, J., Wang, Y., Yang, G. & He, G. (2011). Stable chloroplast transformation of immature scutella and inflorescences in wheat (*Triticum aestivum* L.). *Acta Biochimica et Biophysica Sinica*, Vol.43, No.4, (April 2011), pp. 284-291, ISSN 1745-7270

Daniell, H., Nameirakpam, D.S., Mason, H. & Streatfield, S.J. (2009). Plant-made vaccine antigens and biopharmaceuticals. *Trends in Plant Science*, Vol.14, No.12, (December 2009), pp. 669-679, ISSN 1360-1385

De Muynck, B., Navarre, C. & Boutry, M. (2010). Production of antibodies in plants : status after 20 years. *Plant Biotechnology Journal*, Vol.8, No.5, (June 2010), pp. 529-563, ISSN 1467-7652

Douchkov, D., Nowara, D., Zierold, U. & Schweizer, P. (2005). A high-throughput gene silencing system for the functional assessment of defense-related genes in barley epidermal cells. *Molecular Plant-Microbe Interactions*, Vol.18, No.8, (August 2005), pp. 755-761, ISSN 0894-0282

Dunwell, J.M. (2009). Transgenic Barley, Wheat and Oats: Future Prospects. In: *Methods in Molecular Biology. Transgenic Barley, Wheat and Oats*, Jones, H.D. & Shewry, P.R. (Eds.), pp. 333-345, Humana Press, ISBN 978-1-58829-961-1, New York

Erbayraktar S, Grasso G, Sfacteria A, Xie QW, Coleman T, Kreilgaard M, Torup L, Sager T, Erbayraktar Z, Gokmen N, et al. (2003). Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo. *Proceedings of the National Academy of Sciences USA*, Vol.100, No.11, (May 2003), pp. 6741–6746, ISSN 0027-8424

Erlendsson, L.S., Muench, M.O., Hellmann, U., Hrafnkelsdottir, S.M., Jonsson, A., Balmer, Y., Mäntylä, E. & Örvar, B.L. (2010). Barley as a green factory for the production of functional Flt3 ligand. *Biotechnology Journal*, Vol.5, No.2, (February 2010), pp. 163–171, ISSN 1860-7314

Eskelin, K., Ritala, A., Suntio, T., Blumer, S., Holkeri, H., Wahlström, E.H., Beaz, J., Mäkinen, K. & Nuutila, A.M. (2009). Production of a recombinant full-length collagen type I alpha-1 and of a 45-kDa collagen type I alpha-1 fragment in barley seeds. *Plant Biotechnology Journal*, Vol.7, No.7, (September 2009), pp. 657–672, ISSN 1467-7652

Faye, L.; Gomord, V. (2010). Success stories in molecular farming–a brief overview. *Plant Biotechnology Journal*, Vol.8, No.5, (May 2010), pp. 525–528, ISSN 1467-7652

Fischer, R. & Schillberg, S. (Eds.) (2004). *Molecular farming – plant-made pharmaceuticals and technical proteins.* WILEY-VCH Verlag GmbH & Co. KGaA, ISBN 978-3527307869, Weinheim

Fitchette, A.C., Cabanes-Macheteau, M., Marvin, L., Martin, B., Satiat-Jeunemaitre, B., Gomord, V., Croocks, K., Lerouge, P., Faye, L. & Hawes, C. (1999). Biosynthesis and immunolocalization of Lewis a-containing N-glycans in the plant cell. *Plant Physiology,* Vol.121, No.2, (October 1999), pp. 333–343, ISSN 0032-0889

Floss, D.M., Sack, M., Arcalis, E., Stadlmann, J., Quendler, H., Rademacher, T., Stoger, E., Scheller, J., Fischer, R. & Conrad, U. (2009). Influence of elastin-like peptide fusions on the quantity and quality of a tobacco-derived human immunodeficiency virus-neutralizing antibody. *Plant Biotechnology Journal*, Vol.7, No.9, (December 2009), pp. 899–913, ISSN 1467-7652

Galili, G., Altschuler, Y. & Levanony, H. (1993). Assembly and transport of seed storage proteins. *Trends in Cell Biology*, Vol.3, No.12, (December 1993), pp. 437–443, ISSN 0962-8924

Geli, M.I., Torrent, M. & Ludevid, D. (1994). Two structural domains mediate two sequential events in [Gamma]-zein targeting: protein endoplasmic reticulum retention and protein body formation. *Plant Cell*, Vol.6, No.12, (December 1994), pp. 1911–1922, ISSN 1040-4651

Gibbs, B.F., Alli, I. & Mulligan, C. (1996). Sweet and Taste-modifying Proteins: A Review. *Nutrition Research*, Vol.16, No.9, pp. 1619-1630, ISSN 0271-5317

Gomord, V., Fitchette, A.C., Menu-Bouaouiche, L., Saint-Jore-Dupas, C., Plasson, C., Michaud, D. & Faye, L. (2010). Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotechnology Journal*, Vol.8, No.5, (June 2010), pp. 564–587, ISSN 1467-7652

Green, C. (1999). Thaumatin: a natural flavour ingredient. In: *Low-Calorie Sweeteners: Present* and Future, Corti, A. (Ed.), pp. 129–32, Karger, ISBN 3805569386, Freiburg i.B.

Guglielminetti, L., Yamaguchi, J., Perata, P. & Alpi, A. (1995). Amylolytic activities in cereal seeds under aerobic and anaerobic conditions. *Plant Physiology*, Vol.109, No.3, (November 1995), pp. 1069–1076, ISSN 0032-0889

Harholt, J., Bach, I.C., Lind-Bouquin, S., Nunan, K.J., Madrid, S.M. & Brinch-Pederson, H. (2010). Generation of transgenic wheat (*Triticum aestivum* L.) accumulating heterologous endo-xylanase or ferulic acid esterase in the endosperm. *Plant Biotechnology Journal*, Vol.8, No.3, (April 2010), ISSN 1467-7652

Hiatt, A., Cafferkey, R. & Bowdish, K. (1989). Production of antibodies in transgenic plants. *Nature*, Vol.342, No.6245, (November 1989), pp. 76–78, ISSN 0028-0836

Hensel, G., Valkov, V., Middlefell-Williams, J. & Kumlehn, J. (2008). Efficient generation of transgenic barley: the way forward to modulate plant-microbe interactions. *Journal of Plant Physiology*, Vol.165, No.1, (January 2008), pp. 71-82, ISSN 0176-1617

Hensel, G., Kastner, C., Oleszczuk, S., Riechen, J. & Kumlehn, J. (2009). *Agrobacterium*-mediated gene transfer to cereal crop plants: Current protocols for barley, wheat, triticale and maize. *International Journal of Plant Genomics*, Article ID 835608, 9 pages. doi:10.1155/2009/835608, ISSN 1687-5370

Hensel, G., Himmelbach, A., Chen, W., Douchkov, D.K. & Kumlehn, J. (2011). Transgene expression systems in the *Triticeae* cereals. *Journal of Plant Physiology*, Vol.168, No.1, (January 2011), pp. 30–44, ISSN 0176-1617

Holme, I.B., Brinch-Pedersen, H., Lange, M. & Holm, P.B. (2006). Transformation of barley (*Hordeum vulgare* L.) by *Agrobacterium tumefaciens* infection of in vitro cultured ovules. *Plant Cell Reports*, Vol.25, No.12, (December 2006), pp. 1325-1335, ISSN 0721-7714

Holme, I.B., Brinch-Pedersen, H., Lang, M. & Holm, P.B. (2008). Transformation of different barley (*Hordeum vulgare* L.) cultivars by *Agrobacterium tumefaciens* infection of in vitro cultured ovules. *Plant Cell Reports*, Vol.27, No.12, (December 2008), pp. 1833-1840, ISSN 0721-7714

Hood, E.E., Witcher, D.R., Maddock, S., Meyer, T. & Baszczynski, C. et al. (1997). Commercial production of avidin from transgenic maize: characterization of transformant, production, processing and purification. *Molecular Breeding*, Vol.3, No.4, (August 1997), pp. 291–306, ISSN 1380-3743

Horvath, H., Huang, J., Wong, O., Kohl, E., Okita, T., Kannangara, C.G. & von Wettstein, D. (2000). The production of recombinant proteins in transgenic barley grains. *Proceedings of the National Academy of Sciences USA*, Vol.97, No.4, (February 2000), pp. 1914-1919, ISSN 0027-8424

Huang, N., Rodriguez, R.L. & Hagie, F. (2006). Expression of human milk proteins in transgenic plants. US6,991,824. US patent

Huang, N., Rodriguez, R.L. & Hagie, F.E. (2010). Expression of human milk proteins in transgenic plants. US7,718,851, US patent

Ihlow, A., Schweizer, P. & Seiffert, U. (2008). A high-throughput screening system for barley/powdery mildew interactions based on automated analysis of light micrographs. *BMC Plant Biology*, 8:6 (23 January 2008), ISSN 1471-2229

Joensuu, J. J., Kotiaho, M., Teeri, T. H., Valmu, L., Nuutila, A. M., Oksman-Caldentey, K. M. & Niklander-Teeri, V. (2006). Glycosylated F4 (K88) fimbrial adhesin FaeG expressed in barley endosperm induces ETEC-neutralizing antibodies in mice. *Transgenic Research*, Vol.15, No.4, (June 2006), pp. 359–373, ISSN 0962-8819

Joensuu, J.J., Brown, K., Conley, A,J., Clavijo, A., Menasse, R. & Brandle, J.E. (2008). Plant recombinant antibodies for the prevention of foot-and-mouth disease virus. *In Vitro Cellular & Developmental Biology-Plant*, Vol.44, No.4, (August 2008), pp. 349-350, ISSN 1054-5476

Joensuu, J.J., Brown, K.D., Conley, A.J., Clavijo, A., Menassa, R. & Brandle, J.E. (2009). Expression and purification of an anti-foot-and-mouth disease virus single chain variable antibody fragment in tobacco plants. *Transgenic Research*, Vol.18, No.5, (October 2009), pp. 685–696, ISSN 0962-8819

Joensuu, J.J., Conley, A.J., Lienemann, M., Brandle, J.E., Linder, M.B. & Menassa, R. (2010). Hydrophobin fusions for high-level transient protein expression and purification in *Nicotiana benthamiana*. *Plant Physiology*, Vol.152, No.2, (February 2010), pp. 622–633, ISSN 0032-0889

Kamenarova, K., Gecheff, K., Stoyanova, M., Muhovski, Y., Anzai, H. & Atanassov, A. (2007). Production of human lactoferrin in trangenic barley. *Biotechnology & Biotechnological Equipment*, Vol.21, No.1, (January 2007), pp. , ISSN 1310-2818

Knudsen, S. & Müller, M. (1991). Transformation of the developing barley endosperm by particle bombardment. *Planta*, Vol.185, No.3, (October 1991), pp. 330-336, ISSN 0032-0935

Kukuruzinska, M.A. & Lennon, K. (1998). Protein N-glycosylation: molecular genetics and functional significance. *Critical Reviews in Oral Biology and Medicine*, Vol.9, No.4, pp. 415–448, ISSN 1045-4411

Kumlehn, J., Serazetdinova, L., Hensel, G., Becker, D. & Loerz, H. (2006). Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*. *Plant Biotechnology Journal*, Vol.4, No.2, (March 2006), pp. 251-261, ISSN 1467-7652

Kumlehn, J. & Hensel, G. (2009). Genetic transformation technology in the *Triticeae*. *Breeding Science*, Vol.59, No.5, (December 2009), pp. 553–60, ISSN 1347-3735

Lantzsch, H.J., Hillenbrand, S., Scheuermann, S.E. & Menke, K.H. (1992). Comparative study of phosphorus utilization from wheat, barley and corn diets by young rats and

pigs. *Journal of Animal Physiology and Animal Nutrition*, Vol.67, No.1-5, (January-August 1992), pp. 123–132, ISSN 1439-0396

Leonard, R., Kolarisch, D., Paschinger, K., Altmann, F. & Wilson, I.B.H. (2004). A genetic and structural analysis of the N-glycosylation capabilities of rice and other monocotyledons. *Plant Molecular Biology*, Vol.55, No.5, (July 2004), pp. 631–644, ISSN 0167-4412

Levanony, H., Rubin, R., Altschuler, Y. & Galili, G. (1992). Evidence for a novel route of wheat storage proteins to vacuoles. *Journal of Cell Biology*, Vol.119, No.5, (December 1992), pp. 1117-1128, ISSN 0021-9525

Lin, M., Rose-John, S., Grotzinger, J., Conrad, U. & Scheller, J. (2006). Functional expression of a biologically active fragment of soluble gp130 as an ELP-fusion protein in transgenic plants: purification via inverse transition cycling. *Biochemical Journal*, Vol.398, No.3, (September 2006), pp. 577-583, ISSN 0264-6021

Linder, M., Selber, K., Nakari-Setälä, T., Qiao, M., Kula, M.R. & Penttilä, M. (2001). The hydrophobins HFBI and HFBII from *Trichoderma reesei* showing efficient interactions with nonionic surfactants in aqueous two-phase systems. *Biomacromolecules*, Vol.2, No.2, (June 2001), pp. 511–517, ISSN 1525-7797

Lott, J.N.A. (1984). Accumulation of seed reserves of phosphorus and other minerals. In: *Seed Physiology*, Murray, D.R. (Ed.), pp. 139–166, Academic Press, ISBN 0125119011, New York

Ludevid Mugica, M.D., Bastida Virgili, M., Llompart Royo, B., Marza´ bal Luna, P. & Torrent Quetglas, M. (2007). Production of proteins. EP1819725. European patent application.

Ludevid Mugica, M.D., Torrent Quetglas, M. & Ramassamy, S. (2009). Production of peptides and proteins by accumulation in plant endoplasmic reticulum-derived protein bodies. US7,575,898. US patent.

MagnICON, Icon Genetics. *magnICON*: The desktop system for protein expression. In: Icon Genetics, 06.04.2011, Available from: http://www.icongenetics.com/html/tech3_3a.htm

Ma, J.K.C., Hikmat, B.Y., Wycoff, K., Vine, N.D., Chargelegue, D., Yu, L., Hein, M.B. & Lherner, T. (1998). Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. *Nature Medicine*, Vol.4, No.5, (May 1998), pp. 601–606, ISSN 1078-8956

Ma, J.K., Drake, P.M., Chargelegue, D., Obregon, P. & Prada, A. (2005). Antibody processing and engineering in plants, and new strategies for vaccine production. *Vaccine*, No.23, Vol.15, (March 2005), pp. 1814–1818, ISSN 0264-410X

Maltagen Forschung GmbH. Transgenic barley. In: Maltagen Forschung GmbH, 06.04.2011, Available from: http://www.maltagen.de/en_index.html

Mantyla, E. & Orvar, B.L. (2007). Non-denaturing process to purify recombinant proteins from plants. US2007/0169223 A1, US patent application

Mega, T. (2004). Conversion of the carbohydrate structures of glycoproteins in roots of Raphanus sativus using several glycosidase inhibitors. *Journal of Biochemistry*, Vol.136, No.4, (October 2004), pp. 525–531, ISSN 0021-924X

Meyer, D.E. & Chilkoti, A. (1999). Purification of recombinant proteins by fusion with thermally-responsive polypeptides. *Nature Biotechnology*, Vol.17, No.11, (November 1999), pp. 1112–1115, ISSN 1087-0156

Murray, F., Brettell, R., Matthews, P., Bishop, D. & Jacobsen, J. (2004). Comparison of *Agrobacterium-mediated* transformation of four barley cultivars using the *GFP* and *GUS* reporter genes. *Plant Cell Reports*, Vol.22, No.6, (January 2004), pp. 397-402, ISSN 0721-7714

Nadolska-Orczyk, A., Przetakiewicz, A., Kopera, K., Binka, A. & Orczyk, W. (2005). Efficient method of *Agrobacterium*-mediated transformation for triticale (*x Triticosecale* Wittmack). *Journal of Plant Growth Regulation*, Vol.24, No.1, (March 2005), pp. 2–10, ISSN 0721-7595

Nelson, T.S., Shieh, T.R., Wodzinski, R.J. & Ware, J.H. (1968). The availability of phytate phosphorus in soya bean meal before and after treatment with a mould phytase. *Poultry Science*, Vol.47, pp. 1842–1848, ISSN 1525-3171

Nelson, T.S., Shieh, T.R., Wodzinski, R.J. & Ware, J.H. (1971). Effects of supplemental phytase on the utilisation phytate phosphorus by chicks. *Journal of Nutrition*, Vol.101, No.10, (October 1971), pp. 1289–1294, ISSN 0022-3166

Onate, L., Vicente-Carbajosa, J., Lara, P., Diaz, I. & Carbonero, P. (1999). Barley BLZ2, a seed-specific bZIP protein that interacts with BLZ1 in vivo and activates transcription from the GCN4-like motif of B-hordein promoters in barley endosperm. *Journal of Biological Chemistry*, Vol.274, No.14, (April 1999), pp. 9175-9182, ISSN 0021-9258

ORF Genetics. ISOkine, DERMOkine, The Orfeus™ expression system. In: ORF Genetics, 06.04.2011, Available from: http://www.orfgenetics.com/

Orvar, B.L. (2005). Enhancing accumulation of heterologous polypeptides in plant seeds through targeted suppression of endogenous storage proteins. WO 2005/021765, patent

Orvar, B.L. (2006). Traceability of transgenic plant seeds in upstream and downstream processing. WO 2006/016381, patent

Pompa, A. & Vitale, A. (2006). Retention of a bean phaseolin/maize gamma-zein fusion in the endoplasmic reticulum depends on disulfide bond formation. *Plant Cell*, Vol.18, No.10, (October 2006), pp. 2608-2621, ISSN 1040-4651

Popelka, J.C. & Altpeter, F. (2003). *Agrobacterium tumefaciens*-mediated genetic transformation of rye (*Secale cereale* L.). *Molecular Breeding*, Vol.11, No.3, (April 2003), pp. 203–211, ISSN 1380-3743

Raboy, V. (1990). Biochemistry and genetics of phytic acid synthesis. In: *Inositol Metabolism in Plants*, Morré D.J., Boss, W.F., Loewus, F.A. (Eds.), pp. 55–76, Wiley-Liss, ISBN 0471567086, New York

Rechinger, K.B., Simpson, D.J., Svendsen, I. & Cameronmills, V. (1993). A role for gamma-3 hordein in the transport and targeting of prolamin polypeptides to the vacuole of developing barley endosperm. *Plant Journal*, Vol.4, No.5, (November 1993), ISSN 0960-7412

Rubio-Somoza, I., Martinez, M., Diaz, I. & Carbonero, P. (2006). HvMCB1, a R1MYB transcription factor from barley with antagonistic regulatory functions during seed

development and germination. *Plant Journal*, Vol.45, No.1, (January 2006), pp. 17-30, ISSN 1365-313X

Ritala, A., Wahlström, E., Holkeri, H., Hafren, A., Mäkeläinen, K., Baez, J., Mäkinen, K. & Nuutila, A.-M. (2008). Production of a recombinant industrial protein using barley cell cultures. *Protein Expression and Purification*, Vol.59, No.2, (June 2008), pp. 274–281, ISSN 1046-5928

Saito, Y., Kishida, K., Takata, K., Takahashi, H., Shimada, T., Tanaka, K., Morita, S., Satoh, S. & Masumura, T. (2009). A green fluorescent protein fused to rice prolamin forms protein body-like structures in transgenic rice. *Journal of Experimental Botany*, Vol.60, No.2, (February 2009), pp. 615–627, ISSN 0022-0957

Schauer, R. (2000). Achievements and challenges of sialic acid research. *Glycoconjugate Journal*, Vol.17, No.7-9, (Juli 2000), pp. 485–499, ISSN 0282-0080

Scheller, J., Henggeler, D., Viviani, A. & Conrad, U. (2004). Purification of spider silk-elastin from transgenic plants and application for human chondrocyte proliferation. *Transgenic Research*, Vol.13, No.1, (February 2004), pp. 51–57, ISSN 0962-8819

Schillberg, S., Zimmermann, S., Voss, A. & Fischer, R. (1999). Apoplastic and cytosolic expression of full-size antibodies and antibody fragments in Nicotiana tabacum. *Transgenic Research*, Vol.8, No.4, (August 1999), pp. 255–263, ISSN 0962-8819

Schuenmann, P.H,D., Coia, G. & Waterhouse, P.M. (2002). Biopharming the SimpliRED™HIV diagnostic reagent in barley, potato and tobacco. *Molecular Breeding*, Vol.9, No.2, (June 2002), pp. 113–121, ISSN 1380-3743

Sharma, V.K., Monostori, T., Gobel, C., Hansch, R., Bittner, F., Wasternack, C., Feussner, I., Mendel, R.R., Hause, B. & Schulze J. (2006). Transgenic barley plants overexpressing a 13-lipoxygenase to modify oxylipin signature. *Phytochemistry*, Vol.67, No.3, (February 2006), pp. 264-276, ISSN 0031-9422

Sriraman, R., Bardor, M., Sack, M., Vaquero, C., Faye, L., Fischer, R., Finnern, R. & Lerouge, P. (2004). Recombinant anti-hCG antibodies retained in the endoplasmic reticulum of transformed plants lack core-xylose and core-alpha(1,3)-fucose residues. *Plant Biotechnology Journal*, Vol.2, No.4, (July 2004), pp. 279–287, ISSN 1467-7652

Stahl, R., Horvath, H., Van Fleet, J., Voetz, M., von Wettstein, D. & Wolf, N. (2002). T-DNA integration into the barley genome from single and double cassette vectors. *Proceedings of the National Academy of Sciences USA*, Vol.99, No.4, (February 2002), pp. 2146-2151, ISSN 0027-8424

Stahl, R., Luhrs, R. & Dargatz, H. (2009). Thaumatin from transgenic barley. US 2009/0031458, US patent application

Stoger, E., Vaquero, C., Torres, E., Sack, M., Nicholson, L., Drossard, J., et al. (2000). Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Molecular Biology*, Vol.42, No.4, (March 2000), pp. 583–590, ISSN 0167-4412

Stoger, E., Parker, M., Christou, P. & Casey, R. (2001). Pea legumin overexpressed in wheat endosperm assembles into an ordered paracrystalline matrix. *Plant Physiology*, Vol.125, No.4, (April 2001), pp. 1732–1742, ISSN 0032-0889

Streatfield, S.J. & Howard, J.A. (2003). Plant-based vaccines. *International Journal of Parasitology*, Vol.33, No.5-6, (May 2003), pp. 479–493, ISSN 0020-7519

Tingay, S., McElroy, D., Kalla, R., Fieg, S., Wang, M., Thornton S. & Brettell, R. (1997). *Agrobacterium tumefaciens*-mediated barley transformation. *Plant Journal*, Vol.11, No.6, (June 1997), pp. 1369-1376, ISSN 1365-313X

Torrent, M., Llompart, B., Lasserre-Ramassamy, S., Llop-Tous, I., Bastida, M., Marzabal, P., Westerholm-Parvinen, A., Saloheimo, M., Heifetz, P.B. & Ludevid, M.D. (2009a). Eukaryotic protein production in designed storage organelles. *BMC Biology*, Vol.7, 5, (January 2009), ISSN 1741-7007

Torrent, M., Llop-Tous, I. & Ludevid, M.D. (2009b). Protein body induction: a new tool to produce and recover recombinant proteins in plants. In: *Recombinant Proteins From Plants Methods and Protocols*, L. Faye and V. Gomord, (Eds.), pp. 193–208, Humana Press, ISBN 978-1-58829-978-9, New York, USA

Usayran, N. & Balnave, D. (1995). Phosphorus requirements of laying hens fed on wheat-based diets. *British Poultry Science*, Vol.36, No.2, pp. 285–301, ISSN 0007-1668

Vasil, V., Castillo, A.M., Fromm, M.E. & Vasil, I.K. (1992). Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. *Nature Biotechnology*, Vol.10, No.6, (June 1992), pp. 667-674, ISSN 1087-0156

Varki, A. (2007). Glycan-based interactions involving vertebrate sialic-acidrecognizing proteins. *Nature*, Vol.446, No.7139, (April 2007), pp. 1023–1029, ISSN 0028-0836

Ventria Biosciences. (2011). Ventria Bioscience Products. In: Ventria Bioscience, 06.04.2011, Available from: http://www.ventria.com/products/

Vickers, C.E., Xue, G. & Gresshoff, P.M. (2006). A novel cis-acting element, ESP, contributes to high-level endosperm-specific expression in an oat globulin promoter. *Plant Molecular Biology*, Vol.62, No.1-2, (September 2006), pp 195–214, ISSN 0167-4412

Vitale, A. & Hinz, G. (2005). Sorting of proteins to storage vacuoles: how many mechanisms? *Trends in Plant Science*, Vol.10, No.7, (July 2005), pp. 316-323, ISSN 1360-1385

Wan, Y. & Lemaux, P.G. (1994). Generation of large numbers of independently transformed fertile barley plants. *Plant Physiology,* Vol.104, No.1, (January 1994), pp. 37-48, ISSN 0032-0889

Weber, H. (2002). Fatty acid derived signals in plants. *Trends in Plant Science*, Vol.7, No.5, (May 2002), pp. 217–224, ISSN 1360-1385

Wilhelmson, A., Laitila, A., Vilpola, A., Olkku, J., Kotaviita, E., Fagerstedt, K. & Home, S. (2006). Oxygen deficiency in barley (*Hordeum vulgare*) grain during malting. *Journal of Agricultural Food Chemistry*, Vol.54, No.2, (January 2006), pp. 409–416, ISSN 0021-8561

Wilhelmson, A., Laitila, A., Vilpola, A., Olkku, J., Kotaviita, E., Fagerstedt, K. & Home, S. (2007). Oxygen deficiency in barley (*Hordeum vulgare*) grain during malting. *Journal of Agricultural Food Chemistry*, Vol.54, No.2, (January 2007), pp. 409–416, ISSN 0021-8561

Wilson, I.B.H., Harthill, J.E., Mullin, N.P., Ashford, D.A. & Altmann, F. (1998). Core alpha 1,3-fucose is a key part of the epitope recognized by antibodies reacting against plant N-linked oligosaccharides and is present in a wide variety of plant extracts. *Glycobiology*, Vol.8, No.7, (July 1998), pp. 651–661, ISSN 0959-6658

Wilson, I.B.H., Zeleny, R., Kolarich, D., Staudacher, E., Stroop, C.J.M., Kamerling, J.P. & Altmann, F. (2001). Analysis of Asn-linked glycans from vegetable foodstuffs: widespread occurence of Lewis a, core alpha 1,3 fucose and xylose substitutions. *Glycobiology*, Vol.11, No.4, (April 2001), pp. 261–274, ISSN 0959-6658

Woof, J. & Burton, D. (2004). Human antibody-Fc receptor interactions illuminated by crystal structures. *Nature Reviews Immunology*, Vol.4, No2, (February 2004), pp. 89–99, ISSN 1474-1733

Zimmermann, J., Saalbach, I., Jahn, D., Giersberg, M., Haehnel, S., Wedel, J., Macek, J., Zoufal, K., Glünder, G., Falkenburg, D., & Kipriyanov, S.M. (2009). Antibody expressing pea seeds as fodder for prevention of gastrointestinal parasitic infections in chickens. *BMC Biotechnology*, 9:79 (11 September 2009), ISSN 1472-6750

Zimny, J., Becker, D., Brettschneider, R. & Lörz, H. (1995). Fertile transgenic Triticale (*xTriticosecale* Wittmack). *Molecular Breeding*, Vol.1, No.2, (June 1995), pp. 155-164, ISSN 1380-3743

10

Genetic Transformation of Forest Trees

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1. Introduction

Forests are complex ecosystems capable of providing a wide range of economic, social and environmental benefits. They help to regulate the water cycle, stabilize soils and moderate the climate of the Earth by absorbing and storing carbon dioxide. In addition to these environmental services, forests provide habitat to numerous species and are an important source of food, medicines and wood for humankind. In many countries, forests contribute importantly to their economic and social development through employment, the production and trade of forest products, and the protection and hosting of sites and landscapes of high cultural, spiritual or recreational value.

As a result of the increase in human population and economic activities, larger volumes of forest products, particularly wood, were required, and the natural processes were insufficient to restore the damage imposed on the forests. At present, the total forest area in the world is estimated to be nearly 4,000 million hectares, which cover about 30 percent of the global land area. Although the forest cover is still extensive, the problem of deforestation continues at an alarming rate: according to the Food and Agriculture Organization of the United Nations (FAO), in the 15 years from 1990 to 2005 the world lost 3 percent of its total forest area, representing an average decline of about 0.2 percent per year (FAO, 2007). Deforestation results from a combination of factors, including, among others, increased global demand for forest products, land-use change (e.g. conversion of forest into agriculture land) and the expansion of urban areas. A reduction in forest area can also happen through natural disasters, but deforestation is by far the most important cause of forest loss (FAO, 2007). Deforestation rates differ considerably from region to region, and figure 1 presents estimate data that illustrate the extent of losses and gains of forest land area around the world in the last two decades. It is interesting to note that deforestation is preponderant in developing countries in Latin America (with the notable exception of Cuba), Africa and Southeast Asia. The primary causes of this is the land-use change to agriculture and the production of fuelwood and charcoal through processes that are inefficient and lead to over-exploitation. Between years 2000 and 2010, however, net loss of forest area in the world decreased slightly, which is probably an indicator of the natural expansion of forests primarily due to efforts made to ensure the conservation of

biodiversity, the improvement in the establishment of forest plantations, and the genetic improvement of forest species. China, the United States and several European countries have increased their forest area mainly through the establishment of plantations, which may help reduce the harvest pressure on wild forests (FAO, 2010).

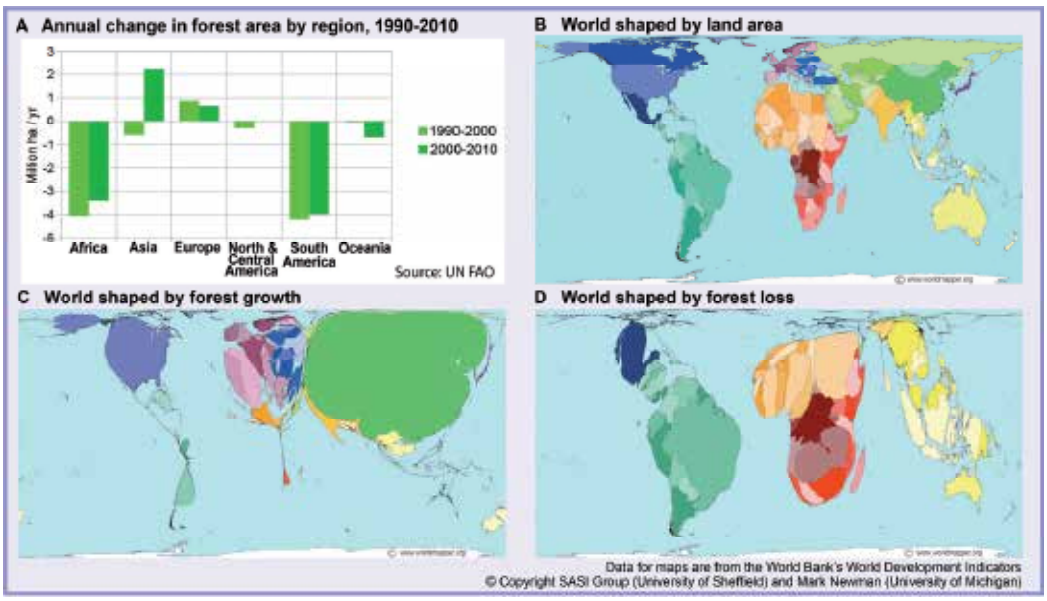

Fig. 1. Extent of forest loss and gain in the world. Territory size in C and D shows the proportion of worldwide net forest growth and loss that occurred there between 1990 and 2000.

2. Traditional tree breeding

Wood possesses physical, chemical and structural properties which have made it valuable to humanity since the earliest prehistoric times and is a renewable resource vital to the actual world economy. During the past century forestry underwent a major transition from foraging to an agricultural cropping mode on a global scale (Sedjo, 2003). The current level of demand for wood is probably exceeding what forests can supply, and this is clearly a major factor in their degradation. The establishment of plantations that can efficiently supply wood and paper pulp products and hence reduce demand for timber from virgin forests is a potential method for decreasing deforestation rates. For plantations to meet this demand, however, they must be much more productive than natural forests, thus it is essential to generate forest tree breeding programs for the selection of genetically superior individuals from large populations and their multiplication through the use of seeds or by asexual propagation (Fenning & Gershenzon 2002).

Morphological and physiological traits directly related to the tree architecture, and ultimately to productivity, such as height, diameter, branch thickness or bifurcation frequency, are typical targets for genetic improvement. Breeding programs also incorporate various characteristics that enable trees to withstand a number of environmental factors, like resistance to pests, diseases, drought and other biotic and abiotic stresses (Cornelius, 1998;

Martinez-Ruiz et al., 2003). Evidently, the physical, mechanical and chemical properties, which strongly influence the suitability of wood for its direct use as timber or as a supply for the pulp and paper industry, are also relevant targets for tree improvement. The most important among these properties are wood density, lignin content, dimensional stability (shrinkage and swelling), bending and compression strength and growth stresses (Malan et al., 1996; Pryor & Willing, 1983; Raymond & Apiolaza, 2004; Turner, 2001; Zobel, 1992). More specific features, such as the content of resin and essential oils or the production of secondary metabolites are also traits of economic importance in forestry.

For the development of a forest tree breeding program, it is essential that genetic variability occurs among individuals within a population. One or more characteristics can be modified in the average population by selection and multiplication of phenotypically superior individuals (Cornelius, 1998). Since the observed variability may be due not only to the genetic background, but also to the effect of the environment, one of the major challenges in all breeding programs is to recognize and effectively separate one from the other.

A tree improvement program involves the selection of the most desirable trees from natural stands or plantations, breeding or mating of these elite trees and testing the resulting progeny. This three-step process is then continuously repeated to increase the extent to which each generation (breeding generation) exhibits the desirable traits, that is, to increase the genetic gain.

Selection of the raw material

Seed stands are an important seed source for tree breeding programs. These are groups of trees identified as having superior characteristics that are formed from either natural forests or established plantations. Although seed can be obtained from natural stands, selection is more effective in plantations, since the variation due to environmental effects is less in the latter. The development of seed stands involves the selection of plantings or natural stands with an above the average phenotypic quality within the ecological zone, the improvement of the stand by removing undesirable individuals, their management to encourage early and abundant production of seed and the application of measures to reduce contamination by pollen from foreign trees, lowering the possibility of developing local breeds adapted to the site of introduction. They represent a stage prior to the formation of seed orchards and are generally not subjected to progeny testing, thus their true genetic value is not known (Lantz, 2008; Niembro, 1985; Quijada, 1980).

Breeding and propagation of the selected genotypes

Replication of the phenotypicaly superior trees is performed in a seed orchard environment. In contrast to seed stands, seed orchards are established from the outset for the specific purpose of seed production (Zobel & Talbert, 1988). They usually consist of families of superior genetic quality which are isolated to avoid or reduce pollination from external sources. Seed orchards are managed intensively to produce abundant seeds with the highest genetic gain in a short period of time. They have helped to achieve significant improvements in aspects such as tree shape, adaptability, disease resistance, growth and wood quality (Quijada, 1980).

Progeny tests

The artificial selection of trees with desirable phenotypic characteristics is assisted by progeny tests. These tests are used to estimate the genetic value of the parent trees based on the behavior of their progeny. Offspring from the selected parents are planted in

randomized, replicated tests usually established in different years and locations. Parents whose progeny perform better, on the average across all tests, are considered genetically superior. The recombinative fitness of the parents, their specific combining ability and the heritability of certain traits can be assessed through these tests (Quijada, 1980a; Roulund & Olesen, 1992; Zobel & Talbert, 1988).

These traditional practices in forestry and tree improvement remain relevant to forestry and the existing conventional programs are limited by the long reproductive cycle, long juvenile period (up to 20 years), low fertility, high levels of heterozygosity, various levels of ploidy, polyembryony, complex intraspecific incompatibility relationships, severe inbreeding depression, and the difficulty to effectively distinguish between phenotypic expression and environmental effects. Regarding the time factor, depending on the species, 5 to 20 years would be necessary for a tree to reach the reproductive maturity, 10 to 100 years to produce a marketable crop and 8 to 10 years to complete a breeding cycle (Lantz, 2008). For that reason, biotechnology plays an important role as a potential tool for the improvement of trees in much less time than was previously needed, either directly with genetic engineering, or by other procedures developed using the knowledge generated.

Biotechnology is a collection of various disciplines, including conventional methods of breeding and cultivation, which allow the management of biological systems for human benefit. Biotechnological methods are being developed worldwide to complement conventional breeding programs in commercial forests, in order to obtain continuous increases in production without increasing the land use (Burdon, 1994; Gomez-Lim & Litz 2004). Besides, the new era of biotechnology offers techniques that overcome the biological barriers that are common in the woody species. These techniques include: *in vitro* cultivation of cells and tissues, genotypic selection, genetic engineering, and molecular markers.

3. Genetic transformation

Innovations in the propagation methods and the introduction of fast-growing exotic species have increased industrial wood production and even reshaped regional and international patterns of generation and trade of forest products. Additionally, industrial forestry is advancing on two fronts with achievements in tree improvement as a result of traditional breeding techniques and with important research efforts oriented towards the production and commercialization of transgenic trees (Fenning & Gershenzon, 2002). Endogenous genes already present in the tree genome can be modified to improve certain traits, such as fiber quality and quantity, while exogenous genes can be transferred from unrelated organisms to confer entirely novel traits, such as resistance to herbicides, diseases or pests. Although most of the productivity gains to date have been accomplished largely by traditional selection and breeding, transgenic trees are becoming increasingly common worldwide. Genetically modified (GM) trees can potentially make the breeding results observable more rapidly and reduce the develoment times, thus increasing productivity in plantations and reducing the exploitation pressure on natural forests (Fenning & Gershenzon, 2002; van Frankenhuyzen & Beardmore, 2004).

3.1 Biotechnological tools for the genetic transformation of trees

Compared to genetic transformation of bacteria, where the transgene is integrated into a single cell and then it passes to the next generation, the genetic transformation of forest trees is not an easy task to achieve because it requires the establishment of protocols for the

regeneration of whole plants from individual cells (Figure 2). Other problematic barriers to the genetic improvement of trees, either by traditional breeding or genetic transformation, are their large size and long breeding cycles. The foreign DNA can be introduced using different approaches, and the following is a description of the techniques that have been used in different studies performed in the field of genetic transformation of forest trees (see also Table 1).

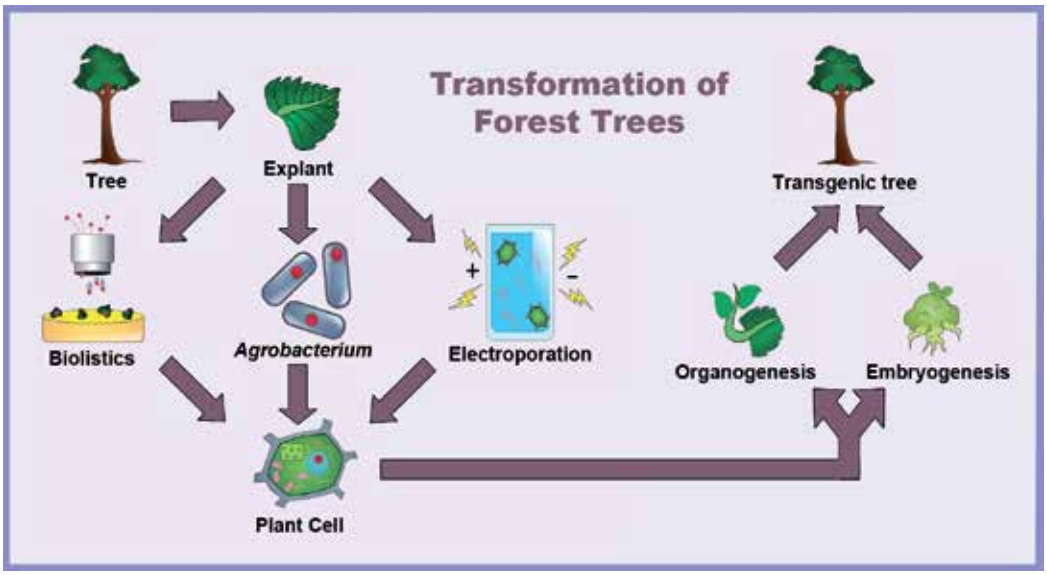

Fig. 2. Schematic diagram of the tree transformation process.

Agrobacterium-mediated gene transfer

Among the several techniques used for transformation, *Agrobacterium* is the most widely used transformation tool, accounting for 80% of the transgenic plants produced so far (Broothaerts et al., 2005). *Agrobacteria* are plant pathogenic organisms capable of infecting a wide variety of dicotyledonous species causing tumoric diseases on infected host plants. *Agrobacterium tumefaciens* and *A. rhizogenes* harbor an extra-chromosomal genetic component, called the Ti (for tumor-inducing) or Ri (for root-inducing) plasmid. During infection, *Agrobacterium* inserts a region of this plasmid, known as the T-DNA (for transferred-DNA), into plant cells, and this DNA fragment is then integrated into the plant chromosomes. The T-DNA contains genes which expression causes aberrant growth of plant cells through the synthesis of growth hormones. These genes can be replaced by any gene(s) of interest without loss of DNA transfer and integration functions. In this way, these novel genes will be transferred to plant cells during *A. tumefaciens* infection (reviewed by Gelvin, 2003).

For the efficient production of transgenic plants the optimization of the *Agrobacterium*-plant interaction is probably the most important aspect to be considered. The use of this system is restricted by the host-range of these bacteria, since some plant species are not susceptible to the infection. Conifers are more difficult to transform with *A. tumefaciens* compared to hardwood species, and mature tissues are in general more recalcitrant to the infection. In addition, even though the DNA transfer has been demonstrated in several woody plants, in

Species	Technique	Vector	Transgen	Plant regeneration	Reference
Pinus radiata	Biolistic transformation	pRC 101	*nptII* and *uidA* genes	Yes (Embryogenesis)	Walter *et al*. 1998
Pinus radiata	Biolistic transformation	pMYC3425+pRN2 or pMYC3425+pCW132 or pMYC3425+pAW16	*cry*1Ac and *npt*II or *cry*1Ac and *npt*II or *cry*1Ac, *npt*II and *uid*A genes respectively	Yes (Embryogenesis)	Grace *et al*. 2005
Quercus suber L.	Cocultivation with *Agrobacterium tumefaciens*	pBINUbiGUSINT	*nptII* and uidA genes	Yes (Embryogenesis)	Álvarez & Ordás, 2007
Picea abies [L.] Karst	Biolistic transformation	pASCCR-BAR	*ccr* gen fused in antisense orientation	Yes (Embryogenesis)	Wadenbäck *et al*., 2008
Paulownia elongata S.Y. Hu	Biolistic transformation	pBI121	*nptII* and *gus* genes	Yes (Organogenesis)	Castellanos-Hernández *et al.* 2009
Castanea dentata	Cocultivation with *Agrobacterium tumefaciens*	pCAMBIA 2301	*nptII* and *uidA* genes	Yes (Embryogenesis)	Andrade *et al.* 2009
Populus tremula x *Populus tremuloides* *Populus tremula* x *Populus alba*	Cocultivation with *Agrobacterium tumefaciens*	pG3KGB and pG3MKGB,	*nptII, gfp* and *bar* genes	Yes (Organogenesis)	Li J. *et al.* 2009
Populus alba x *Populus berolinensis*	Cocultivation with *Agrobacterium tumefaciens*	pROK2	*nptII* and *JERFs* genes	Yes (Organogenesis)	Li. Y. *et al*. 2009
Leucaena leucocephala	Cocultivation with *Agrobacterium tumefaciens*	pCAMBIA3201	*bar* and *uidA* genes	Yes (Zigotic inmature embryos)	Jube & Borthakur 2009
Prunus serotina	Cocultivation with *Agrobacterium tumefaciens*	PsAGRNAi	*nptII* and *PsAG* genes	Yes (Organogenesis)	Liu & Pijut 2010
Betula platyphylla Suk.	Cocultivation with *Agrobacterium tumefaciens*	pCAMBIA-2301	*nptII, gus, bgt* genes	Yes (*In vitro* propagation)	Zeng *et al.* 2010

Species	Technique	Vector	Transgen	Plant regeneration	Reference
Quercus robur	Cocultivation with *Agrobacterium tumefaciens*	pBI121 or pUbiGUSINT	*nptII* and *gus* genes or *nptII* and *uidA* genes respectively	Yes (Embryogenesis)	Vidal *et al.* 2010
Hevea brasiliensis	*Cocultivation with Agrobacterium tumefaciens*	pCAMBIA 2301-GFP or pCAMBIA 2300-GFP	*uidA, nptII* and *gfp* genes or *uidA, nptII, gfp* and *EcGSH1* genes respectively	Yes (Embryogenesis)	Leclercq *et al.* 2010
Populus	*Cocultivation with Agrobacterium tumefaciens*	pSKI015 or pSKI074	*bar* or *nptII* respectively	Yes	Busov *et al.* 2010

Table 1. Recent reports of transgenic forest tree species and the method of transformation used. *See van Frankenhuyzen and Beardmore (2004), Ahuja (2009) and Harfouche *et al*. (2011) for more information of transformed and regenerated forest trees.

many cases no subsequent plant regeneration has been obtained. The first transgenic tree, a hybrid poplar (a woody angiosperm), was produced more than 20 years ago (Fillatti et al., 1987) using *A. tumefaciens*. The first transgenic conifer plants were produced based on the use of *A. rhizogenes* in European larch (Huang et al., 1991). Since then, this genetic transformation system has been successfully applied to other forest tree species, including aspen, cottonwood, eucalyptus, walnut, pine and spruce (Henderson & Walter, 2006).

Biolistic-mediated transformation

Biolistic transformation has become the method of choice for introducing genes into cell organelles. The method involves bombarding target cells with microscopic (1 µm diameter) DNA-coated tungsten or gold microprojectiles, which are accelerated mainly through compressed gases (helium, nitrogen or carbon dioxide). Exogenous DNA may integrate into the genome of the cells if they are in a competent physiological state and the physical conditions for delivery are appropriate for the species concerned (Klein et al., 1987). This technique has been used to produce transgenic plants from recalcitrant coniferous or monocotyledonous species, but the transformation efficiency remains generally low and usually results in a high number of transgene inserts in the genome. For these reasons, *Agrobacterium*-mediated protocols are usually preferred over direct DNA transfer techniques.

Protoplast transformation

Protoplasts are produced by the enzymatic digestion of the cell walls of plant cells usually isolated from the leaf mesophyll, and are often grown in a liquid suspension culture. Protoplasts can be transformed by direct DNA uptake, following polyethylene glycol pre-treatment, by microinjection or by electroporation. Although many studies have resulted in successful transient expression of a transgene in cell-derived protoplasts (Bekkaoui et al., 1995), very few have described the regeneration of transgenic trees (Chupeau et al., 1994).

This is probably due to the lack of suitable methods to recover whole plants from protoplasts of most tree genotypes.

Regeneration of transgenic woody plants

Plants are regenerated through one of two methods: organogenesis (direct or indirect) or somatic embryogenesis. The former involves the generation of organs, such as shoots and roots, from various plant tissues or undifferentiated cell masses (calli), whereas the latter leads to the production of embryos from somatic tissues. Regardless of the approach used, the process of *in vitro* regeneration is often genotype-dependent and its feasibility has to be assessed on a case by case basis.

Direct transformation of mature material is not easily achieved because of the low transformation competence and regeneration potential of adult tree tissues. Moreover, juvenile and adult tissues show marked differences in their responses to organogenesis and embryogenesis induction in tissue culture, with a progressive loss of competence during the transition to the mature phase. Mature to juvenile phase manipulation in the tree tissue has been a common practice for clonal propagation, either by grafting, rooting of cuttings, micropropagation or somatic embryogenesis, occasionally resulting in either rejuvenation or reinvigoration, i.e., the transient appearance of juvenile characteristics, which can be advantageous for genetic transformation (von Aderkas & Bonga, 2000). While *Agrobacterium tumefaciens*-mediated transformation is most successful with hardwood species using organogenic or embryogenic technologies, biolistic transformation can be used most successfully with embryogenic cultures of both softwoods and hardwoods. This means that the development of GM trees is highly dependent on the availability of a reliable, reproducible propagation system (Campbell et al., 2003).

3.2 Targets for forest-tree engineering

Tree breeding programs are generally aimed to increase the volume of wood produced or to enhance its properties and quality for the desired end-uses. The approaches used to achieve this goal include the biochemical modification of wood characteristics and trunk structure to increase its growth rate and alter its shape. Other targets for the improvement of tree performance relate to the enhancement of the root system and canopy performance, and to all aspects of tree development and the interaction with its biotic and abiotic environment (Altman, 2003; Campbell et al., 2003). In the following sections, the use of genetic transformation to perform modifications directed at the improvement of these characteristics will be described citing some examples of its application to woody species.

Modification of lignin content and composition

Cell walls can account for up to 95% of the mass of woody plants, where the main components are cellulose, hemicelluloses and lignin. The tensile strength of wood fibers is primarily determined by cellulose and hemicelluloses, while lignin mediates adhesion between the fibers. Cellulose comprises approximately 30-60% of softwood (gymnosperm) cell walls and approximately 60-65% of hardwood (angiosperm) cell walls. Hemicelluloses comprise approximately 15-35% of both softwood and hardwood cell walls, although the percentage may exceed 40 in some hardwoods. Lignin accounts for 22-37% and 14-35% of the cell wall mass of softwoods and hardwoods, respectively (BeMiller, 2001; Mai et al., 2004). This makes lignin the second most abundant organic compound on Earth after cellulose, accounting for approximately 25% of plant biomass. Lignin confers mechanical

strength to the cell wall and due to its hydrophobicity, it waterproofs the vascular elements, playing an important role in the conduction of water and solutes. Furthermore, because of its cross-linking with other cell wall components, it minimizes the accessibility of cellulose and hemicellulose to microbial enzymes. Hence, the presence of lignin is associated with reduced digestibility of the plant biomass, providing a defensive barrier against pathogens and herbivores (Boudet & Grima-Pettenati, 1996; Campbell et al., 2003).

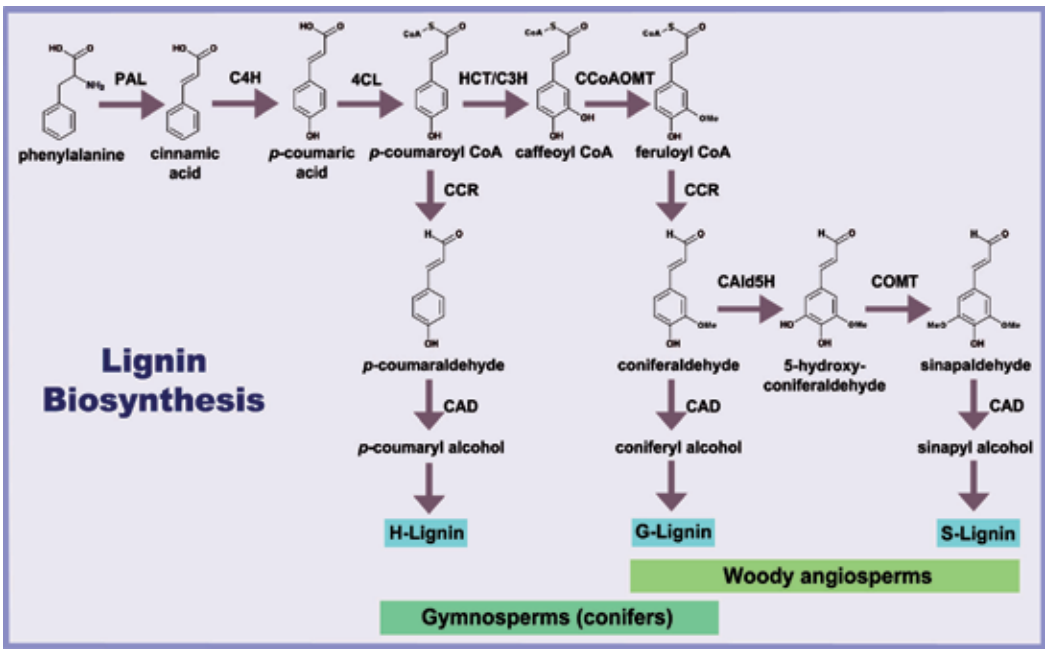

Fig. 3. Schematic diagram of the lignin biosynthetic pathway in forest trees.

Despite the fact that lignins play so important roles in plants, in the manufacture of pulp and paper, cellulose microfibrils are the component of the cell wall that is desired, and they need to be liberated from the lignin matrix through expensive and polluting processes. Additionally, residual lignin components are susceptible to oxidation and will cause a yellowing of the resultant pulp or paper if they are not thoroughly removed or bleached. Therefore, it is highly desirable to develop means by which lignin content is decreased, or make lignins more extractable, while maintaining basic structural integrity and the resistance of wood to hervibores and pathogens (Boudet & Grima-Pettenati, 1996; Campbell et al., 2003). Trees with reduced lignin would also improve the efficiency of their conversion into biofuels, especially during the pre-treatment step used in fermentation systems for the production of liquid biofuels from lignocellulosics (Hinchee et al., 2009).

Lignins result from the oxidative coupling of three monomers, namely p-coumaryl, coniferyl and sinapyl alcohols (also named monolignols), which give rise to p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of lignin, respectively (Figure 3). Lignins exhibit a high degree of structural variability depending on the species, the tissue, the cells and the environmental conditions. Gymnosperm (softwood) lignin is predominantly lignin with G units, but also contains a smaller amount of H units. Angiosperms (hardwoods) typically possess G-S lignin. The presence of S units makes hardwoods, in general, a better choice for

paper production because the lignin is more easily extractable using chemical techniques (Peña & Séguin, 2001).

The biosynthesis of lignins proceeds from intermediate metabolism through a long sequence of reactions involving (1) the shikimate pathway which supplies phenylalanine and tyrosine, (2) the phenylpropanoid pathway from phenylalanine to the cinnamoyl CoAs which are the general precursors of different phenolic compounds and (3) the lignin specific pathway which channels the cinnamoyl CoAs towards the synthesis of monolignols and lignins (Figure 3; Boudet & Grima-Pettenati, 1996). It has become clear that lignin content and composition can be modified in genetically engineered trees by manipulation of several key enzymes, especially phenylalanine ammonia lyase (PAL), cinnamoyl alcohol dehydrogenease (CAD), O-methyltransferase (OMT), peroxidases, and laccases. However, many of the enzymes and reactions in the lignin biosynthetic pathway still need to be characterized (Altman, 2003; Boudet & Grima-Pettenati, 1996; Campbell et al., 2003).

Hu et al. (1999) were among the first to demonstrate the potential of genetic engineering for modifying lignin in trees for industrial applications. Aspen (*Populus tremuloides*) was transformed with antisense 4-coumarate:coenzyme ligase (4CL) constructs that resulted in a 45% reduction in lignin content. They also demonstrated that this genetic modification had the added advantage of indirectly increase cellulose content in about 15%. Later, the same group reported that the co-transformation of the antisense 4CL and sense coniferaldehyde 5-hydroxylase (CAld5H) into the same species produced trees with up to 52% less lignin and 30% more cellulose than the wild-type control trees, showing that the genetic modification of these genes had an additive effect (Li et al 2003). A more limited reduction in the lignin content (about 10%) was obtained from the down-regulation of 4CL in hybrid poplar (*Populus tremula* X *Populus alba*) and it led to reduced growth and serious physiological abnormalities (Voelker et al., 2010). Lignin reduction in this species was associated with a loss in wood strength and stiffness (Voelker et al., 2011). Similarly, the RNA interference suppression of coumaroyl 3'-hydroxylase (C3'H) in hybrid poplar trees (*Populus alba* X *grandidentata*) caused reduction in lignin content, and the growth characteristics of these tress were significantly impaired, resulting in smaller stems and reduced root biomass when compared to wild-type trees, as well as altered leaf morphology and architecture (Coleman et al., 2008).

The use of a transgenic approach to reduce the lignin content or alter its composition has been achieved not only in angiosperm tree species, but also gymnosperms like the Norway spruce (*Picea abies* [L.] Karst) expressing the gene encoding cinnamoyl CoA reductase (CCR) in antisense orientation, showing a reduction in lignin content of up to 8% (Wadenback et al., 2008). In another gymnosperm, the conifer *Pinus radiata*, the suppression of 4CL using a RNA interference (RNAi) construct substantially affected plant phenotype and resulted in dwarfed plants with a "bonsai tree-like" appearance. Micro-structural changes included the formation of weakly lignified tracheids that displayed signs of collapse (Wagner et al., 2009).

Studies in populations of forest tree hybrids have shown a negative correlation of biomass growth and lignin content, implying that selection for improved growth rate could be accompanied by a reduction in lignin content (Novaes et al., 2010). However, it is clear from some of the above-mentioned experiences with transgenic trees, that a minimum amount of lignin is needed for anchoring the cellulose and hemicellulose components together for proper cell wall genesis and maintenance of structural integrity, and that a concentration below this tolerable limit would probably lead to cell wall disorganization and growth alterations (Koehler & Telewski, 2006).

Increasing lignin content can also be a target of improvement for those interested in using wood as fuel, since that modification would increase the thermal energy of wood. Pure cellulose has a calorific value of ~8,000 British thermal units per pound (BTU/lb), equivalent to ~18,600 kilo Joules per kilogram (kJ/kg), whereas that of pure lignin is ~11,000 BTU/lb (~25,600 kJ/kg). There is a high correlation between the heating value of wood and the lignin content. Increasing lignin content from 25% to 35% would increase the calorific value of wood by approximately 450 BTU/lb (1050 kJ/kg). This might be accomplished by achieving the opposite of the lignin reduction strategies mentioned above, namely the over-expression of a limiting enzyme in the biosynthetic pathway (Hinchee et al., 2009; White, 1987).

Alteration of the tree form, performance, and growth rate

The main objective of modifying phytohormones level in forest trees was to increase tree size, biomass production or wood quality. Induction of the GA 20-oxidase gene from *Arabidopsis* in hybrid aspen has resulted in an increase of bioactive gibberellin levels and subsequently faster growth in diameter and height, larger leaves, more numerous and longer xylem fiber and increasing biomass (Eriksson et al., 2000). This gene could be used to increase biomass production in forest trees or the use of its antisense can reduce tree size, which makes harvesting easier. In walnut, the expression of chalcone synthase decreases flavonoids synthesis and enhances the production of adventitious roots (Diouf, 2003). Expression of *iaaM* and *iaaH* auxin-biosynthetic genes from *Agrobacterium tumefaciens*, as well as *rolC* and *rolB* genes from *Agrobacterium rhizogenes*, has been shown to alter the growth patterns and development of transgenic forest trees. In addition, peroxidase genes and genes involved in directing cellulose biosynthesis have also been shown to affect stem elongation of several forest trees. It should be also noted that undesired alterations, such as reduced apical dominance and the breaking of axillary buds, could also occur (Altman, 2003).

Nitrogen availability is one of the main constraints for plant growth and limits production without fertilizer supplies. Development depends not only on the inorganic nitrogen available in the soil, but also on recycling within the plant, particularly in situations with limited nitrogen. However, many genes encoding proteins playing a key role in nitrogen fixation and assimilation have been isolated and characterized. Glutamine synthetase (GS) plays a significant role in both nitrogen uptake and recycling, as it catalyses the incorporation of ammonium into glutamine, the precursor to glutamate. Glutamine is also the precursor for all other plant N-containing compounds. In an attempt to alter tree growth, GS has been and important target for genetic engineering. One of these, the gene encoding glutamine synthetase under the control of 35S promoter was introduced in poplar. (Gallardo et al., 1999). The generated transgenic trees showed increased protein and chlorophyll content and a significantly greater net growth in height. Sulphur is an essential element found mostly in its reduced form as the amino acids cysteine and methionine. In plants, cysteine is used either in the synthesis of proteins, or can be further metabolized to methionine, glutathione (GSH) and phytochelatins. Given the biological significance of sulphur in plant development, it has also been a key target for genetic engineering in trees, particularly GSH formation (Diouf, 2003).

Herbicide resistance

Herbicide-resistant transgenic crops are considered one of the major successes of genetic engineering, being one of the major products of the first generation of agricultural

biotechnology. They are intended to reduce weed control costs, increase control flexibility, facilitate the use of low-tillage (and thus reduced erosion) cropping systems, and enable broad-spectrum, environmentally benign herbicides to be more readily employed. In herbaceous plants, there is a constant need for extensive weed control throughout the growing season. Direct competition with weeds also occurs in trees and it is especially important early in the tree's life cycle. Thus, although constitutive over-expression of herbicide-resistance genes is highly desirable throughout the life cycle of herbaceous crops, transgenic trees do not necessarily need to express resistance after they establish control of the site (Altman, 2003; Campbell et al., 2003). The first report on genetic transformation of forest trees was a herbicide-resistant poplar obtained through the introduction of the *aroA* gene, which confers resistance to glyphosate (Fillatti et al., 1987). Since then, transgenic trees with resistance to that and other herbicides, including chlorsulphuron, chloroacetanilide and phosphinothricine (glufosinate), have been generated (Campbell et al., 2003; Diouf, 2003).

Herbivore and pathogen resistance

Damage to forest trees caused by both native and introduced pests and pathogens is of global importance. These biotic stresses significantly affect forest growth and productivity, with substantial economic consequences. By virtue of selecting and propagating superior individuals and families, domestication inevitably involves a narrowing of genetic diversity. One of the consequences of this is that the domesticated population will not posses all of the alleles that are present in wild progenitors that confer resistance to herbivores and pathogens. Furthermore, as the domesticated population grows larger, and the more uniform it is planted, there is selection pressure on herbivores and pathogens to overcome any resistance mechanism that the domesticated population may possess. This is particularly true for forest trees because genotypes remain in the environment for a period of time that usually encompasses many more generations of the herbivore or pathogen. Consequently, a major goal of breeding programmes is to introduce alleles that confer robust and durable herbivore and pathogen resistance. Different insects feed on different tree parts, and their damage can sometimes be a limiting factor for tree growth and survival. In practice, the use of insecticides is rather limited in forestry, due in part to the large forest areas and tree size. Thus, insecticide application is usually restricted to nurseries and young or small plantations. Genetic engineering for insect control has been achieved in several forest trees using either the Bt toxin (from *Bacillus thuringiensis*) or insect digestive-system inhibitor genes. The Bt toxin binds to the epithelial glycoproteins of the intestine of insects, especially the midgut, and causes fatal leakage of fluids between the intestine and the hemocoel. This toxin specifically affects insects belonging to the lepidopteran, dipteran and coleopteran orders of insects, which include a number of major herbivores of forest tree species (Campbell et al., 2003; Diouf, 2003). The other approach is the expression of genes involved in the proteinase inhibitor system (Altman 2003; Campbell et al., 2003). Genetic transformation using gene coding for Bt or proteinase inhibitors could lead to reduced damage and chemicals used in the environment.

Restriction of gene-flow and early flowering

The long delay in the onset of flowering in forest trees, which lasts up to 30 or 40 years in some species, is an important constrain for inbreeding as a means for identifying and fixing beneficial recessive mutations and introgression/backcrossing as a means to increase the

frequency of rare alleles in breeding populations. The opposite (the ability to prevent the floral transition) is also desirable in trees for other reasons. Forest trees grown under intensive culture usually flower earlier than in the wild and produce large quantities of pollen and seed. It would be desirable that domesticated trees flower later or not at all so that additional resources for vegetative growth are available. Moreover, flowering is a major constraint to the use of genetic engineering in tree improvement. Because most forest trees have an abundance of wild or feral relatives, outcross, and display long-distance gene flow via pollen and sometimes seed, there is likely to be considerable public concern about large-scale use of genetically engineered trees and the spread of transgenes to the ecosystem. Since some forest trees are very close to their wild-type relatives, gene flow within and among genetically engineered forest trees can be rather extensive. Furthermore, gene flow from transgenic to wild-type plants may be especially problematic in forest trees because they produce large amounts of pollen and seeds which are easily dispersed over relatively long distances. Thus, current efforts are aimed at the use of developmental stage-dependent promoters, the co-engineering for reproductive sterility, and the use of naturally sterile or low-fertility tree hybrids (e.g., triploid hybrid poplars). Limiting the use of transgenic trees to nurseries, thus harvesting them before they reach their reproductive age, is another potential precaution. Advanced research efforts towards these goals include engineering for flower sterility through flower-specific expression of cytotoxic structural genes, and using sense/antisense or promoter suppression of specific homeotic reproductive development genes. In addition, induction of early flowering is beneficial in terms of reducing the tree's breeding cycle, allowing early characterization of transgene inheritance in young, small, transgenic seedling progeny (Altman, 2003). The major obstacle to engineering sterility in forest trees is simply demonstrating that a tree is reliably sterile under field conditions, and for many species, inefficient transformation, regeneration and field-testing capabilities are serious impediments. Transgenic trees with sterility constructs have been generated and some established in field tests, but the results cannot be observed until the trees reach maturity. These studies usually need to employ trees that lack nearby wild relatives, or provide other containment procedures (e.g. physical isolation or biological buffer zones), so that gene dispersal into wild populations is minimal. This points to another reason why the ability to induce early flowering is important: to speed the development and verification of sterility transgenes, preferably while in the greenhouse (Campbell et al., 2003). It has been shown that over-expression in juvenile tissues of flowering meristem-identity genes such as *LEAFY* (*LFY*), *APETALA1* (*AP1*) or *FLOWERING LOCUS T* (*FT*) from *Arabidopsis thaliana* and homologues from other plants leads to early flowering in different tree species (Campbell et al., 2003; Cervera et al., 2009).

Abiotic stress tolerance

In general, most natural forest-tree species are well adapted to their environment, exhibiting high ecological competence. However, forestation with plantation-improved or imported and exotic tree species will probably reveal their sensitivity to several ecological factors. Cold, drought, salinity, and heavy metal toxicity are the main stresses specifically affecting trees, which are subjected to many annual changes during their life cycle. Genetic engineering for cold tolerance would allow the use of cold-sensitive species in northern areas, as well as providing better protection of native plants from chilling damage. Drought and salinity tolerance is particularly important for forestation in arid and semiarid areas to prevent forest losses and desertification. Drought stress is primarily osmotic stress, which causes the

disruption of homeostasis and ion distribution in the cell. Salt stress is an increasingly important issue throughout the world, and it is imposed by two factors: water deficit due to osmotic stress, and the accumulation of ions that negatively affect biochemical processes. A number of genes have been tested in attempts to increase salt tolerance in trees (El-Khatib et al, 2004; Kawazu, 2004; Tang et al., 2007) The use of plants to remove contaminants from the environment is known as phytoremedation, and this technology has recently been applied to several environmental problems, including disposal of municipal wastewater, biofiltration of farm and industrial runoff, and the remediation of soils spoiled by industrial processes. Because this technology is less costly, less invasive, more aesthetic, and often yields a usable product (e.g. biomass), it has many advantages over traditional, engineering-based methods. Several plant species have been considered for phytoremediation efforts, but trees have most recently been identified as particularly useful vehicles because they produce large amounts of biomass, have far-reaching roots and are perennial, although leaves may need to be collected for incineration (Altman, 2003; Diouf, 2003; FAO, 2010; Giri et al., 2004).

4. Distribution and commercialization of GM trees

It is clear from the previous section that the production and commercialization of GM trees on a large scale offer numerous potential benefits. However, some concerns have been raised by environmental groups about their potential dangers, and GM trees have been banned in forest plantations certified by the Forest Stewardship Council (FSC) regardless of the source of genes, traits imparted, or whether for research or commercial use. Some researchers argue that this ban on research is counterproductive because it makes it difficult for certified companies to participate in the research field needed to assess the value and biosafety of GM trees. Furthermore, genetic modification could be an important tool for translating new discoveries from tree genomes into improved growth, quality, sustainability, and pest resistance (Strauss et al., 2001). The first genetically engineered tree, reported by Fillatti et al. (1987), was developed by a team of scientists from the University of Wisconsin, the Forest Service of the United States (US), and the biotechnology company Calgene (now part of Monsanto). Since then, dozens of other forest tree species have been genetically engineered for research purposes, though none have seen commercial use. The only commercialized tree in the US to date is papaya, a horticultural tree which was made virus resistant via genetic engineering and is now in widespread use in Hawaii (Gonsalves, 2006). This case has involved practically no environmental risk because papaya is an introduced species in Hawaii, thus lacking close wild relatives and because the Pacific Ocean is an effective barrier to transgene escape. A virus-resistant plum tree has already been deregulated by the US Department of Agriculture (USDA) and authorized by the US Food and Drug Administration (FDA) and is awaiting final approval by the US Environmental Protection Agency (EPA). Despite this success, no genetically engineered forest trees have yet been commercialized in the US (Sedjo, 2010). A transgenic poplar (*Populus nigra*) transformed with the Bt gene *Cry1Ac* was developed in China and used in field testing as early as 1994. In 2000, the Chinese regulatory authority allowed the establishment of about one million trees on about 300 hectares, though this release is more oriented toward forestation in parts of China where pests restrain the establishment of forests than toward the commercial production of wood (Sedjo 2005).

From a technical point of view, the use of genetic engineering would make it possible to modify forest tree species introducing the desired traits faster than through a traditional

breeding approach. However, given the regulatory restrictions associated with the commercialization of transgenic trees, the added costs and time needed to determine their long-term impact on the environment may countervail any advantages that the genetic transformation has over traditional breeding. For these reasons, an extensive commercialization of GM trees is not anticipated in the near future (Sedjo, 2010).

5. Ethics and biosafety

Regulatory issues related to transgenic plants concentrate on health, safety, and environmental risks. Health and safety concerns arise when humans or animals consume transgenic plants or their byproducts, which is generally not a problem for forest trees. The concerns about the environmental effects of the transgenic plants include fears that the GM plant itself might become a pest or, of greater concern, the possibility that a transferred gene might "escape" and alter the genetic composition of a wild relative, perhaps increasing the competence of the native plant and turning it into an invasive pest. In addition, an escaped gene might affect a non-transformed species and compromise its usefulness as a raw material for developing improved hybrids in the traditional way (van Frankenhuyzen & Beardmore, 2004; Walter, 2004). Although genetic containment systems have long been requested by ecologists and other scientists to reduce a number of undesired effects of genetically engineered crops (NRC, 2004; Snow et al., 2005), there has been strong pressure on companies and governments against the use of any forms of 'Terminator-like' containment technology (ETC, 2006). In agriculture, these concerns are primarily about control of intellectual property and the forced repurchase of seed by farmers. But in the forestry area, there has also been activism against containment technology because of a lack of confidence that it will be fully effective, concerns about loss of biodiversity associated with the modification or loss of floral tissues (Cummins & Ho, 2005), and legal uncertainties and liability risks from the dispersal of patented genes. These biological concerns occur despite the intention to use such technology mainly in plantations that, due to breeding, high planting density and short life spans, already produce few flowers and seeds compared with long-lived and open-grown trees. The powerful inverse association between forest stand density and degree of tree reproduction is widely known (Daniel et al., 1979). There is also an abundance of means to avoid and mitigate such effects at gene to landscape levels (Johnson & Kirby, 2004; Strauss & Brunner, 2004). Government regulations against the dispersal of genes from research trials also pose very substantial barriers to field research to study the efficiency of containment mechanisms (Strauss et al., 2004; Valenzuela & Strauss, 2005). The production by genetic engineering of trees that are unable to produce viable pollen or seeds, has been proposed (Strauss et al., 1995), but the genetic containment technology is, itself, difficult and highly controversial, requiring special social conditions even to carry out research. Another approach proposed to impede transgene escape is their targeting into chloroplast or mitochondrial genomes in species where these organelles are maternally inherited and therefore, the introduced traits would not be transmitted by pollen. Although most of angiosperm tree species show maternal inheritance of the chloroplast genome, most conifers exhibit a strictly paternal inheritance, thus this may be an option for preventing transgene escape only in angiosperm trees. However, this methodology is not infallible, since low levels of paternal inheritance may still occur in angiosperms (Ahuja, 2009; Ruf et al., 2007). Even though a plastid transformation system has already been developed for poplar, improvements are still needed (Okumura et al., 2006).

GM trees transformed with the purpose of providing resistance to pest and pathogens have been of particular concern. They may impose a selection pressure for the development of pests resistant to the defense mechanism introduced. The production of resistance-related components by these trees may not only suppress target organisms, but may also affect beneficial insects and plant symbionts as well as other micro-organisms involved in decomposition and nutrient cycling (Hoenicka & Fladung, 2006). Regarding the selection of resistant pests, the use of mixed populations of insect-resistant GM and susceptible non-GM trees shows resistance of the overall stand. Such strategy might help reduce the selection pressure on pests to overcome the introduced resistance mechanism in the transgenic trees and minimize the risk of mass disease outbreaks (Fenning & Gershenzon, 2002; Hu et al., 2001). It is important to note, however, that despite conventional tree breeding is a widely accepted and practiced technology, it is not completely risk-free. Traditional breeding is an intrinsically imprecise process since the new variations, created through recombination or mutations, are usually not well characterized at the genetic level. This appears to be much less controllable than genetic engineering, where only one or a few characterized genes may be either added or their expression altered (Henderson & Walter 2006). Specific studies addressing GM tree-related effects on the environment have reported that no changes in the ectomycorrhizal fungal community were found after transgenic poplars were used in the field for eight years and no unintended impacts of transgenic pine trees were observed for above-ground invertebrate communities over a period of two years (Schnitzler et al., 2010; Stefani et al., 2009). Furthermore, available information on the performance and safety of GM trees in field trials around the world reveals that none of them has reported any substantive harm to biodiversity, human health or the environment (Walter et al., 2010).

Transgenic tree germplasm is generally regulated at the country level throughout the world, as it is for other transgenic crops. Current concerns about GM trees are similar to those about agricultural crops, even though the majority of activities on genetic transformation are experimental and regulated under very strict conditions. The use of generic engineering in forestry will only succeed when the potential environmental impacts of genetically engineered tress are assessed and balanced against the costs of not pursuing this technology.

6. Conclusion

For the world to be supplied with the wood it needs without further degradation of forest areas, it is necessary to look for alternatives for the sustainable production of forest goods. One of these alternatives is the establishment of plantations, which productivity has increased considerably due to the development and improvement of forest management practices and to the introduction of new germplasm generated through traditional breeding. However, the generation of these new genotypes has been slow, because of the long life cycle of trees and the long time they require to reach reproductive maturity, which delay genetic crossing and the phenotypic evaluation of progeny performance.

Biotechnology, and particularly genetic engineering, is a valuable tool that can help to accelerate the process of tree improvement, especially through the introduction of traits that can be rapidly determined in juvenile plants. Forest tree biotechnology has taken advantage of the techniques developed for the genetic transformation of agricultural crops, but in spite of the great progress achieved so far, there are still important constraints that limit the generation of transgenic trees: the restricted susceptibility to *Agrobacterium* infection, the effect of the genotype in the development of efficient protocols for plant regeneration, and

the limited knowledge of tree physiology for the selection of potential target genes. In that context, transgenic trees are important not only as an alternative to increase wood production and quality, but also as tools for the study of different aspects of tree biology, which in concert with the recent developments in genomics and gene cloning techniques, will accelerate our ability to discover and introduce new value-added traits.

The research carried out in the area of molecular biology of forest trees is not comparable to the efforts implemented for similar studies in annual crops, mainly in terms of total funding resources and number of scientific groups. Nevertheless, although trees are not as easy for laboratory work as the herbaceous plants due to their size and life cycle, they are being increasingly used for research and many physiological and developmental processes specific to trees are being studied at the molecular level.

The use of transgenic trees may be assumed to raise fewer concerns to the general public than food crop plants, since the final products are not ingested and, therefore, no effect on the human health is expected. However, there is a great interest in the potential environmental effects of the GM trees, including the risks associated with the spread of transgenes to native populations. Several alternatives to restrict this potential transgene flow have been proposed, including the reproductive sterility and the transformation of plastids.

There is no doubt that the evaluation not only of the real commercial value, but also of the environmental safety of the GM trees is important, and the concerns of the public must be addressed through extensive field testing before they are planted on a commercial scale. Ironically, however, the legal framework created to regulate the release of untested GM plants into the environment makes it almost impossible to establish field trials where the potential impacts on the environment can be assessed.

Forest tree biotechnology offers a great potential and a significant progress in the field has been made to date. This area of research is expected to advance rapidly over the coming years, but this development must be accompanied by appropriate regulations and social acceptance if the transgenic trees are expected to enter the market.

7. References

Ahuja, M.R. (2009). Transgene stability and dispersal in forest trees. *Trees,* Vol. 23, No.6, (December 2009), pp. 1125-1135, ISSN 0931-1890.

Altman, A. (2003) From plant tissue culture to biotechnology: Scientific revolutions, abiotic stress tolerance, and forestry. *In Vitro Cellular & Developmental Biology Plant,* Vol. 39, No. 2, (March-April 2003), pp. 75-84, ISSN 1054-5476.

Álvarez, R. & Ordás, R.J. (2007). Improved genetic transformation protocol for cork oak (*Quercus suber* L.). *Plant Cell Tissue Organ Culture,* Vol.91, No.2, (November, 2007), pp. 45-52, ISSN 0167-6857.

Andrade, G.M.; Nairn, C.J.; Le, H.T. & Merckle, S.A. (2009). Sexually mature transgenic American chestnut trees via embryogenic suspension-based transformation. *Plant Cell Reports*, Vol.28, No.9, (September, 2009), pp. 1385-1397, ISSN 0721-7714.

Bekkaoui, F.; Tautorus, T.E. & Dunstan, D.I. (1995). Gymnosperm protoplasts, I*n: Somatic embryogenesis in woody plants* Vol. 1., Jain S.M., Gupta P.K. & Newton R.J. (editors), pp. 167–191, Kluwer Academic Publishers, ISBN 0792330706, Dordrecht, Netherlands.

BeMiller, J.N. (2001)Plant cell walls: Economic significance. Encyclopedia of Life Sciences, pp. 1-5, ISSN 9780470015902.

Boudet, A.M. & Grima-Pettenati, J. (1996) Lignin genetic engineering. *Molecular Breeding*, Vol. 2, No. 1, (March 1996), pp. 25-39, ISSN 1380-3743.

Broothaerts, W.; Mitchell, H.J.; Weir, B.; Kaines, S.; Smith, L.M.; Yang, W.; Mayer, J.E.; Roa-Rodriguez, C. & Jefferson, R.A. (2005). Gene transfer to plants by diverse species of bacteria. *Nature*, Vol.433, No.7026, (February, 2005), pp. 629-633, ISSN 0028-0836.

Burdon, R.D. (1994). The role of biotechnology in forest tree breeding. *Forest Genetic Resources, FAO*, Vol.22, No.1, pp. 2-5.

Busov, V.; Yordanov, Y.; Gou, J.; Meilan, R.; Ma, C.; Regan, S. & Strauss, S. (2011). Activation tagging is an effective gene tagging system in Populus. Tree Genetics & Genomes, Vol.7, No.1, (February, 2011), pp. 91-101, ISSN 1614-2942.

Campbell, M.M.; Brunner, A.M.; Jones, H.M. & Strauss, S.H. (2003). Forestry's fertile crescent: the application of biotechnology to forest trees. *Plant Biotechnology Journal*, Vol.1, No.3, (May, 2003), pp. 141-154, ISSN 1467-7652.

Castellanos-Hernández, O.A.; Rodríguez-Sahagún, A.; Acevedo-Hernández, G.J.; Rodríguez-Garay, B.; Cabrera-Ponce, J.L. & Herrera-Estrella, L.R. (2009). Transgenic *Paulownia elongata* S. Y. Hu plants using biolistic-mediated transformation. *Plant Cell Tissue Organ Culture,* Vol.99, No.2, (November, 2009), pp. 175-181, ISSN 0167-6857.

Cervera, M.; Navarro, L. & Peña, L. (2009) Gene stacking in 1-year-cycling APETALA1 citrus plants for a rapid evaluation of transgenic traits in reproductive tissues. *Journal of Biotechnology*, Vol. 140, No. 3-4, (March 2009), pp. 278-282, ISSN 0168-1656.

Chupeau, M.C.; Pautot, V. & Chupeau, Y. (1994). Recovery of transgenic trees after electroporation of poplar protoplasts. *Transgenic Research,* Vol.3, No.1, (January, 1994), pp. 13-19, ISSN 0962-8819.

Coleman, H.D.; Samuels, A.L.; Guy, R.D. & Mansfield, S.D. (2008) Perturbed lignification impacts tree growth in hybrid poplar- a function of sink strength, vascular integrity, and photosynthetic assimilation. *Plant Physiology*, Vol. 148, No. 3, (November 2008), pp. 1229-1237, ISSN 0032-0889.

Cornellius, J. (1998). Introducción al mejoramiento genético vegetal, In: *Selección y manejo de fuentes semilleras en América central y República Dominicana*, Jara L.F., pp. 9-21, PROSEFOR-Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), ISBN 9977-57-315-8, Turrialba, Costa Rica.

Cummins, J. & Ho, M.W. (2005). Terminator Trees, In: *Institute of Science in Society (ISIS),* Accessed February 14, 2011, Available from: March 1, 2005 <www.i-sis.org.uk/full/TerminatorTreesFull.php>

Daniel, T.W.; Helms, J.A. & Baker, F.S. (1979). *Principles of silviculture.* McGraw-Hill, ISBN 0-07-015297-7, New York, USA.

Diouf, D. (2003) Genetic transformation of forest trees. *African Journal of Biotechnology*, Vol. 2, No. 10, (October 2003), pp. 328-333, ISSN 1684-5315.

El-Khatib, R.T.; Hamerlynck, E.P.; Gallardo, F. & Kirby, E.G. (2004) Transgenic poplar characterized by ectopic expression of a pine cytosolic glutamine synthetase gene

exhibits enhanced tolerance to water stress. *Tree Physiology*, Vol. 24, No. 7, (July 2004), pp. 729-736, ISSN 0829-318X.

Eriksson, M.E.; Israelsson, M.; Olsson, O. & Moritz, T. (2000) Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nature Biotechnology*, Vol. 18, No. 7, (July 2000), pp. 784-788, ISSN 1087-0156.

ETC [Action Group on Erosion, Technology and Concentration]. (January 20, 2006). *Terminator Threat Looms: Intergovernmental meeting to tackle suicide seeds issue.* ETC Group News Release, Accessed January 20, 2011, Available from: <www.etcgroup.org/en/node/36>

FAO. (2007). State of the World's Forests 2007. Food and Agriculture Organization of the United Nations, Rome, Italy, 144 pp., ISBN 978-92-5-105586-1.

FAO (2010) Forests and genetically modified trees. Food and Agriculture Organization of the United Nations, Rome, Italy, 235 pp,

Fenning, T.M. & Gershenzon, J. (2002) Where will the wood come from? Plantation forests and the role of biotechnology. *Trends in Biotechnology*, Vol. 20, No. 7, (July 2002), pp. 291-296, ISSN 0167-7799.

Fillati, J.J.; Sellmer, J.; McCown, B.; Haissig, B. & Comai, L. (1987). *Agrobacterium*-mediated transformation and regeneration of *Populus*. *Molecular and General Genetics,* Vol. 206, No.2, (February, 1987), pp. 192-199, ISSN 0026-8925.

Gallardo, F.; Fu, J.; Canton, F.R.; Garcia-Gutierrez, A.; Canovas, F.M. & Kirby, E.G. (1999) Expression of a conifer glutamine synthetase gene in transgenic poplar. *Planta*, Vol. 210, No. 1, (November 1999), pp. 19-26, ISSN 0032-0935.

Gelvin, S.B. (2003). *Agrobacterium*-mediated plant transformation: the biology behind the ''gene-jockeying'' tool. *Microbiology and Molecular Biology Reviews,* Vol.67, No.1, (March, 2003), pp. 16-37, ISSN 1092-2172.

Giri, C.C.; Shyamkumar, B. & Anjaneyulu, C. (2004) Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. Trees, Vol. 18, No. 2, pp. 115-135, ISSN 09311890.

Gomez-Lim, M.A. & Litz, R. (2004). Genetic transformation of perennial tropical fruits. *In Vitro Cellular & Developmental Biology-Plant,* Vol.40, No.5, (September, 2004), pp. 442-449, ISSN 1054-5476.

Gonsalves, D. (2006) Transgenic papaya: Development, release, impact and challenges. *In: Advances in Virus Research*, Vol. 67, pp. 317-354, ISSN 0065-3527.

Grace, L.J.; Charity, J.A.; Gresham, B.; Kay, N. & Walter Ch. (2005). Insect-resistant transgenic *Pinus radiata*. *Plant Cell Reports,* Vol.24, No. 2, (May 2005) pp.103-111, ISSN 0721-7714.

Henderson, A.R. & Walter, C. (2006). Genetic engineering in conifer plantation forestry. *Silvae Genetica*. Vol.55, No.6, (January, 2007), pp. 253-262, ISSN 0037-5349.

Hinchee, M.; Rottmann, W.; Mullinaux, L.; Zhang, C.; Chang, S.; Cunningham, M.; Pearson, L. & Nehra, N. (2009) Short-rotation woody crops for bioenergy and biofuels applications. *In vitro Cellular & Developmental Biology-Plant*, Vol. 45, No. 6, (December 2009), pp. 619-629, ISSN 1054-5476.

Hoenicka, H. & Fladung, M. (2006). Biosafety in Populus spp. and other forest trees: from non-native species to taxa derived from tradicional breeding and genetic engineering. *Trees,* Vol.20 , No.2, (March, 2006), pp. 131-144, ISSN 0931-1890.

Hu, W.-J.; Harding, S.A.; Lung, J.; Popko, J.L.; Ralph, J.; Stokke, D.D.; Tsai, C.-J. & Chiang, V.L. (1999) Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. *Nature Biotechnology*, Vol. 17, No. 8, (August 1999), pp. 808-812, ISSN 1087-0156.

Hu, J.J.; Tian, Y.C.; Han, Y.F.; Li, L. & Zhang, B.E. (2001) Field evaluation of insect-resistant transgenic *Populus nigra* tree. *Euphytica*, Vol. 121, No. 2, pp. 123-127, ISSN 0014-2336

Huang, Y.; Diner, A.M. & Karnorsky, D.F. (1991) *Agrobacterium rhizogenes* mediated genetic transformation and regeneration of a conifer: *Larix decidua*. *In vitro Cellular & Developmental Biology-Plant*, Vol. 27, No. 4, (October 1991), pp. 201-207, ISSN 1054-5476.

Johnson, B. & Kirby, K. (2004). Potential impacts of genetically modified trees on biodiversity of forestry plantations: a global perspective, In: *The bioengineered forest: challenges to science and society,* Strauss S.H. & Bradshaw H.D. (editors), pp. 190–207, Resources for the Future, ISBN 1-891853-71-6, Washington DC, USA.

Jube, S. & Borthakur, D. (2009). Development of an Agrobacterium-mediated transformationprotocol for the tree-legume Leucaena leucocephala using immature zygotic embryos. *Plant Cell Tissue Organ Culture,* Vol.96, No.3, (March, 2009), pp. 325-333, ISSN 0167-6857.

Kawazu, T. (2004) Development of environmental-stress-tolerant eucalyptus and forest plantations. *Japan TAPPI Journal*, Vol. 58, No. 1, pp. 55-61, ISSN 0022-815X.

Klein, T.M.; Wolf, E.D.; Wu, R. & Sanford, J.C. (1987). High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature,* Vol.327, No.6117, (May, 1987), pp. 70-73, ISSN 0028-0836.

Koehler, L. & Telewski, F.W. (2006) Biomechanics and transgenic wood. *American Journal of Botany*, Vol. 93, No. 10, (October 2006), pp. 1433-1438, ISSN 0002-9122.

Lantz, C.W. (2008) Genetic improvement of forest trees. In: *Woody Plant Seed Manual, USDA FS Agriculture Handbook 727*, F.T. Bonner & R.P. Karrfalt (editors), pp. 39-56, United States Department of Agriculture, Forest Service, ISBN 0160811317.

Leclercq, J.; Lardet, L.; Martin, F.; Chapuset, T.; Oliver, G. & Montoro, P. (2010). The green fluorescent protein as an efficient selection marker for Agrobacterium tumefaciens-mediated transformation in Hevea brasiliensis (Müll. Arg). *Plant Cell Reports,* Vol.29, No.5, (May, 2010) pp.513-522, ISSN 0721-7714.

Li, J.; Brunner, A.M.; Meilan, R. & Strauss S.H. (2009). Stability of transgenes in trees: expression of two reporter genes in poplar over three field seasons. *Tree Physiology*, Vol.29, No.2, (February, 2009), pp. 299-312, ISSN 0829-318X.

Li, L.; Zhou, Y.; Cheng, X.; Sun, J.; Marita, J.M; Ralph, J. & Chiang, V.L. (2003) Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. *Proceedings of the National Academy of Sciences USA*, Vol. 100, No. 8, (April 2003), pp. 4939-4944, ISSN 0027-8424.

Li, Y.; Su, X.; Zhang, B.; Huang, Q.; Zhang, X. & Huang, R. (2009). Expression of jasmonic ethylene responsive factor gene in transgenic poplar tree leads to increased salt

tolerance. *Tree Physiology*, Vol.29, No.2, (February, 2009), pp. 273-279, ISSN 0829-318X.

Liu, X. & Pijut, P.M. (2010). Agrobacterium-mediated transformation of mature Prunus serotina (black cherry) and regeneration of transgenic shoots. *Plant Cell Tissue Organ Culture,* Vol.101, No.1, (April, 2010), pp. 49-57, ISSN 0167-6857.

Mai, C.; Kües, U. & Militz, H. (2004) Biotechnology in the wood industry. *Applied Microbiology and Biotechnology*, Vol. 63, No. 5, (February 2004), pp. 477-494, ISSN 0175-7598.

Malan, F.S.; Retief, R.J. & Dyer, S.T. (1996). Improvement of South African timber resource: concerns and propoused strategies. *South African Forestry Journal*, Vol.175, No.1, pp. 61-65, ISSN 0038-2167.

Niembro, R.A. (1985). Preguntas y respuestas más comunes relacionadas con el establecimiento y manejo de áreas semilleras. In: *Boletín Técnico No. 22. Serie Técnica,* pp. 15, División de Ciencias Forestales, Universidad Autónoma Chapingo, ISBN 968884-1307, Texcoco, Mexico.

Novaes, E.; Kirst, M.; Chiang, V.; Winter-Sederoff, H. & Sederoff, R. (2010) Lignin and biomass: a negative correlation for wood formation and lignin content in trees. *Plant Physiology*, Vol. 154, No. 2, (October 2010), pp. 555-561, ISSN 0032-0889.

NRC [National Research Council]. (2004). *Biological confinement of genetically engineered organisms.* The National Academies, Washington, DC, USA.

Okumura, S.; Sawada, M.; Park, Y.W.; Hayashi, T.; Shimamura, M.; Takase, H. & Tomizawa, K. (2006). Transformation of poplar (Populus alba) plastids and expression of foreign proteins in tree chloroplasts. *Transgenic Research*, Vol.15 , No.5, (October, 2006), pp. 637-646, ISSN 0962-8819.

Peña, L. & Séguin, A. (2001). Recent advances in the genetic transformation of trees. *Trends in Biotechnology,* Vol.19, No.12, (December, 2001), pp. 500-506, ISSN 0167-7799.

Pryor, L.D. & Willing, R.R. (1983). *Growing and breeding poplars.* Canberra Publishing and Printing Co. ISBN 978-0959275100, Australia.

Quijada, M.R. (1980). Selección de árboles forestales. In: *mejora genética de árboles forestales*. Estudios FAO, Montes No. 20, pp. 169-176, Roma, Italia.

Quijada, M.R. (1980a). Ensayos de progenie. In: *FAO. 1980. Mejora Genética de Árboles Forestales. Informe sobre el curso de capacitación FAO/DANIDA sobre la mejora genética de árboles forestales celebrado en Mérida, Venezuela, (*January-February 1980), FAO, pp. 224-230, Rome, Italy.

Raymond, C.A. & Apiolaza, L.A. (2004). Incorporating wood quality and deployment traits in *Eucalyptus globulus* and *Eucalyptus nitens*. In: *Plantation Forest Biotechnology for the 21st Century*. Walter C. & Carson M. (editors), pp. 87-89, Research Signpost, ISBN 81-7736-228-3, Kerala, India.

Roulund, H. & Olesen, K. (1992). Mejoramiento forestal a nivel de familia y de individuo. Class Note No. D. 4. Humlebaek, Dinamarca, In: *Mejoramiento Forestal y Conservación de Recursos Genéticos Forestales. Tomo I. CATIE-MIREN-PROSEFOR*, Jara N., L.F. 1995, pp. 75-94, Turrialba, Costa Rica.

Ruf, S.; Karcher, D.; Bock, R. (2007) Determining the transgene containment level provided by chloroplast transformation. *Proceedings of the National Academy of Sciences USA*, Vol. 104, No. 17, (April 2007), pp. 6998-7002, ISSN 0027-8424.

Schnitzler, F.R.; Burgess, E.P.; Kean, A.M.; Philip, B.A.; Barraclough, E.I.; Malone, L.A. & Walter, C. (2010) No unintended impacts of transgenic pine (*Pinus radiata*) trees on above ground invertebrate communities. *Environmental Entomology*, Vol. 39, No. 4, (August 2010), pp. 1359-1368, ISSN 0046-225X.

Sedjo, R.A. (2003) Biotech and planted trees: some economic and regulatory issues. AgBioForum, Vol. 6 No. 3, pp. 113-119, ISSN 1522-936X.

Sedjo, R. A. (2005) Will developing countries be the early adopters of genetically engineered forests? *AgBioForum*, Vol. 8, No. 4, pp. 205-212, ISSN 1522-936X.

Sedjo, R. A. (2010) Transgenic trees for biomass: the effects of regulatory restrictions and court decisions on the pace of commercialization. *AgBioForum*, Vol. 13, No. 4, pp. 391-397, ISSN 1522-936X.

Snow, A.A.; Andow, D.A.; Gepts, P.; Hallerman, E.M.; Power, A.; Tiedje, J.M. & Wolfenbarger, L.L. (2005). Genetically engineered organisms and the environment: current status and recommendations. *Ecological Applications,* Vol.15, No.2, (April, 2005), pp. 377-404, ISSN 1051-0761.

Stefani, F.O.P.; Moncalvo, J.M.; Séguin, A.; Bérubé, J.A. & Hamelin, R. (2009) Impact of an 8-year-old transgenic poplar plantation on the ectomycorrhizal fungal community. *Applied and Environmental Microbiology*, Vol. 75, No. 23, (December 2009), pp. 7527-7536, ISSN 0099-2240.

Walter, C.; Fladung, M. & Boerjan, W. (2010) The 20-year environmental safety record of GM trees. *Nature Biotechnology*, Vol. 28, No. 7, (July 2010), pp. 656-658, ISSN 1087-0156.

Strauss, S.H.; Rottmann, W.H.; Brunner, A.M.; Sheppard, L.A. (1995) Genetic engineering of reproductive sterility in forest trees. *Molecular Breeding*, Vol. 1, No. 3, (September 1995), pp. 5-26, ISSN 1380-3743.

Strauss, S.H. & Brunner, A.M. (2004). Tree biotechnology in the 21st century: Transforming trees in the light of comparative genomics, In: *The BioEngineered Forest: Challenges to Science and Society*, Strauss S.H. & Bradshaw H.D. (editors), pp. 76-97, Resources for the Future, ISBN 1-891853-71-6, Washington, D.C. USA.

Strauss, S.H.; Campbell, M.M.; Pryor, S.N.; Coventry, P. & Burley, J. (2001). Plantation certification and genetic engineering: FSC's ban on research is counterproductive. *Journal Forestry*, Vol.99, No.12, (December, 2001), pp. 4-7, ISSN 0022-1201.

Strauss, S.H.; Brunner, A.M.; Busov, V.B.; Ma, C. & Meilan, R. (2004). Ten lessons from 15 years of transgenic *Populus* research. *Forestry,* Vol.77, No.5, (January, 2004), pp. 455-465, ISSN 0015-752X.

Tang, W.; Newton, R.J.; Li, C. & Charles, T.M. (2007) Enhanced stress tolerance in transgenic pine expressing the pepper *CaPF1* gene is associated with the polyamine biosynthesis. *Plant Cell Reports*, Vol. 26, No. 1, (January 2007), pp. 115-124, ISSN 0721-7714.

Turner, P. (2001). Strategic and tactical options for managing the quality and value of eucalypt plantation resource. "Developing the Eucalypt of the future". Proceedings

IUFRO International Symposium Actas in CD: pp. 17, Valdivia, Chile, September 2001.

Valenzuela, S. & Strauss, S.H. (2005). Lost in the woods. *Nature Biotechnology,* Vol.23, No.5, (May, 2005), pp. 532-533, ISSN 1087-0156.

Van Frankenhuyzen, K. & Beardmore, T. (2004). Current status and environmental impact of transgenic forest trees. *Canadian Journal of Forest Research*, Vol. 34, No.6, (June 2004), pp. 1163-1180, ISSN 0045-5067.

Vidal, N.; Mallón, R.; Valladares, S.; Meijomín, A.M. & Vieitez A.M. (2010). Regeneration of transgenic plants by *Agrobacterium*-mediated transformation of somatic embryos of juvenile and mature *Quercus robur*. *Plant Cell Reports,* Vol.29, No.12, (December 2010) pp.1411-1422, ISSN 0721-7714.

Voelker, S.L.; Lachenbruch, B.; Meinzer, F.C.; Jourdes, M.; Ki, C.; Patten, A.M.; Davin, L.B.; Lewis, N.G.; Tuskan, G.A.; Gunter, L.; Decker, S.R.; Selig, M.J.; Sykes, R.; Himmel, M.E.; Kitin, P.; Shevchenko, O. & Strauss, S.H. (2010) Antisense down-regulation of 4CL expression alters lignification, tree growth, and saccharification potential of field-grown poplar. *Plant Physiology*, Vol. 154, No. 2, (October 2010), pp. 874-886, ISSN 0032-0889.

Voelker, S.L.; Lachenbruch, B.; Meinzer, F.C. & Strauss, S.H. (2011). Reduced wood stiffness and strength, and altered stem form, in young antisense *4CL* transgenic poplars with reduced lignin contents. *New Phytologist,* Vol.189, No.4, (March 2011), pp. 1096-1109, ISSN 1469-8137.

Von Aderkas, P. & Bonga, J.M. (2000). Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. *Tree Physiology*, Vol.20, No.14, (August, 2000), pp. 921-928, ISSN 0829-318X.

Wadenbäck, J.; von Arnold, S.; Egertsdotter, U.; Walter, M.H.; Grima-Pettenati, J.; Goffner, D.; Gellerstedt, G.; Gullion, T. & Clapham D. (2008). Lignin biosynthesis in transgenic Norway spruce plants harboring an antisense construct for cinnamoyl CoA reductase (CCR). *Transgenic Research*, Vol. 17, No. 3, (June 2008). pp.379-392, ISSN 0962-8819.

Wagner, A.; Donaldson, L.; Kim, H.; Phillips, L.; Flint, H.; Steward, D.; Torr, K.; Koch, G.; Schmitt, U. & Ralph, J. (2009) Suppression of 4-coumarate-CoA ligase in the coniferous gymnosperm *Pinus radiata*. *Plant Physiology*, Vol. 149, No. 1, (January 2009), pp. 370-383, ISSN 0032-0889.

Walter, C. (2004) Genetic engineering in conifer forestry: technical and social considerations. *In Vitro Cellular & Developmental Biology Plant,* Vol. 40, No. 5, (September 2004), pp. 434-441, ISSN 1054-5476.

Walter, C.; Grace, L.J.; Wagner, A.; White, D.W.R.; Walden, A.R.; Donaldson, S.S.; Hinton, H.; Gardner, R.C. & Smith, D.R. (1998). Stable transformation and regeneration of transgenic plants of *Pinus radiata* D. Don. *Plant Cell Reports*, Vol. 17, No. 6-7, (April 1998) pp.460-468, ISSN 0721-7714.

White, R.H. (1987) Effect of lignin content and extractives on the higher heating value of wood. *Wood and Fiber Science*, Vol. 19, No. 4, (October 1987), pp. 446-452, ISSN 0735-6161.

Zeng, Fan-Suo; Zhan, Ya-Guang; Zhao, Hong-Cui; Xin, Ying; Qi, Feng-Hui & Yang, Chuan-Ping. (2010). Molecular characterization of T-DNA integration sites in transgenic birch. *Trees,* Vol.24 , No. 1, (May 2010), pp. 753-762, ISSN 0931-1890.

Zobel, B. & Talbert, J. (1988). Técnicas de Mejoramiento Genético de Árboles Forestales. First Edition, North Carolina State University, Editorial Limusa, ISBN 968-18-2856-9, México.

Zobel, B. (1992). Silvicultural effects on wood properties. *IPEF International. Piracicaba,* Vol.2, No.1, pp. 31-38, ISSN 0100-8137.

11

Agrobacterium-Mediated Transformation of Indonesian Orchids for Micropropagation

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1. Introduction

Indonesia is the most biodiverse country in the world after Brazil, and its biodiversity is reflected by the vast species of flora found there. The Orchidaceae are one of the largest and most diverse families in the plant kingdom (Arditti, 1992). In Indonesia, orchids are very popular ornamental crops, both as cutting flowers and as potted plants. Many orchid species belonging to the Orchidaceae family can be found only in Indonesia. Most of these species are classified as tropical orchids, which comprise the greatest part of the orchids' diversity. Orchid flowers have fabulous variations based on size, shape, structure, odor, color, and floriferousness.

Indonesian orchids are very unique and exotic. The black orchid (*Coelogyne pandurata* Lindley), endemic to the provinces of East Kalimantan and Papua, is a very important species. The uniqueness of this orchid is in its flowering characteristics, i.e., a very short blooming period (3-5 days) and difficulties with pollination (Arditti, 1992; Wibowo, 2010). Another important Indonesian orchid is the moth orchid (*Phalaenopsis amabilis (L.)* Blume) which has been used as a parent for creating new hybrids. The pandanus orchid (*Vanda tricolor* Lindley) has spread over Java, Bali, and Sulawesi islands. This orchid is quite popular in those regions, not only because of it odor, but also because of its value as an ornamental plant (Figure 1). The destruction of habitat and difficulties in cultivation, however, are threatening these species (Arditti, 1992). Thus, there is a need to counteract the decrease of the populations of these orchids to conserve them as a genetic resource, either using conservation methods both *in situ* and *ex situ* or micropropagation methods.

Micropropagation methods can be applied using biotechnology. Modern biotechnology is important in agriculture, particularly in the economically important horticulture industry, and methods such as genetic transformation have become increasingly important tools for

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A B C

Fig. 1. A. The Black Orchid (*Coelogyne pandurata* Lindley); B. The Moth Orchid (*Phalaenopsis amabilis*); C. The Pandanus Orchid (*Vanda tricolor var Suavis* Merapi Form), bars: 1 cm.

improving cultivars and studying gene function in plants. This is particularly true in orchids, which are highly valued ornamental plants that are continually being genetically altered. Of the reports published to date on the genetic transformation of orchids, most have focused on transformation techniques targeting specific genes and areas related to crop improvement. We have used the *BREVIPEDICELLUS (BP)/KNAT1* gene, which is a member of the family of class 1 *KNOTTED*-like homeobox (*KNOX)* genes in *Arabidopsis thaliana,* for transformation studies in Indonesian orchids. One reason to use it is that the *KNAT1* gene is required for the maintenance of the indeterminate state of cells during which the plant produces multiple shoots. Micropropagation of orchids conducted by genetic transformation is potentially very valuable. We hope that the *BP/KNAT1* gene can be used to improve shoot formation for mass propagation of these orchids.

2. Orchids are an important horticultural plant

Horticulture, literally "garden cultivation", is a branch of agriculture concerned with cultivation of crops that also includes agronomy and forestry. Traditionally, horticulture deals with garden crops such as fruits, nuts, vegetables, culinary herbs and spices, beverage crops, and medicinal, as well as ornamental plants.

In Indonesia, orchids are very popular ornamental crops, and many people enjoy keeping orchids in their homes as decorative plants. On the other hand, orchids such as Dendrobium, Cymbidium, Eulophia, and Habenaria are used in traditional medicine as restoratives and to treat various diseases (Puri, 1970). The market for orchids is worldwide, providing opportunities to grow orchids not only as a hobby, but also for commercial purposes. Horticulturally important species are clustered into taxonomic sections including Phalaenanthe, Spatulata (Ceratobium), Latourea, Formosae (Nigrohirsutae), Dendrobium (Eugenanthe), and Callista (Schelpe and Stewart, 1990). The breeding of orchids is common since most species have high crossability with other orchids, including hybridization between different genera.

3. Orchid breeding

Enhancing productivity and qualitative traits are the main objectives in general horticultural breeding programs. Common goals in breeding new varieties of orchids mainly concern

flower size, flower shape, floriferousness, flower color, early flowering, compact growth (dwarfing), resistance to pathogens, and flower longevity. However, breeding programs are successful if the products are marketable regarding extrinsic (color, size, shape) and intrinsic (odor, longevity) traits. Breeding of ornamental plants can be achieved by several methods, either through conventional breeding or biotechnology.

3.1 Classical breeding

Classical or conventional breeding methods using crosses, such as intraspecific and interspecific hybridization of orchid species, are a common way to create new varieties. The French hybrid variety registered in 1934, Dendrobium Pompadour (PPPC), is comprised of three chromosome sets of *D. phalaenopsis* and one chromosome set of *D. discolor*. The genome symbols for each Dendrobium section are P and C for Phalaenanthe and Ceratobium, respectively. Those sections are key contributors to both the cut flower and potted varieties of the new hybrid species. Different ploidies can be found within one species within the same family, and so hybridization between them can generate new species, such as in the family Brassicaceae (Figure 2).

Variability in the percentage of viable progeny is generally determined by the genome or cytogenetic relatedness; more similar genome constitutions have more normal meiotic

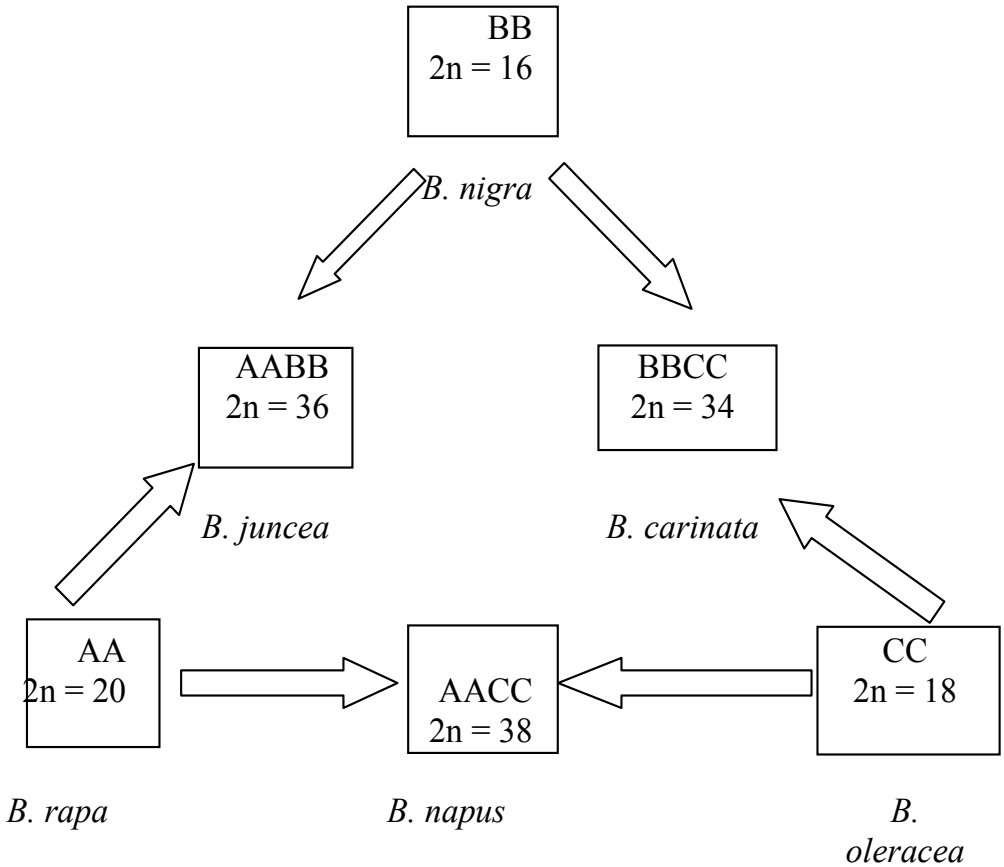

Fig. 2. Genome constitution of species belonging to the genus *Brassica*

pairings (Kanemoto and Wilfret, 1980). The development of new F1 hybrids of orchids allows high flower quality to be achieved, which is an important factor not only for hobbyists but also for commercial purposes.
Creating new varieties can be conducted without hybridization. Polyploidization is the simplest way to induce variability in horticultural plants. Chromosome doubling occurs in existing polyploid plants, but it is more likely to produce a positive effect from a diploid base (Levin, 2002). The induction of chromosome doubling is generally performed *in vitro*. Protocorm-like bodies can be treated with autoclaved 0.1% (w/v) colchicine in orchid seed germination medium for 5, 7, and 10 days at 100 rpm under continuous light before being transferred onto solid medium (Sanguthai et al., 1973).

3.2 Modern breeding through biotechnology

Plant genetic transformation is potentially a powerful tool for orchid breeding as it can break the species barrier and bring in favorable traits from other gene pools that are not easily accessible through traditional breeding techniques. Transformation of orchids has been accomplished mainly using *Agrobacterium*. *Agrobacterium*-mediated genetic engineering is currently the most widely used plant genetic engineering strategy, and was first demonstrated in rice in 1994 (Hiei et al., 1994). The greatest motivation for using *Agrobacterium* appears to be molecular and cytogenetic analyses showing that higher frequencies of transgene loci produced by microprojectile bombardment and other direct DNA delivery methods are more complex compared to transgene loci produced via *Agrobacterium* (Pawlowski and Somers, 1996). Complex transgene loci are associated with problems of transgene expression (Finnegan and McElroy, 1994; Matzke and Matzke, 1995; Pawlowski et al., 1998; Kohli et al., 1999) and inheritance (Christou et al., 1989; Choffnes et al., 2001). In other words, *Agrobacterium*-mediated transformation is a simple and inexpensive method that produces the simplest transgene locus structure. Therefore, this method is readily adopted for plant genetic engineering.

Point of view	Classical breeding	Modern breeding (Biotechnology methods)
Source of gene desired	limited	unlimited
Insertion of gene desired	indirect	direct
Application	easy	difficult
Expected result	unfixed	fixed
Time consumed	longer	shorter

Table 1. Comparison between classical and modern (biotechnology) breedings

Transformation using *Agrobacterium* has become common in horticultural plants such as in lettuce (Michelmore et al., 1987), cabbage (Metz et al., 1995; Jin et al., 2000; Lee et al., 2000), kale (Cogan et al., 2001), orchids (Semiarti et al., 2007 and 2010; Chin and Mii, 2011), and petunias (Klee et al., 1987). With genes that can modulate auxin concentrations, transgenic techniques have been used to manipulate endogenous levels of auxin in plants. Insertion of the *iaa*M gene into petunia causes increased apical dominance and reduced stem growth (Klee et al., 1987). Expression of the *iaa*M gene, if under the control of appropriate gene promoters, can enhance the formation of adventitious roots of cuttings of hard-to-root horticultural crops (Li et al., 2004). Zhang and colleagues (2000) reported that endogenously

produced cytokinin can regulate senescence caused by flooding stress, thereby increasing plant tolerance to flooding. A bacteria ethylene-forming enzyme (EFE), cloned into transgenic plants, causes a high rate of ethylene production compared to untransformed plants (Araki et al., 2000). Dwarf morphology was observed in transgenic tobacco that resembled the phenotype of a wild-type plant exposed to excess ethylene. Dwarf Phalaenopsis orchid plants were also produced by over-expression of the gibberellin 2-oxidase gene (Chin and Mii, 2011). Moreover, it is possible to use transgenic technology to manipulate the kinds of hormones in plants to improve performance, such as stress tolerance, in horticultural crops.

Wakita and colleagues (2001) reported that the nutritional value of sweet potato was improved by transgenic changes to the fatty acid composition. Transgenic tomato plants expressing the ACC deaminase gene withstand flooding stress (low oxygen) better than untransformed plants and are less subject to the deleterious effects of root hypoxia on plant growth (Grichko and Glick, 2001). The transgenic plants have greater shoot fresh and dry weight, produce lower amounts of ethylene, and have higher amounts of leaf chlorophyll content.

During 1998-2001, more than 40 orchid genes were discovered. However, only a few of these sequenced genes could be directly applied to orchid production and improvement (Kuehnle, 2007). Some of the published orchid genes are summarized in Table 2.

Gene name	Gene origin	Reference
DCAC	*Dendrobium crumenatum*	Yang et al., 1996
DOH1	*Dendrobium* Madame Thong-In	Yu and Goh, 2000
DOMAD1	*Dendrobium* Madame Thong-In	Yu and Goh, 2000
Ovg14	*Dendrobium* Madame Thong-In	Yu and Goh, 2000
DSCKO1	*Dendrobium*	Yang et al., 2003

Table 2. Some genes discovered in orchids.

Using the orchid genes or other sequenced genes, genetic transformation of orchids can be conducted using three major approaches including particle-bombardment, direct gene transfer to protoplasts, and *Agrobacterium*-mediated transformation. Several studies regarding orchid transformation are summarized in Table 3.

Species	Transformation method	Reference
Phalaenopsis	*Agrobacterium*-mediated	Semiarti et al., 2007
Phalaenopsis	*Agrobacterium*-mediated	Semiarti et al., 2010
Phalaenopsis	*Agrobacterium*-mediated	Belarmino and Mii, 2000
Phalaenopsis	*Agrobacterium*-mediated	Chin and Mii, 2011
Dendrobium	*Agrobacterium*-mediated	Semiarti et al., 2008
Dendrobium	Particle-bombardment	Kuehnle and Sugii, 1992
Vanda tricolor	*Agrobacterium*-mediated	Semiarti et al., 2009
Cymbidium	Particle-bombardment	Yang et al., 2000

Table 3. Several studies on orchid transformation.

3.3 Cultivation and propagation

Another modern biotechnological procedure in orchid breeding is plant tissue culture, which is widely used for orchid cultivation and propagation. The orchid seed has no

endosperm, so the probability of growing naturally in its habitat is very low, necessitating plant tissue culture. Plant tissue culture techniques use rich nutrient culture medium and aseptic conditions with growth in transparent bottles. The orchid seed obtains its nutrients from the media and can grow normally. The phenotype of the plantlet varies from its parent. If we use explants from leaves, shoots, or other vegetative organs, we can produce identical offspring. As we know, orchids need a long time to reproduce, but using plant tissue culture techniques, we can produce offspring in large quantities in a very short time. This is very useful for breeders and orchid conservationists. Plant tissue culture is also a tool that facilitates genetic engineering, so it is being continuously refined and should be studied by orchid researchers.

3.4 Standard techniques of orchid micropropagation

Several aspects that need to be considered in plant tissue culture are culture conditions, media components, and aseptic conditions. Ideal culture conditions for orchid micropropagation depend on the type of medium, pH, illumination, photoperiod, and temperature. Both liquid and solid media can be used, but proliferation is faster and more extensive in liquid media; however, differentiation is always better in solid media. The optimum pH for orchid micropropagation is approximately 5-7. Suitable illumination for orchid tissue is from darkness to 2000 ft-c (ft-candles). Photoperiods used for orchid tissue culture vary from none (constant illumination or darkness) to short and long days (12-18 hours). Orchid tissue culture is usually maintained at a temperature of 22 to 26°C (Arditti and Ernst, 1993). The medium components for orchid micropropagation are mostly the same as those for other plant culture media. It must contain macro elements (C, H, O, N, S, P, K, Ca, Mg, and Fe), microelements, hormones, myo-inositol, vitamins, amino acids, adsorbents, solidifying agents, and complex organic additives (Arditti and Ernst, 1993; George and Sherrington, 1984).

In principle, all plant cells can be cultured because they all have totipotency and autonomic properties. However, in practice, only meristematic cells can be used as explants for plant tissue culture. Micropropagation of orchids was first performed by German orchid expert Hans Thomale in 1957, who used *Orchis maculata* shoot-tips as explants. The parts of the orchid usually used as explants are the shoot-tip, root-tip, leaf, flower bud and segments, stems, cells, and protoplasts (Arditti and Ernst, 1993). The use of phytohormones in the orchid medium is very important, as different combinations of phytohormones can induce different organs to grow. The most common combination in orchid media is auxin and synthetic cytokinin, while giberelin is very seldom used. Combinations of indole-3-acetic acid (IAA) and indole-3-butanoic acid (IBA) can induce root growth in the *Dendrobium* orchid's protocorm-like body (Khatun et al., 2010). The family of cytokinin-like kinetins, including 6-benzyl amino purine (BAP) and dimethyl allyl phosphate (DMAP), are known to induce shoots (Arditti and Ernst, 1993). Thus, the phytohormones used depend on the aim of the orchid culture.

Orchids, like any other plant cultured in medium, need acclimatization before being planted in a pot. Plant tissue culture uses rich media to support orchid growth, which while good for the orchid, has the effect of limiting its ability to produce food for itself. Acclimatization is needed to adapt the orchid to its new environment and to change it from heterotrophic into autotrophic conditions (Arditti and Ernst, 1993). In their natural habitat, orchids cannot always obtain optimum conditions to grow, so we need to adapt orchids to their new environment slowly. This is usually done in a greenhouse.

4. *Agrobacterium*-mediated genetic transformation of Indonesian orchids

4.1 Transformation of *Phalaenopsis amabilis* (L.) Blume

Phalaenopsis amabilis (L.) Blume is one of the national flowers of Indonesia, along with the padma (*Rafflessia arnoldi*) and jasmine (*Jasminum sambac*). This orchid is distributed widely in some islands such as Java, Kalimantan, Sulawesi, Maluku, Papua, and also the Philippines and North Australia. Due to its long-lasting flowering, the well-known *P. amabilis*, with its large white flowers, is one of the most important ancestor species of *Phalaenopsis* hybrids. Hybrids of this orchid are of great economic value as house and garden plants as well as cut flowers. These hybrids are usually clonally propagated. A problem in this respect is that seedlings initially form only a single vegetative shoot (Dressler, 1981). However, additional shoots induced from cut protocorm-like bodies (PLBs) can be efficiently obtained using new *Phalaenopsis* medium, which contains a high concentration of nitrogen (Islam et al., 1998).

To improve the potential of the orchid's micropropagation, we developed a genetic transformant of *P. amabilis* using *Agrobacterium tumefaciens* that harbors T-DNA with a meristem-related gene, *BP/KNAT1*. We started with intact protocorms (developing orchid embryos) of *P. amabilis* that were maintained in a pre-culture NP medium with the addition of 100 g l^{-1} tomato extract (Semiarti et al., 2010). Regenerated plants under the same conditions showed the highest frequency of shooting. A kanamycin resistance gene under the control of the 35S promoter was used as a selective marker. In addition, T-DNA vectors containing the *Arabidopsis* class 1 *KNOX* gene, *BP/KNAT1,* were successfully introduced into protocorms. The protocorms transformed with *BP/KNAT1* produced multiple shoots. Both the presence and expression of the transgene in transformed plants were confirmed by molecular analysis.

To introduce the *KNAT1* gene into *P. amabilis,* the entire coding region of *BP/KNAT1*cDNA was cloned in a pG35S binary vector to generate pG35SKNAT1. To generate pG35S, two DNA fragments, one containing the promoter for 35S RNA from the cauliflower mosaic virus (P35S) and the other the terminator of the nopaline synthase gene, were amplified from pTH-2 (Chiu et al., 1996) by PCR. These two amplified fragments were introduced into the multiple cloning sites of the binary vector pGreen. Disarmed octopine type *A. tumefaciens* strain LBA 4404 was used for transformation. Nucleic acids of the orchids were isolated from the protocorms and leaves of wild-type or putative transgenic plants by the method of Semiarti et al. (2001). cDNA was synthesized from the mRNA using the TimeSaver cDNA synthesis kit (Amersham Biosciences, USA). The cDNA was used as a template for RT-PCR analysis.

Transformation was started by preparation of overnight cultures of *A. tumefaciens* that were diluted 1:4 (v/v) using NP (Ishii et al., 1998) liquid medium supplemented with 15% (v/v) coconut water, 2% (w/v) sucrose, 5 μM benzyladenine and 0.01% silvet L-77 (Nippon Unicar Co. Ltd., Tokyo). Prior to modification, orchid protocorms were cultured for 4 days in modified NP solid medium containing 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 100 mg l^{-1} tomato extract, then immersed in the diluted culture of *A. tumefaciens* for 30 min. These protocorms were transferred onto sterile filter paper to dry and then onto modified NP medium containing 1 mg l^{-1} 2,4-D without antibiotics. After 4 days, the explants were transferred onto solid NP medium containing 1 mg l^{-1} 2,4-D and 300 mg l^{-1} carbenicillin, which inhibits the growth of *A. tumefaciens* , and cultured for 4 weeks. Protocorms that grew were rinsed thoroughly with liquid NP medium containing 200 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin, then transferred onto medium containing 5 μM N^6-(Δ^2-isopenthenyl)

adenine (2-IP), 0.15 μM synthetic auxin (NAA), 200 mg l^{-1} kanamycin, and 300 mg l^{-1} carbenicillin. The developing protocorms were transferred onto new medium every 3 weeks for further selection of transformants. Developing shoots were screened by PCR using a *BP/KNAT1* specific primer to confirm that they were transformants. When the shoot and roots had grown sufficiently, the plantlets were transferred onto NP medium supplemented with 100 mg l^{-1} kanamycin and 50 mg l^{-1} carbenicillin.

Genomic DNA from the putative 35S::*BP/KNAT1* transformants was analyzed by PCR using primers KNAT1F1 and KNAT1R1, which are specific for the *BP/KNAT1* gene. First, we transformed the construct pG35S, which contains the kanamycin resistance gene. The experiments were performed twice. The protocorms that had been cocultivated for 4 days with *A. tumefaciens* harboring pG35S produced shoots at frequencies of 1.7 and 1.5% on kanamycin-containing medium (Table 4). We obtained 35 shoots out of 2,150 protocorms on kanamycin- and/or carbenicillin-containing medium after cocultivation with *A. tumefaciens* harboring pG35S in these experiments. The thirty-five shoots were independent, since each protocorm produced only one shoot (Figure 3).

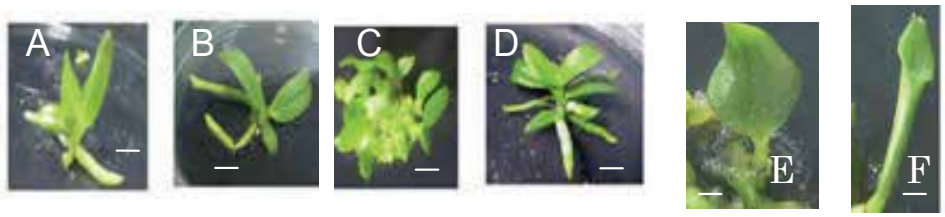

Fig. 3. Phenotype of non-transformant (NT), vector transformant, and 35S::KNAT1 transformant orchid plants. A. NT, B. vector-only transformed plant, C. 35S::KNAT1 multishoot transformant (6 months old), D. 35S::KNAT1 transformant (9 months old), E. abnormal trumpet-like leaf shape, and F. quadrat shape of leaf. Bars: 5 mm in A-D, 1 mm in E and F.

Antisense *DOH1* expression also causes abnormal multiple shoot development in *Dendrobium* orchids, indicating a role for *DOH1*, another member of the class 1 *KNOX* family, in basic plant architecture (Yu et al., 2001).

	Exp	Number of protocorms examined	Number of protocorms producing shoots	Frequency of transformation (%)*	Number of regenerated plants
Non-transformant	1	100	0	0/0	0
PG35S	1	1150	20	1.7	20
PG35S::KNAT1	2	1000	15	1.5	15

Table 4. Frequency of *Agrobacterium*-mediated transformation in *P. amabilis.* * Ratio of the number of protocorms producing shoots to total number of transformed protocorms.

In addition, both *DOH1* sense and antisense transformants exhibit defects in leaf development (Yu et al., 2000; 2001). Since the transformation frequency using pG35SKNAT1 was one-seventh of that using pG35S, the *BP/KNAT1* gene might somehow affect the efficiency of transformation due to multishoot production (Figure 4).

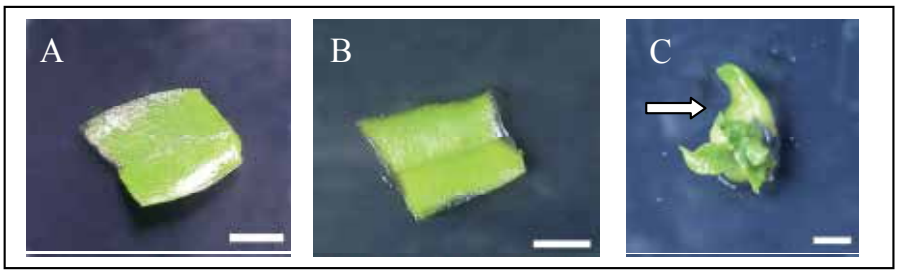

Fig. 4. Multishoot generation from leaf culture of 35S::KNAT1 orchid transformant. A-C. leaf disc of non-transformant (A), transformant with vector only (B), and 35S::KNAT1 transformant (C). Bars: 5 mm. Arrow is pointing to multishoots.

Although the function of members of the class 1 *KNOX* family is not known in *P. amabilis*, further studies using transformed *P. amabilis* plants are also expected to lead to a better understanding of the function of genes that are involved in developmental processes, including shoot and leaf development. In this experiment, we showed that *Phalaenopsis* protocorms transformed with the *KNOX* gene of *Arabidopsis* produced multiple shoots with trumpet-like, medio-laterally unopened blades of leaves that were not observed in rice (Figure 3). First, we thought that some of these phenotypes might be due to over-expression and ectopic expression of the *KNOX* gene. Alternatively, these phenotypes might be produced by suppression of the function of endogenous genes by ectopic expression of the *Arabidopsis KNOX* gene. Finally, however, the phenotype gradually returned to normal after acclimation in a pot under greenhouse conditions. These phenomenon should be investigated in future studies by functional analysis of the class 1 *KNOX* genes of *P. amabilis*. Production of multishoots will have a good impact on micropropagation of orchids through tissue culture.

Maryani (2010, personal communication) introduced the *Wasabi defensin* gene that is responsible for soft rot disease resistance into *P. amabilis* with the *Bar* gene responsible for herbicide resistance. Development of a method for improving *Phalaenopsis* orchids through genetic modification could be extremely valuable for horticulture and, indirectly, also for conservation, as well as contributing to understanding the functions of genes in *Phalaenopsis* orchids.

The core component of genetic modification of orchids is the need to create efficient and reproducible gene transformation systems. A reproducible methodology for the genetic transformation of orchids, and better recognition of the factors affecting the transformation process, are needed in order to support this objective. Previous studies have reported orchid transformation either directly through the delivery of marker genes such as those encoding *Escherichia coli* β-glucuronidase (*GUS*) and *Aequorea victoria* green fluorescent protein (*GFP*) into plant cells by particle bombardment (Anzai et al., 1996), or indirectly through the use of *A. tumefaciens* (Belarmino and Mii, 2000; Chia et al., 1994; Mishiba et al., 2005; Chan et al., 2005; Sjahril et al., 2006; Sjahril and Mii, 2006). Recently, we developed a convenient method for genetic modification of *Phalaenopsis amabilis* orchids using *A. tumefaciens* (Semiarti et al., 2007) in which intact protocorms (young orchid seedlings) were used for transformation.

This method is simple, reproducible, and applicable to other species. However, the transformation efficiency was ≤2%, and further studies are needed to improve this.
For *in vitro* germination of orchid seeds, organic substances such as coconut water and tomato extract are commonly used as media supplements. The presence of anti-oxidants such as vitamin C, sugars, and other compounds in tomato extracts may promote the germination and growth of protocorms (Arditti and Ernst, 1993). Perl et al. (1996) determined that a combination of polyvinylpolypyrrolidone (PVPP) and dithiothreitol (DTT) as anti-oxidants improved plant viability. Tissue necrosis in *A. tumefaciens* -treated embryogenic calli of grapevine plants was inhibited completely using these anti-oxidants, while the virulence of *A. tumefaciens* remained unaffected. These treatments enabled the recovery of stable transgenic grapevine plants resistant to hygromycin.
In order to improve the frequency of *Agrobacterium*-mediated transformation of *P. amabilis*, we pre-cultured the protocorms in medium containing an extract from fully-ripe tomatoes and/or coconut water and investigated the effect of this pre-culturing treatment on improving the efficiency of regeneration of transformed shoots. Adult plants of *P. amabilis* (L.) Blume from Java were obtained from Royal Orchids (Prigen, East Java, Indonesia). Seeds were derived from cross-pollinated plants that had been sown on modified, new *Phalaenopsis* (NP) medium (Islam et al., 1998) and maintained under continuous white light. Adult plants were maintained in a glasshouse at room temperature. Seeds were sown on modified NP medium with various concentrations of coconut water (50–150 ml l^{-1}) and/or tomato extract (50–200 mg l^{-1}) and grown for 3 weeks to produce protocorms, which were used for transformation. Coconut (*Cocos nucifera* from Java) and the tomato (*Lycopersicon esculentum*) cultivar 'Arthaloka' from West Java were obtained from local markets. Tomato fruit extract was prepared by cutting tomatoes into 1-cm^3 cubes, homogenizing them, and filtering the homogenate through a steel mesh with a 150-μm pore size. The nutrient compositions of the coconut water sample and the tomato extract were analyzed by high performance liquid chromatography.
To determine the growth rates of orchid embryos and protocorms, the sizes, colors, and shapes of the embryos or protocorms were evaluated as described by Dressler (1981). At Stage 0, each intact seed (270–400 μm long) with its embryo (100–200 μm long) is coated by a layer of net-like cells, the testa. At Stage 1, the testa spreads apart and the embryo swells into an ovoid-shaped mass of cells. At Stage 2, the seed coat cracks and the mass of cells grows outside the coat (0.5–1.0 mm long). At Stage 3, the mass of cells elongates gradually into a cone-shaped body (1.0–1.4 mm long). At Stage 4 (the protocorm), root hairs emerge from the basal portion of the cone-shaped body, which turns green. At Stage 5, the photosynthetic protocorm forms a leafy shoot at its apex and forms new root hairs. After Stage 5, seed germination is complete, two leaves gradually emerge, and roots are formed.

4.2 Plasmid vector and bacterial strain

Using the binary plasmid vector pBI121 (Clontech Laboratories Inc., Otsu, Japan), containing a kanamycin resistance gene and the 35S CaMV promoter with the 3' *nos* terminator, a PCR-amplified fragment containing the entire coding region of the *GFP* gene was used to generate a plasmid that we designated pBI121-p35S::GFP. This construct was introduced into the disarmed, octopine-type *A. tumefaciens* strain LBA4404 (Hoekema et al., 1983). Nucleic acids were isolated from the protocorms and leaves of wild-type or putative transgenic plants by the method of Semiarti et al. (2001) using the QIAGEN DNA

purification kit for isolation of genomic DNA and QIAGEN RNeasy mini kit (QIAGEN GmbH, Germany) for isolation of total RNA. mRNA was isolated from total RNA using Dynabeads Oligo $(dT)_{25}$ (DYNAL, Norway), and cDNA was synthesized from the mRNA using the TimeSaver cDNA synthesis kit (Amersham Biosciences, USA). The cDNA was used as a template for RT-PCR analysis.

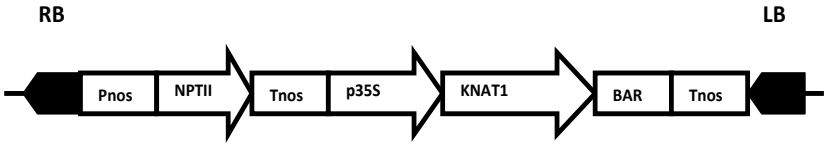

Fig. 5. Schematic structure of 35S::KNAT1 containing T-DNA. LB, left border; RB, right border; 35S: CaMV promoter; KNAT1: BREVIPEDICELLUS (BP)/KNAT1 gene; NPTII: neophosphotransferase II; Tnos: nos terminal.

4.3 Transformation and transformant regeneration

Overnight cultures of *A. tumefaciens* were diluted 1:4 (v/v) using NP liquid medium supplemented with 15% (v/v) coconut water, 2% (w/v) sucrose, 5 µM benzyladenine, and 0.01% silvet L-77 (Nippon Unicar Co. Ltd., Tokyo). Prior to modification, orchid protocorms were cultured for 4 days in modified NP solid medium containing 1 mg l^{-1} 2,4-D, then immersed in the diluted culture of *A. tumefaciens* for 30 min. These protocorms were transferred onto sterile filter paper to dry and then onto modified NP medium containing 1 mg l^{-1} 2,4-D without antibiotics. After 4 days, the explants were transferred onto solid NP medium containing 1 mg l^{-1} 2,4-D and 300 mg l^{-1} carbenicillin, which inhibits the growth of *A. tumefaciens* , and cultured for 4 weeks. Protocorms that grew were rinsed thoroughly with liquid NP medium containing 200 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin, then transferred onto medium containing 5 µM 2-isopentenyl adenine (2-IP), 0.15 µM naphthalene acetic acid (NAA), 200 mg l^{-1} kanamycin, and 300 mg l^{-1} carbenicillin. The developing protocorms were transferred onto new medium every 3 weeks for further selection of transformants. Genomic DNA from putative 35S::GFP transformants was analyzed by PCR using the following selective forward (F) and reverse (R) primers to detect both the kanamycin resistance gene (neomycin phosphotransferase II; *NPTII*) and the *GFP* gene: NPTIIF1 (5′-CCTGCCCATTCGACCACCAA-3′) and NPTIIR1 (5′-AGCCCCTGATGCTCTTCGTC-3′) for the *NPTII* gene; and GFPF1 (5′-ATGGTGAGCAAGGGCGAGGA-3′) and GFPR1 (5′-GTCCATGCCGTGAGTGATCC-3′) for the *GFP* gene. PCR was performed with 30 cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 90 s. As an internal control, genomic DNA was amplified using primers for the *ACTIN* gene, as described by Semiarti et al. (2007). To detect *GFP* gene expression in the transformants, seedlings or plant tissues were excited with blue light (495 nm) using a Nikon Diaphot 300 microscope (Nikon Corp., Tokyo, Japan) equipped with a B2 filter, which distinguishes the red autofluorescence of chlorophyll from the fluorescence of GFP. The images were captured using a Nikon Cool Pix 5000 digital camera system with an adaptor for microscopy (Nikon Corp.).

4.4 DNA analysis by Southern hybridization

Genomic DNA from 9-month-old leaves of five independent transgenic lines of *P. amabilis* that expressed GFP fluorescence was digested using the restriction enzymes *Eco* RI and

*Hin*d III. These plants also yielded the predicted 360-bp PCR product using a primer pair designed for the *GFP* coding region. The digested genomic DNA fragments were transferred to a nylon membrane (Amersham Hybond-N+; GE Healthcare, Cambridge, UK) and hybridized with a digoxigenin-labeled probe for the *GFP* gene derived from the plasmid pBI121-GFP (12.6 kbp) using the DIG DNA Labeling Kit (Roche Diagnostics, Tokyo, Japan). The hybridized DNA fragments were visualized using the DIG Luminescent Detection Kit (Roche Diagnostics) according to the manufacturer's instructions.

4.5 Effect of tomato extract on the formation of shoots from protocorms of *P. amabilis*

We tested coconut water and tomato extract as potential supplements to accelerate the growth of *Phalaenopsis* embryos, especially at the early developmental stages, using embryos grown on NP medium with or without either supplement. Based on the growth stage classification described above, we determined the optimal concentration of tomato extract based on the number of growing embryos and protocorms found at each stage (Table 5). The number of seeds developing to Stage 4 was increased at higher concentrations of tomato extract in the NP medium, achieving an optimal number at 100–150 mg l^{-1} tomato extract. Therefore, 100 mg l^{-1} tomato extract was used in the following experiments.

We also analyzed growth rates on NP medium with or without coconut water and tomato extract (Figure 6A). The fastest rate of embryo development was observed on NP medium supplemented with both coconut water and tomato extract. Protocorms cultured on NP medium containing tomato extract alone appeared to change from yellow to green more rapidly than those cultured on NP medium containing coconut water alone. Tomato extract thus appeared to affect the growth rate at all stages of embryo development, including the

Concentration of tomato extract (mg/l)	Total no. embryos examined	Experiment No.	Embryo Stage 0 (%)	Embryo Stage 1 (%)	Embryo Stage 2 (%)	Swollen embryo (protocorm) Stage 3 (%)	Green protocorm Stage 4 (%)	Protocorm with shoot apical meristem Stage 5 (%)
0	806	1	23.9	12.2	3.6	20.8	39.1	0.5
		2	18.7	5.4	7.4	30.5	36.9	1.0
		3	24.4	2.2	4.9	40.1	28.3	0.0
50	1148	1	22.2	3.6	4.3	40.2	29.3	0.4
		2	27.5	6.0	8.8	28.4	28.4	0.9
		3	28.0	6.9	4.0	28.7	31.5	0.9
100	1577	1	24.9	4.7	5.5	13.0	51.1	0.8
		2	31.3	1.9	6.1	9.0	51.2	0.5
		3	36.0	4.1	2.0	15.8	42.1	0.0
150	1417	1	46.0	4.8	3.7	9.5	36.1	0.0
		2	26.2	1.8	5.6	14.6	51.8	0.0
		3	35.5	2.9	4.4	15.0	42.3	0.0
200	1583	1	49.6	3.1	2.8	9.7	34.7	0.0
		2	62.6	7.8	3.6	6.0	20.1	0.0
		3	52.5	5.7	1.1	17.0	23.7	0.0

Table 5. Growth stages of *P. amabilis* cultured on NP medium supplemented with different concentrations of tomato extract for 21 days after sowing. Reproduced from the Journal of Horticultural Science & Biotechnology with permission from the editor.

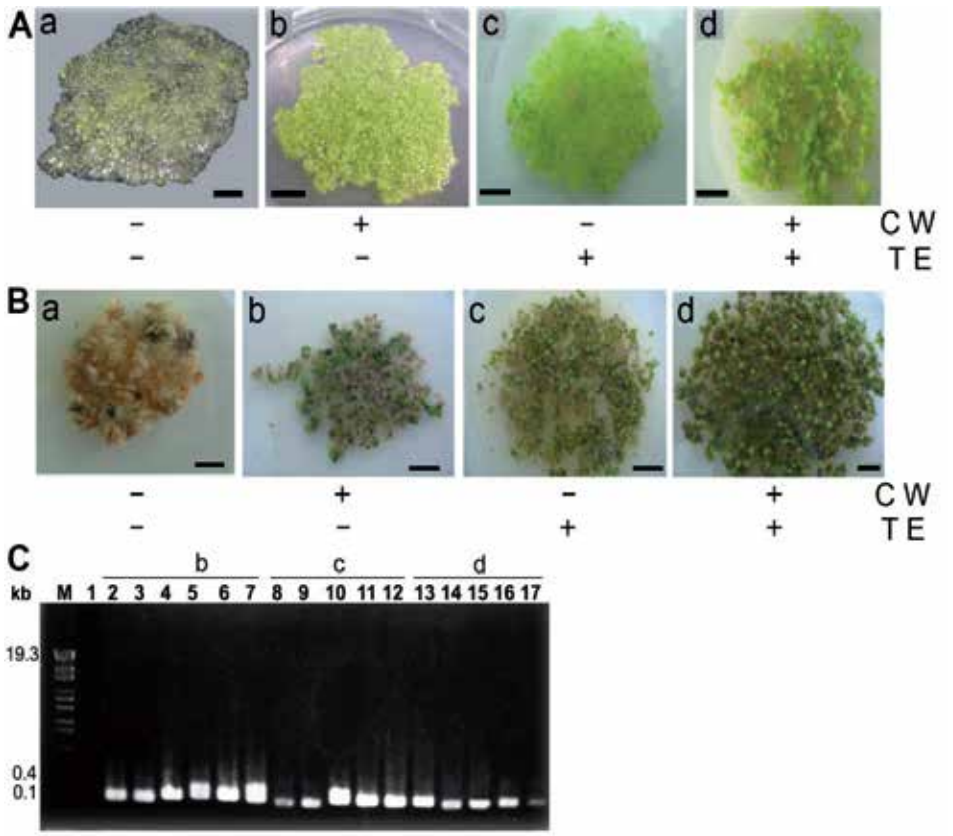

Fig. 6. Main panel A, growth of protocorms of *P. amabilis* on various culture media 3 weeks after sowing. Sub-panel a, NP medium; sub-panel b, NP medium supplemented with 150 ml l^{-1} coconut water (CW); sub-panel c, NP medium supplemented with 100 mg l^{-1} tomato extract (TE); and sub-panel d, NP medium supplemented with 150 ml l^{-1} CW and 100 mg l^{-1} TE. Main panel B, development of shoots from protocorms of *P. amabilis* that had been cultured on NP medium supplemented with coconut water (CW) and/or tomato extract (TE) for 3 weeks. Protocorms were selected from NP medium containing 200 mg l^{-1} kanamycin after *Agrobacterium*-mediated transformation with pBI121 after 5 weeks. Sub-panel a, unregenerated protocorms on medium containing 200 mg l^{-1} kanamycin (Km); sub-panels b–d, kanamycin-resistant seedlings produced from protocorms that had been transformed with pBI121 containing the kanamycin resistance gene (*NPTII*). Main panel C, PCR detection of the kanamycin resistance gene (*NPTII*) in putative transgenic orchid plants harboring pBI121. Fragments from a *Sty*I digest of phage DNA were used as size markers (M). No amplified DNA fragments from the kanamycin resistance gene were seen in DNA from untransformed *P. amabilis* orchid plants (lane 1). The specific 105-bp PCR fragment of the *NPTII* gene was amplified from the DNA of putative transgenic orchid seedlings from sub-panels b (lanes 2–7), c (lanes 8–12), and d (lanes 13–17) in main panel A. Bars: 5 mm (from Semiarti et al., 2010). Reproduced from the Journal of Horticultural Science & Biotechnology with permission from the editor.

Component	Coconut water	Tomato extract
Ash	0.55%	0.31%
Lipid	0.05%	0.47%
Total protein (soluble protein)	0.19% (0.17%)	1.78% (1.46%)
Total sugars (reducing sugars)	3.22% (3.02%)	3.70% (3.39%)
Total carotene	Nd	1.84%
Antioxidants (DPPH)*	Nd	0.024%
Vitamin C	Nd	0.042%
Crude fibre	Nd	1.05%
Phosphate (P_2O_5)	0.013%	0.13%
Inorganic ions		
Mg^{2+}	0.0058%	0.0081%
Mn^{2+}	0.00021%	0.000029%
Na^{+}	0.046%	0.0090%
K^{+}	0.23%	0.16%
pH	5.16	4.34

Table 6. Components of coconut water and tomato extract used in this study. * Total carotene antioxidants measured based on the activity of antioxidants against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals from the sample. Reproduced from the Journal of Horticultural Science & Biotechnology with permission from the editor.

formation of the shoot apical meristem prior to the emergence of the leaf primordia. The tomato extract contained carotene, vitamin C, and other anti-oxidants which were not detected in coconut water (Table 6). These components could affect the growth of the embryo.

Oladiran and Iwu (1992) showed that fully-ripe tomato fruit contains basic nutrients and essential vitamins, as well as trace elements. Among these, carotenoids with cyclic end-groups were essential components of all photosynthetic membranes and played several roles, including protection against photo-oxidation (Cunningham et al., 1996). These are potential candidates for the growth-promoting compounds in the tomato extract, as it is rich in carotenoids. We therefore tested a single carotenoid, lycopene, for its possible effects on growth promotion, but found no significant effect at concentrations typically found in tomato extracts [≤ 0.1% (w/w)], while high concentrations of lycopene inhibited seed growth (data not shown). Further studies on other components found in tomato extract are needed to determine whether any single compound has an effect, or if several compounds have a synergistic effect, on the growth and development of *P. amabilis* seeds.

4.6 Effect of pre-culture of protocorms on NP medium containing tomato extract on the transformation frequency of *P. amabilis* orchids

Protocorms were pre-cultured on NP medium supplemented with coconut water and/or tomato extract prior to transformation to determine the effects of pre-culture supplementation on the frequency of transformation (Table 7). The transformation efficiency was determined based on the percentage of protocorms that produced shoots on the

selective medium out of the total number of protocorms examined. The transformation frequency of regenerated shoots was increased from 1.2% on NP medium with coconut water alone to 13.2% on NP medium containing 100 mg l^{-1} tomato extract alone, and to between 6.8–16.6% on NP medium containing both coconut water and tomato extract (Table 7; Figure 6B, panel D). These results were higher than the frequency of transformed regenerated shoots on medium containing coconut water alone (1.2%; Table 7), confirming the observations made by Semiarti et al. (2007).

In the case of transformation with pBI121-p35S::GFP, transformed regenerated shoots were produced at frequencies of 9.8–13.5% following pre-culture on NP medium supplemented with both coconut water and tomato extract (Table 7). Overall, the transformation frequencies of protocorms pre-cultured on NP medium supplemented with tomato extract alone, or with both coconut water and tomato extract, were higher than those for protocorms pre-cultured on NP medium supplemented with coconut water alone, suggesting that the growth rate of protocorms was related to the pre-culture conditions which are therefore important for the regeneration of transformed shoots.

Several studies have examined the use of rich sources of nutrients, vitamins, and phytohormones, including coconut water, carrot, maize, or potato extracts, as possible supplements for stimulating the germination of various orchid species (Arditti and Ernst, 1993; Raghavan, 1997; Islam et al., 2003; Mishiba et al., 2005; Chansean and Ichihashi, 2007). More studies on other sources of nutrients may be required to establish an optimum method for transformation.

Plasmid	Coconut water	Tomato extract	Total no. protocorms examined	No. protocorms producing shoots (% of total)
None	+	+	1557	0 (0%)
pBI121 (vector)	+	-	1200	14 (1.2%)
pBI121 (vector)	-	+	1200	159 (13.2%)
pBI121 (vector)	+	+	1557	260 (16.6%)
			1500	102 (6.8%)
pBI121-p35S::GFP	+	+	1557	210 (13.5%)
			1500	147 (9.8%)

Table 7. Transformation frequency of *P. amabilis* protocorms following 3 weeks of pre-culture on NP medium supplemented with tomato extract and coconut water. Reproduced from the Journal of Horticultural Science & Biotechnology with permission from the editor.

We used this transformation method on the pandanus orchid (*Vanda tricolor var Suavis* Form Merapi). Protocorms were pre-cultured on NP medium supplemented with coconut water and tomato extract prior to transformation. The transformation frequency of regenerated shoots was 20.3%. This result indicates that the transformation method may be useful for other orchid species.

4.7 Molecular analysis of putative transformants

We examined the genomic DNA from *P. amabilis* plantlets regenerated on agar plates containing 200 mg l^{-1} kanamycin for the presence and expression of the kanamycin resistance gene (*NPTII*) using PCR. The predicted 105-bp fragment was amplified from all putative transformants in each treatment (Figure 7C). The plantlets that regenerated after

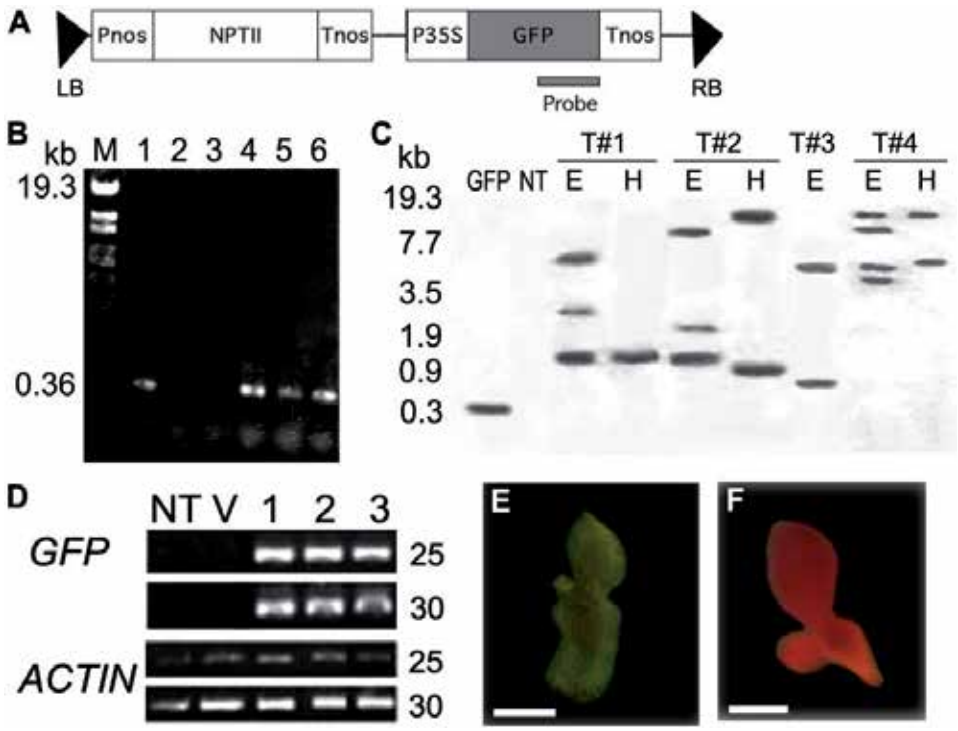

Fig. 7. Analysis of putative *GFP* transformants of *P. amabilis.* A: Schematic representation of the T-DNA region of the binary plasmid pBI121-p35S::GFP. The binary plasmid pBI121-p35S::GFP contained the 720-bp *GFP* gene, which encodes the jellyfish GFP under control of the 35S promoter of cauliflower mosaic virus (CaMV). RB, right border; LB, left border; Pnos, promoter of the nopaline synthase gene; Tnos, polyadenylation site of the nopaline synthase gene; NPTII, neomycin phosphotransferase gene; P35S, 35S promoter of CaMV. A 360-bp fragment from the 3′ end of the *GFP* gene was used as a probe during Southern hybridization. B: PCR analysis of the *GFP* transgene in putative transgenic orchids. Fragments from a *Sty*I digest of λ phage DNA were used as size markers (M), and the specific 360-bp DNA fragment was amplified from the plasmid pBI121-p35S::GFP (lane 1), and fragments were amplified from DNA of an untransformed *P. amabilis* plant (lane 2), a plant transformed with the empty vector pBI121 (lane 3), and three plants independently transformed with pBI121-p35S::GFP (lanes 4-6). No fragment was amplified from DNA from untransformed plants (lane 3) or empty vector-transformed plants (lane 4). C: Southern blot analysis. Lane 1: fragments from a *Sty*I digest of λ phage DNA as the size marker. GFP: GFP probe; NT: genomic DNA from an untransformed plant was digested with *Eco*RI; T#1-4: genomic DNA (5 µg) from transformed plants was digested with *Eco*RI (E) or *Hind*III (H). Fragments were fractionated in a 1% agarose gel, then blotted and hybridized with a digoxigenin (DIG)-labelled *GFP* gene probe. D: expression of the *GFP* gene in putative transgenic *P. amabilis* plants. RT-PCR analysis of transcripts of the *GFP* gene in a wild-type plant (NT), in a plant transformed with the empty vector pBI121 (V), and in pBI121-p35S::GFP-transformed plantlets (1-3). The number of cycles is indicated at the right of each panel. Amplified DNA fragments were separated by electrophoresis in an agarose gel and visualised with ethidium bromide. As a control, the same samples were amplified with primers specific for the *ACTIN* gene transcript. See the Materials

and Methods for details about RT-PCR. E and F: detection of GFP expression in a putative 35S::GFP transformant plantlet (E) and untransformed plantlet (F) under blue light. Bars: 1 cm (from Semiarti et al., 2010). Reproduced from the Journal of Horticultural Science & Biotechnology with permission from the editor.

transformation with the plasmid pBI121-p35S::GFP were examined for the presence of the *GFP* gene by PCR amplification of the 360-bp fragment from the GFP coding region (Figure 7B). Of the 210 plantlets examined, 191 were positive for the *GFP* gene fragment.

To confirm the presence of the *GFP* gene, and to assess the gene copy number in plants that also showed kanamycin resistance, we performed Southern hybridizations. Hybridization using an anti-sense probe for the 3' end of the *GFP* gene (Figure 7A) showed two to four copies of the *GFP* gene in each transgenic line (Figure 7C). Since the genomic DNA of each putative transgenic plant showed uniquely-sized bands hybridizing to the *GFP* anti-sense fragment, this T-DNA fragment was confirmed to be inserted into the genome at different independent sites, and in multiple copies in each putative transgenic plant line.

For further analysis, we purified total poly(A)+ RNA from individual leaves of an untransformed wild-type plant, a plantlet transformed with pBI121, and three lines transformed with pBI121-p35S::GFP. We quantified the relative levels of *GFP* gene transcripts (mRNA) using RTPCR with primers specific for *GFP*. PCR products were detected in all three lines of plantlets transformed with pBI121-p35S::GFP, but not in the untransformed plantlet or the plantlet transformed with pBI121 alone (Figure 7D). Thus, transcripts of the *GFP* gene had accumulated in the leaves of the transformants, confirming expression of the *GFP* transgene in these plants. Plantlets transformed with pBI121-p35S::GFP showed green fluorescence after excitation with blue light (Figure 7E), whereas untransformed plantlets did not (Figure 7F). Taken together, the molecular analyses of the transformants strongly suggests that supplementation using tomato extract during pre-culture in NP medium improved the transformation efficiency of *P. amabilis* by several-fold.

4.8 Transformation of *Vanda tricolor* Lindl. var. Suavis

Vanda tricolor Lindl. var. Suavis is an Indonesian wild orchid that has spread throughout Indonesian territory and is grown in some regions such as Mount Merapi (Central Java and Yogyakarta), Bali, East Java, and West Java. Since the Mount Merapi is a very active volcano with frequent pyrroclastic flows and eruptions, the population of *V. tricolor* in its natural habitat is now extremely rare due to habitat loss. Micropropagation to save the population of this orchid is worthwhile as are *ex situ* conservation efforts. Dwiyani et al. (2011, in press) developed a transformation method based on that previously published by Semiarti et al. (2007) with the use of acetosyringone to improve transformation efficiency of the *V. tricolor* Merapi Form. Acetosyringone was added into the protocorm and *A. tumefaciens* co-cultivation medium.

Vanda tricolor pods (6 months after self pollination) from Mount Merapi were used as seed sources. Orchid pods were washed, sterilized three times by dipping in 70% ethanol, flamed, and then put gently on sterilized petri dishes in a laminar air flow hood. Seeds were then sown in New Phalaenopsis (NP) medium (Islam et al., 1998) enriched with 100 g l^{-1} tomato extract as used by Semiarti et al. (2010). Five weeks after sowing, protocorms were collected to be used for target transformation. A kanamycin test was performed on 8 and 10 week-old protocorms. The disarmed octopine type of *A. tumefaciens* LBA4404 harboring the p35S binary vector containing the *Neophosphotransferase* (*NPTII*) gene as a selectable marker was used for transformation.

Three days before infection, eight week-old germinated protocorms were transferred onto fresh NP medium containing 1 µl l^{-1} of 2,4D. *A. tumefaciens* was cultured overnight in LB liquid medium containing 200 mg l^{-1} kanamycin. Inoculation of *A. tumefaciens* was performed by adding 2 ml *A. tumefaciens* liquid culture into 8 ml NP liquid medium (4 x dilution), 0.01% Tween 20, with or without addition of 25 µl l^{-1} acetosyringone. Precultured protocorms were then immersed in this diluted *A. tumefaciens* liquid culture for 30 minutes, and immediately transferred onto sterilized filter paper for 60 minutes of air drying. These protocorms were then transferred into 0.2% (w/v) gellan gum-solidified NP medium with 1 µl l^{-1} 2,4D added and with or without 25 µl l^{-1} AS for co-cultivation. After 3 days co-cultivation in this medium, protocorms were washed three times with ½ NP liquid medium containing 20 mg l^{-1} meropenem, and then transferred to 0.2% (w/v) gellan gum-solidified NP medium with 5 µM 2-IP and 0.15 µM NAA added (called Shoot Induction Medium or SIM) and 8 mg l^{-1} meropenem to slow the growth of *A. tumefaciens* . Protocorms were cultured in this medium for 3 days.

Three days after maintaining the protocorm in the bacterial-elimination medium, protocorms were washed with ½ NP liquid medium containing 20 mg l^{-1} meropenem three times, then were transferred to selection medium (SIM with 300 mg l^{-1} kanamycin and 8 mg l^{-1} meropenem). Protocorms were mantained in this medium for 5 weeks and subcultured every week or less to eliminate *A. tumefaciens* . Elimination of *A. tumefaciens* was performed by immersing protocorms with ½ NP liquid medium containing 20 mg l^{-1} meropenem for 30 minutes, and then gently washing it using the same medium 2-3 times until it was free of *A. tumefaciens* . After 5 weeks of selection, green protocorms were collected as transformant candidates. Protocorms of *V. tricolor* derived from seeds require 20 weeks after sowing to become plantlets.

The kanamycin test was performed twice on the protocorms of *V. tricolor* Merapi. The first test was performed on eight week-old protocorms and the second on ten week-old protocorms. There were five variations in the concentration of kanamycin applied on each age group of protocorms. Concentrations of 0 mg l^{-1}, 100 mg l^{-1}, 150 mg l^{-1}, 200 mg l^{-1}, and 250 mg l^{-1} were applied on eight week-old protocorms, and concentrations of 0 mg l^{-1}, 200 mg l^{-1}, 300 mg l^{-1}, 400 mg l^{-1}, and 500 mg l^{-1} were used on ten week-old protocorms. The percentage of green (surviving) and brown (dead) protocorms was observed after five weeks of application. As a result, 95% of eight week-old protocorms turned brown at a kanamycin concentration of 250 mg l^{-1}, indicating that the selection medium should have a concentration of kanamycin more than 250 mg l^{-1} (Figure 8). However, for ten week-old protocorms, 20% remained green at a kanamycin concentration of 500 mg l^{-1}. These data indicated that older protocorms were more resistant to kanamycin than the younger ones. Based on these data, we used eight week-old protocorms as the target of transformation and a kanamycin concentration of 300 mg l^{-1} for selection. Besides our results, previous research by Dwiyani (2009, unpublished) also found that more than 50% of *V. tricolor* embryos were resistant to kanamycin. Genetic transformation using the *A. tumefaciens* LBA4404 strain harboring pG35S containing the *NPTII* gene was conducted three times on the eight week-old protocorms. The first transformation used *V. tricolor* from West Java, and the second and the third used *V. tricolor* from Merapi. The difference between the second and the third was the use of pre-culture treatment on the second transformation. Five weeks after growth on selection medium containing 300 mg l^{-1} kanamycin, 100% of control protocorms (without *A. tumefaciens* infection) turned brown in *V. tricolor* Merapi Form (Figure 8).

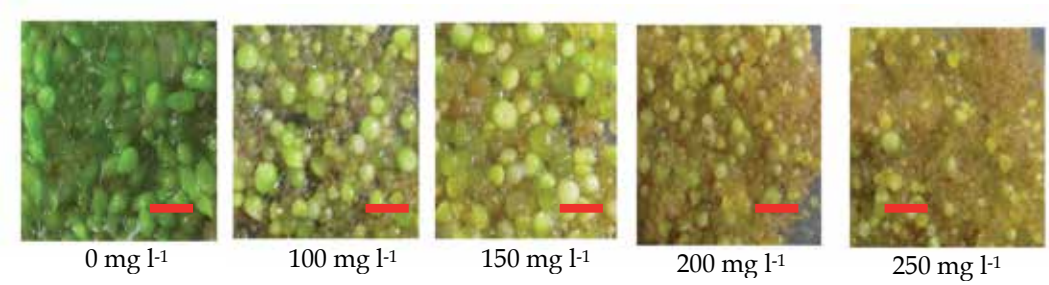

Fig. 8. Kanamycin test on 8 week-old protocorms of *Vanda tricolor* Merapi. From left to right, protocorms after 5 weeks of kanamycin application of 0, 100, 150, 200, and 250 mg l^{-1}. At 250 mg l^{-1}, 5% of protocorms remained green. Bar: 1500 µm. (After Dwiyani et al., 2011, in press).

From the study of Dwiyani et al. described above, two points can be infered: first, the addition of AS into co-cultivation medium is required. The addition of AS into the inoculum increased the percentage of protocorms surviving in the selection medium above that with the addition of AS into co-cultivation only. AS supplementation in this step of transformation (inoculation) might stimulate higher concentrations of AS within the tissues of the treated protocorm, thereby eliciting higher *vir* gene-inducing activity in *A. tumefaciens* (Nan et al., 1997). The presence of AS during transformation is known to induce *vir* gene activity and stimulate T-DNA transfer into plant cells (Zupan and Zambryski, 1995; Gelvin, 2003). This result was in line with Jacq et al. (1993); Robischon et al. (1995); and Mishiba et al. (2005), who found that pre-culture of transformation targets prior to infection resulted in higher transformation efficiency. Cell-cycle progression might be induced during pre-culture (Mishiba et al., 2005), and T-DNA transfer is likely to occur in cells with this condition (Villemont et al., 1997).

The resistancee of *V. tricolor* protocorms to kanamycin varies depending on the developmental stage of the protocorms and the orchid form. Older protocorms are more resistant than younger ones. The West Java form is more resistant than the Merapi form. The addition of AS (especially at the inoculation step) and pre-culture treatment of protocorms prior to infection with *A. tumefaciens* is necessary for *Agrobacterium*-mediated-transformation of *V. tricolor* protocorms.

4.9 Transformation of the black orchid (*Coelogyne pandurata* Lindley)

The black orchid (*Coelogyne pandurata* Lindley), which is endemic to the province of East Kalimantan, Indonesia, is very important. The black orchid is an epiphytic sympodial orchid characterized by a large greenish flower with a black labellum (Figure 1A). This orchid exhibits some pseudobulbs that grow parallel with two leaves each. Five to seven flowers are arranged in a raceme and are fragrant. The diameter of each flower is 7-12 cm. Sepals and petals are green and the labellum (lip) is black. Seeds are microscopic in size, and are located inside the fruit. The uniqueness of this orchid is in the very short blooming period (3-5 days) and its difficulties with pollination (Arditti, 1992; Wibowo, 2010). According to Wirakusumah (2009, personal communication), *in vitro* seed germination of the black orchid needs special conditions such as incubation in the dark prior to germination for 3-4 months. For successful cultivation, *in vitro* seed germination is the key step (Arditti and Ernst, 1993). In order to obtain optimal conditions for *in vitro* seed germination of this orchid, some experiments using various culture media have been carried out (Semiarti et al., 2010), and

we have used 35S::KNAT1 containing T-DNA constructs to get multishoot production similar to our work with *P. amabilis*.

4.10 Developmental phases of the black orchid (*Coelogyne pandurata* Lindley) embryo

The development of black orchid embryos during seed germination can be classified into six phases based on growth and morphology: phase 1) yellowish embryo, phase 2) green embryo, phase 3) bipolar embryo, phase 4) first leaf formed embryo, phase 5) second leaf formed embryo, and phase 6) third leaf formed embryo. The time-course of embryo development shows that the embryo starts to change from yellowish (phase 1) into green (phase 2) at one to two weeks after sowing. At three to four weeks, the green embryo forms a bipolar structure (phase 3), with one side darker than the other. The darker pole of the embryo changes into leaf primordia (phase 4) at the fifth week, a protocorm with two leaves at seven weeks (phase 5), and a protocorm with three leaves at eleven to twelve weeks (phase 6) (Table 8).

At twelve weeks after sowing, based on the growth rate of embryos, the data revealed that ½ NP medium is the best to support and accelerate growth that will result in seed germination. Approximately 86% of protocorms grew up to phase 5 (Table 8). The results show that for embryo development during seed germination in black orchids, a half-strength concentration of complete element-containing medium is the best. It might be that the content of macro- and micro-elements in the half strength medium provides a suitable consentration to promote the development of the embryos, so it is not necessary to use full-strength basic medium. As described by Arditti and Ernst (1993), tissue culture is an empirical science. It is difficult to predict the type of explant, media, and conditions that are suitable for a specific genus or species or clone. It is not possible to explain why certain media and culture conditions lead to success while others fail. In the black orchid, the half-strength NP medium may be the best for seed germination, so that the embryo will respond better to genetic transformation than that if we used full-strength NP medium.

Variation of Medium	No of seeds	Percentage of growing embryos at each phase						Death of protocorms
		Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Phase 6	
½ NP	193	0.00%	0.00%	0.00%	13.47% (26)	86.53% (167)	0.00%	0.00%
NP	112	0.00%	0.00%	0.00%	18.75% (21)	57.14% (64)	4.46% (5)	19.64% (22)
NP+CW	105	0.00%	0.00%	0.00%	1.90% (2)	72.38% (76)	8.57% (9)	17.14% (18)

Table 8. Growth of black orchid embryos on NP medium.

4.11 Insertion of the *KNAT1* gene in the black orchid

For *in vitro* cultivation of orchids, various media are used for seed germination and shoot induction, such as Knudson C (KC), Murashige-Skoog (MS), and Vacin and Went (VW), with the addition of some organic complexes such as coconut water (Arditti and Ernst, 1993; Widiastoety and Syafril, 1994; Demasabu et al., 1998; Untari and Puspaningtyas, 2006). Islam et al. (1998) used *New phalaenopsis* (NP) medium for callus induction of *Phalaenopsis*. Semiarti et al. (2007) also used the NP medium for growing *Phalaenopsis* orchids before and after

genetic transformation treatment using *A. tumefaciens.* Our previous experiments (Semiarti et al., 2007) indicated that the insertion of the *KNAT1* gene into *Phalaenopsis* protocorms induced multishoot production (about 31 shoots from one protocorm), and further results showed that the level of multiplication increased to more than 90 shoots from one embryo. This is a very valuable and promising technique for micropropagation of black orchids. Multishoot occurrence in *KNAT1* transgenic plants has also been reported by Chuck et al. (1996) in transgenic *Arabidopsis* and Nishimura et al. (2000) in *Nicotiana*. In the hybrid orchid *Dendrobium* "Madame Thong In", Yu et al. (2001) obtained multishoots from calli derived from cut-off protocorms transformed with the *DOH1* gene (a *KNAT1* homologous gene in *Dendrobium*). Each shoot could independently grow into a plantlet. Genetic transformation of the *KNAT1* gene under the control of *Cauliflower Mosaic Virus* (CaMV) in a pGreen vector using *A. tumefaciens* strain LBA 4404 into intact protocorms is a useful means of micropropagation.

Genetic transformation of plasmid 35S::KNAT1 and pGreen vector into orchids was carried out according to the method of Semiarti et al. (2007), except that the liquid medium used to rinse the protocorm was half-strength NP medium with 300 mg l^{-1} cefotaxim. SIM (Shoot Induction Medium; 0.15 µM NAA + 5 µM 2-IP) supplemented with 100 mg l^{-1} kanamycin for selecting independent transformants. Into each step 75 mg l^{-1} acetosyringone was added to improve the efficiency of T-DNA insertion as described by Semiarti et al. (2010). The frequency of transformation was measured as the ratio of the number of surviving protocorms per total number of transformed protocorms (Table 9).

No.	Treatment	Kan, 300 mg l^{-1} (+/-)	No. of protocorms	Percentage of Kan-resistant plants	
				Surviving	Death
1.	Non-transformant (NT)	-	100	98% 98/100	2% 2/100
2.	NT	+	237	37.6% 89/237	62.4% 148/237
3.	pGreen	+	707	66.0% 467/707	34.0% 240/707
4.	p35S::KNAT1	+	701	61.6% 475/701	38.4% 296/701

Table 9. Frequency of transformation by pGreen and pKNAT1 in regeneration medium, two months after selection on kanamycin-containing medium.

The expression of the *KNAT1* gene in black orchid transformants can be judged from their ability to form multishoots similar to another natural orchid, *Phalaenopsis amabilis* (Semiarti et al., 2007). Yu et al. (2001) obtained multishoots of a hybrid orchid from callus protocorms that were transformed by the *DOH1* gene, in which each shoot could independently grow into a plantlet. PCR analysis has confirmed the existance of the *KNAT1* gene in the genomes of surviving plants in antibiotic-containing medium for black orchid transformants. For other transformant candidates, confirmation of the transgene is still in progress. Images in Fig. 8B and C show that non-transformant plants produce only one shoot from the embryo, but a transformant produces seven shoots from one protocorm. The addition of cytokinins as phytohormones such as kinetin or 2-penthenyl adenine (2-IP), when combined with NAA (synthetic auxin) in medium, might induce and increase the number of multishoots from

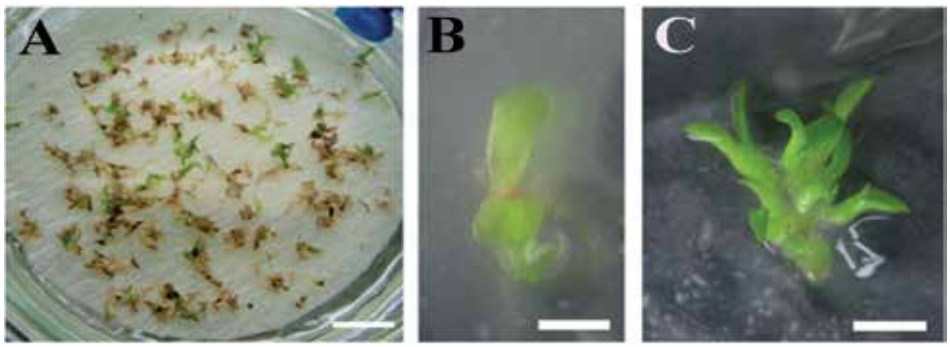

Fig. 8. Black orchid transformants. A. Two-month old candidates of transformants grown on kanamycin-containing medium in a selection plate. B. a shoot grows from a non-transformant protocorm. C. multishoots grow from one 35S::KNAT1 transformant protocorm. Bars: 1 cm in A, 0.5 cm in B-C.

transformants. Further results of transformation with *P. amabilis* indicate that maintaining transformants on NP medium with 3 μM 2-IP + 0.15 μM NAA induces high levels of multishoot production, up to 91 shoots per embryo. It is worth trying this method in black orchids. The high propensity for shoot production will strongly support both conservation efforts and agribusiness of this orchid.

5. Conclusions and future prospects

Agrobacterium-mediated transformation of the *KNAT1* gene into *P. amabilis* orchids resulted in multishoot production of transformants, which is helpful for micropropagation of Indonesian orchids such as *P. amabilis*, *Vanda tricolor*, and *Coelogyne pandurata.* Three-week-old intact protocorms are convenient for use in *Agrobacterium*-mediated transformation. This method is a highly effective technique to enhance the efficiency of micropropagation of Indonesian orchids, which is useful due to the rareness of their populations in their natural habitat. Utilizing advanced orchid biotechnology may facilitate the improvement of Indonesian orchid production for commercial use or for orchid conservation. These tools will also be helpful for creating new traits in orchids.

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7. References

Anzai, H., Ishii, Y., Shichinohe, M., Katsumata, K., Nojiri, C., Morikawa, H. & M. Tanaka. (1996). Transformation of Phalaenopsis by particle bombardment. Plant Tissue Cult. Lett. 13: 265-271.

Araki, S., M. Matsuoka, M. Tanaka, & T. Ogawa. (2000). Ethylene formation and phenotypic analysis of transgenic tobacco plants expressing a bacterial ethylene-forming enzyme. Plant and Cell Physiology 41(3):327-334

Arditti J. (1992). Fundamentals of Orchid biology. John Wiley and Sons, Inc. New York. P. 691

Arditti, J, & R. Ernst. (1993). *Micropropagation of orchids*. Jhon Willey & Son Inc. New York,pp.6-8, 25-65.

Belarmino M.M, & M. Mii. (2000). *Agrobacterium*-mediated genetic transformation of a *Phalaenopsis* orchid. Plant Cell Reports 19:435-442

Chan YL, Lin KH, Sanjaya, Liao LJ, Chen WH, Chan MT (2005) Gene stacking in *Phalaenopsis* orchid enhances dual tolerance to pathogen attack. *Transgenic Res* 14: 279–288

Chansean, M. & S. Ichihashi. (2007). Conservation of wild orchids in Cambodia by simple aseptic culture method. Proceedings of Nagoya International Orchid Conference 2007, Nagoya, Japan. 13-20.

Chin, D.P., & M. Mii. (2011). Dwarf Phalaenopsis Plant Produced by Overexpression of Gibberellin2-Oxidase gene. Proceeding of NIOC, Nagoya Japan, 11 Maret 2011.

Chiu W-l, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a viral reporter in plants. *Curr Biol* 6:325-330

Chia TF, Chan YS, & Chua NH. (1994). The firefly luciferase gene as a non-invasive reporter for *Dendrobium* transformation. *Plant J* 6: 441-446

Choffnes, D.S., R. Philip, & L.O. Vodkin. (2001). A transgene locus in soybean exhibits a high level of recombination. In Vitro Cellular & Developmental Biology-Plant 37:756-762

Christou, P., W.F. Swain, N.S. Yang, & D.E. McCabe. (1989). Inheritance and expression of foreign genes in transgenic soybean plants. Proceedings of the National Academy of Science of the United State of America 86:7500-7504.

Chuck G, Lincoln C, & Hake S (1996) *KNAT1* induces lobed leaves with ectopic meristems when overexpressed in *Arabidopsis*. *Plant Cell* 8:1277-1289

Cogan, N., Harvey, E., Robinson, H., Lynn, J., Pink, d., Newbury, H.J., & Puddephat, I. (2001). The effects of anther culture and plant genetic background on *Agrobacterium rhizogenes*-mediated transformation of commercial cultivars and derived double-haploid *Brassica oleracea*. Plant Cell Reports 20:755-762.

Cunningham, F. X. Jr., Pogson, B., Sun, Z.,Mcdonald, K. A., Dellapenna, D. & E. Gantt. (1996). Functional analysis of the B and E lycopene cyclase enzymes of Arabidopsis reveals a mechanism for control of cyclic carotenoid formation. The Plant Cell, 8, 1613-1626.

Demasabu, Sofia, B. Dodo, dan D. Kojoh (1998), Penggunaan limbah Air Kelapa dan Bahan Substitusi Agar pada kultur jaringan pisang, krisan, dan anggrek. *Prosiding Seminar Nasional Sains dan Teknologi II, tanggal 17-18 November 2008. Universitas Lampung.*

Dressler RL (1981) The Orchids: Natural History and Classification. Harvard University Press, USA, pp 332.

Dwiyani, R. A. Purwantoro, A. Indrianto, & E. Semiarti. (2011). Improvement of Genetic Transformation Technique in *Vanda tricolor* Orchid Using Acetosyringone.

Finnegan, J. & D. McElroy. (1994). Transgene inactivation: Plants fight back!. Bio/Technology 12:883-888

George, E.F, & P.D. Sherrington. (1984). *Plant Propagation by Tissue Culture*. Exergetics Ltd. Hants, pp, 184-200.

Grichko, V.P., & B.R. Glick. (2001). Flooding tolerance of transgenic tomato plants expressing the bacterial enzyme ACC deaminase controlled by the 35S,rolD or PRB-1b promoter. Plant Physiology and Biochemistry 39:19-25

Hiei, Y., S. Ohta, T. Komari, & T. Kumashiro. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* & sequence analysis of the boundaries of the T-DNA. The Plant Journal 6:271-282

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vectorstrategy based on separation of vir- and T-region of the *Agrobacterium tumefasciens* Ti-plasmid. *Nature* 303:179-180

Ishii Y, Tanaka M, Takamura T, Goi M (1998) Callus induction and somatic embryogenesis of *Phalaenopsis*. *Plant Cell Rep* 17:446-450

Islam MO, Ichihasi S, & Matsui S. (1998). Control of growth and development of protokorm like body derived from callus by carbon sources in *Phalaenopsis*. *Plant Biotechnol* 15:183-187.

Islam, M. O., Rahman, A. R. M. M., Matsui, S. & A. K. M. A. Prodhan. (2003). Effects of complex organic extracts on callus growth and PLB regeneration through embryogenesis in the Doritaenopsis orchid. Japan Agricultural Research Quarterly, 37, 229-235.

Jacq B, Lesobre O, Sangwan RS, & Sangwan-Norreel BS. (1993). Factors influencing T-DNA transfer in *Agrobacterium*-mediated transformation of sugarbeet. *Plant Cell Rep* 12: 621-624

Jin, R.G., Lui, Y.B., Tabashnik, B.E., & Borthakur, D. (2000). Development of transgenic cabbage (*Brassica oleracea* var. *capitata*) for insect resistance by *Agrobacterium tumefaciens*-mediated transformation. In Vitro Cell. Develop. Biol. Plant 36:231-237

Kanemoto H. & G.J. Wilfret. (1980). Inter- and multi-sectional *Dendrobium* hybrid. In: M.R. Sukshom Kashemsanta (ed.), Proc. 9th World Orchid Conf. 9th World Orchid Conference Committee, Bangkok, pp 255-261

Khatun. M.M., Khatun, H., Khanam, D., & Al-Amin, Md. (2010). *In Vitro* Root Formation And Plantlet Development In Dendrobium Orchid. *Bangladesh J. Agril. Res. 35(2): 257-265*

Klee, H.J., R.B. Horsch, M.A. Hinchee, M.B. Hein, & N.L. Hoffmann. (1987). The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. Gene and Development 1:86-96

Kohli, A., D. Gahakwa, P. Vain, D.A. Laurie, & P. Christou. (1999). Transgene expression in rice engineered through particle bombardment: Molecular factors controlling stable expression and transgene silencing. Planta 208:88-97

Kuehnle A.R., & Sugii N. (1992). Transformation of *Dendrobium* orchid using particle bombardment of protocorms. Plant cell Reports 11:484-488

Kuehnle. A.R. (2007). Orchids. Dendrobium. In: Anderson (ed.). Flower Breeding and Genetics, 539-560

Lee, Y.H., Lee, S.B., Suh, S.C., Byun, M.O., & Kim, H.I. (2000). Herbicide resistant cabbage (*Brassica oleracea* ssp. *capitata*) plants by *Agrobacterium*-mediated transformation. J.Plant Biotechnol. 2:35-41

Levin, D.A. (2002). The Role of Chromosomal Change in Plant Evolution. Oxford Series in Ecology and Evolution. Oxford University Press. 230p.

Li, Y., Duan, H., Wu, Y.h., McAvoy, R.J., Pei, Y., Zhao, D., Wurst, J., Li, Q., & Luo, K. (2004). Transgenics of Plant hormones and Their Potential Application in Horticultural Crops. In: Liang and Skinner (ed.). Genetically modified Crops, pp 101-117

Matzke, M.A. & A.J.M. Matzke. (1995). How and why do plants inactive homologous(trans)genes? Plant Physiology 107:679-685

Metz, T.D., Dixit, R., & Earle E.D. (1995). *Agrobacterium tumefaciens*-mediated transformation of broccoli (*Brassica oleracea* var *italic*) and cabbage (*Brassica oleracea* var *capitata*). Plant Cell Reports 15:287-292

Michelmore, R., Marsh E., Seely S., & Landry B. (1987). Transformation of lettuce (*Lactuca sativa*) mediated by *Agrobacterium tumefaciens*. Plant Cell Reports 6:439-442

Mishiba K, Chin DP, & Mii M. 2005. *Agrobacterium*-mediated transformation of *Phalaenopsis* by targeting protocorms at an early stage after germination. *Plant Cell Rep* 24: 297-303

Nishimura A, Tamaoki M, Sakamoto T, Matsuoka M (2000) Over- expression of tobacco *knotted1*-type class1 homeobox genes alters various leaf morphology. *Plant Cell Physiol* 41: 583–590

Oladiran, A. O. & Iwu, L. N. (1992). Changes in ascorbic acid and carbohydrate contents in tomato fruits infected with pathogens. Plant Foods for Human Nutrition, 42, 373-382.

Pawlowski, W.P. & D.A. Somers. (1996). Transgenic inheritance in plant genetically engineering using microprojectile bombardment. Molecular Biotechnology 6:17-30

Pawlowski, W.P., K.A. Torbert, H.W. Rines, & D.A. Somers. (1998). Irregular patterns of transgene silencing in allohexaploid oat. Plant Molecular Biology 38:597-607

Robichon MP, Renou JP, & Jalouzot R. (1995). Genetic transformation of *Pelargonium x hortorum*. *Plant Cell Rep* 15:63-67

Perl A, Lotan O, Abu-Abied M, & D. Holland. (1996). Establishment of an Agrobacterium-mediated transformation system for grape (Vitis vinifera L.): the role of antioxidants during grape-Agrobacterium interactions. Nat Biotechnol 1996 Nov;14(11):1521.

Puri, H.S. (1970). American Orchid. Society Bulletin 39:723

Raghavan, V. (1997) Molecular Embryology of Flowering Plants 1st Edition Cambridge Univ. Press, Cambridge, UK. 525-532.

Sanguthai O., Sanguthai S., & H. Kamemoto. (1973). Chromosome doubling of a Dendrobium hybrid with colchicines in meristem culture. Na Okika o Hawaii 2:12-16

Schelpe S, & J. Stewart. (1990). *Dendrobium*, an introduction to the species in cultivation. Orchid Sundries Ltd, Dorset, Great Britain.

Semiarti E, Ueno Y, Tsukaya H, Iwakawa H, Machida C, Machida Y (2001) The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana* regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. *Development* 128:1771-1783.

Semiarti, E, Indrianto, A., Purwantoro, A, Suseno, N., Isminingsih, S., Yoshioka, Y., Iwakawa, H., Machida, Y. & Machida C. (2007). *Agrobacterium*-mediated transformation of the wild orchid species *Phalaenopsis amabilis*. Plant Biotechnology 24(3).

Semiarti, E., A. Indrianto, A. Purwantoro, I.N.A. Martiwi, Y.M.L. Feroniasanti, F. Nadifah, I.S. Mercuriana, R.1, H. Iwakawa, Y.Yoshioka, Y. Machida, & C. Machida. (2010). High-frequency genetic transformation of *Phalaenopsis amabilis* orchid using tomato extract-enriched medium for the pre-culture of protocorms. Journal of Horticultural Science and Biotechnology 85(3):205-210

Sjahril, R. & Mii, M. (2006). High-efficiency Agrobacterium-mediated transformation of Phalaenopsis using meropenem, a novel antibiotic to eliminate Agrobacterium. J. Hort. Sci. Biotech. 81: 458-464.

Sjahril, R., Chin, D.P., Khan, R.S., Yamamura, S., Nakamura, I. Amemiya, & Y. Mii, M. (2006). Transgenic *Phalaenopsis* plants with resistance to *Erwinia carotovora* produced by introducing wasabi defensin gene using *Agrobacterium* method. Plant Biotechnology 23: 191-194

Untari, R. & D.W. Puspitaningtyas (2006), Pengaruh Bahan Organik dan NAA terhadap Pertumbuhan Anggrek Hitam (Coelogyne pandurata Lindl.) dalam kultur in vitro. *Biodiversitas, 7(3), 344-348.*

Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, & Sangwan-Norreel BS. (1997). Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: evidence of an S-phase control mechanism for T-DNA transfer. *Planta* 201: 160-172

Wakita, Y., M. Otani, T. Hamada, M. Mori, K. Iba, & T. Shimada. (2001). A tobacco microsomal ω-3 fatty acid desaturase geneincreases the linolenic acid content in transgenic sweet potato (*Ipomoea batatas*). Plant Cell Reports 20:244-249

Wibowo, R.U.W. (2010), Polinasi pada Tanaman Anggrek Hitam (*Coelogyne pandurata* Lindl.), Fakultas Biologi UGM.

Widiastoety, D. dan Syafril. (1994), Pengaruh Air Kelapa terhadap pembentukan protocorm like bodies (plb) dari anggrek Vanda dalam medium cair, *Jurnal Holtikultura, 4 (2), 71-73*4), Pengaruh Air Kelapa terhadap pembentukan protocorm like bodies (plb) dari anggrek Vanda dalam medium cair, *Jurnal Holtikultura, 4 (2), 71-73*

Yang J, Lee H.J., Shin D.H., Oh S.K., Seon J.H., Paek K.Y., & Han K.H. (2000). Genetic transformation of *Cymbidium* orchid by particle bombardment. Plant Cell Reports 18:978-984

Yang X.H., Pua E.C., & Goh. C.J. (1996). Isolation of a cDNA clone encoding 1-aminocyclopropane-1-carboxylase synthase from *Dendrobium crumenatum* PGR96-088 (Accession No. U64031). Plant Physiol. 112:1383

Yang, S.H, Yu H, Goh CJ (2003) Functional characterisation of a cytokinin oxidase gene DSCKO1 in *Dendrobium* orchid. Plant Mol. Biol. 51: 237-248.

Yu H., & C.J. Goh. (2000). Differential gene expression during floral transition in an orchid hybrid *Dendrobium* Madame Thong-In. Plant Cell reports 9:926-931

Yu, H., Yang S.H. Goh CJ (2000) *DOH1,* a class 1 *knox* gene, is required for maintenance of the basic plant architecture and floral transition in orchid. *Plant Cell* 12:2143-2160

Yu H, Yang SH, Goh CJ (2001) *Agrobacterium*-mediated transformation of a *Dendrobium* orchid with the class1 *knox* gene *DOH1. Plant Cell Rep* 20:301-305

Zhang, J., T. Van Toai, L, Huynh, & J. Preiszner. (2000). Development of Flooding-tolerant *Arabidopsis thaliana* by autoregulated cytokinin production. Molecular Breeding 6:135-144

12

Transient Transformation of Red Algal Cells: Breakthrough Toward Genetic Transformation of Marine Crop *Porphyra* Species

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1. Introduction

Genetic transformation is a powerful tool not only for elucidating the functions and regulatory mechanisms of genes involved in various physiological events but also for establishing organisms that efficiently produce biofuels and medically functional materials or carry stress tolerance in face of uncertain environmental conditions (Griesbeck et al., 2006; Torney et al., 2007; Bhatnagar-Mathur et al., 2008). Eukaryotic algae classified into microalgae and macroalgae (seaweeds) are highly diverse photosynthetic plants that are utilized as human food and animal feed as well as sources of valuable compounds such as fatty acids, pigments, vitamins and polysaccharides (Hallmann, 2007; Sugawara et al, 2011). Because of their importance in ecology and industry, algae are now considered promising organisms for economical and industrial applications and are thus a target of genetic transformation (Walker et al., 2005; Hallmann, 2007; Blouin et al., 2011). To date, genetic transformation has succeeded in microalgae; thus, stably transformed microalgae are now employed to produce recombinant antibodies, vaccines, or bio-hydrogen as well as to analyze the gene functions targeted for engineering (Sun et al., 2003; Zorin et al., 2009; Specht et al., 2010; Wu et al., 2010). However, it has proven difficult to establish transgenic macroalgae, which has hampered understanding their gene functions in various physiological regulations and also their utilization in biotechnological applications.

The red macroalga *Porphyra yezoensis* is the most popular sea crop in Japan with economical and pharmaceutical importance as the source of foods such as "nori" and pharmacological regents such as the sulfated polysaccharide "porphyran", which has anti-tumor and anti-allergic activities (Noda et al., 1990; Zemke-White & Ohno, 1999; Ishihara et al., 2005). Recently, non-beneficial climate change due to global warming has decreased the quality and yield of *P. yezoensis* at algal farms by enhancing discoloration and red rot disease caused by fungal infection (Kakinuma et al., 2008; Park et al., 2000). Although breeding of *P. yezoensis* by traditional selection and crossing methods has progressed to obtain strains showing high growth rates and economically valuable characteristics, these methods have limitations in terms of the isolation of strains carrying heat-stress tolerance or disease

resistance. Development of a molecular breeding method based on genetic transformation is expected to resolve this problem using knowledge about gene functions in *P. yezoensis*.
Despite the difficulty of genetic transformation, genetic information about *P. yezoensis* is now accumulating by a collection of ESTs from leafy gametophytes and filamentous sporophytes (Nikaido et al., 2000; Asamizu et al., 2003) and also by ongoing full-length cDNA and whole genome analyses. Genetic information provides a sophisticated way to isolate *P. yezoensis* genes of interest; however, utilization of this knowledge – in both basic biological research and molecular breeding of *P. yezoensis* – is hindered by the lack of a genetic transformation system.
Given this situation, it is clear that the development of a genetic transformation system for *P. yezoensis* is critical for sustainable production as well as the enhancement of biological study. In our laboratory much effort has been expended on the establishment of a genetic transformation system; as a result we have identified several factors preventing the establishment of a genetic transformation system in macroalgae: for example, the lack of efficient methods for foreign gene transfer and expression systems and the absence of valid selectable markers for isolation of genetically transformed cells. Thus, it is considered worthwhile to develop a transient gene expression system, because some of the above inhibiting factors, such as defects in the transfer and expression of foreign genes, could be removed by using non-integrated plasmids containing genes for expression. Since we have recently succeeded in developing an efficient transient gene expression system in Bangiophyceae including *Porphyra* species, the establishment and successful application of this system in a molecular biological study are summarized here.

2. Development of a transient gene expression system in *P. yezoensis* cells

2.1 Problems in previously published experiments

Expression of foreign genes in macroalgae has already been attempted using bacterial *lacZ* (β-galactosidase) and *uidA* (β-glucronidase, *GUS*) genes under direction by promoters of the cauliflower mosaic virus 35S RNA (*CaMV 35S*) and simian virus 40 (*SV40*) genes (Kübler et al., 1994; Kuang et al., 1998; Huang et al., 1996; Gan et al., 2003; Jiang et al., 2003). The *CaMV 35S* and *SV40* promoters are typical eukaryotic class II promoters with a TATA box and thus are generally employed to drive transgenes in dicot plant and animal cells, respectively (Kang & An, 2005; Funabashi et al., 2010). However, we have observed a quite low activity of the *CaMV 35S* promoter in *P. yezoensis* cells as shown in Figs. 1 and 2 (Fukuda et al., 2008), which is strongly supported by the fact that the TATA box is not found in the core promoters of *P. yezoensis* genes (unpublished observation), suggesting differences in the promoter structure and transcriptional regulation of protein-coding genes between red algae and dicot plants. We believe that the same could be predicted for the SV40 promoter. Thus, it seems to be necessary to reconfirm the results of reporter expression using the *CaMV 35S* and *SV40* promoters in red algae like *P. miniata*, *P. tenera*, *Kappaphycus alvarezii* and *Gracilaria changii* in previously published literatures (Kurtzman & Cheney, 1991; Kübler et al., 1993; Okauchi & Mizukami, 1999; Cheney et al., 2001; Gan et al., 2003).
In addition, the use of the *GUS* reporter gene is also a problem, since we have already confirmed that the *GUS* reporter gene is not functional in red algal cells by histochemical analysis and enzymatic activity test (see below). This is supported by previous reports indicating background levels of the GUS enzymatic activity in red and brown macroalgal cells when the GUS reporter gene was driven by *CaMV 35S* and endogenous beta-tubulin promoters (Liu et al., 2003; Gong et al., 2005).

According to the above findings, previous reports of successful expression of the *GUS* reporter gene under the direction of the *CaMV 35S* and *SV40* promoters are not likely to be replicated. Thus, an important step is to enable the effective expression of the *GUS* reporter genes in *P. yezoensis* cells, which should eventually lead to the establishment of a transient gene expression system via the development of procedures for introduction and expression of foreign genes as described below.

2.2 Codon-optimization of the reporter gene

Inefficient expression of foreign genes in algal cells is often due to the incompatibility of the codon usage in their coding regions, inhibiting the effective use of transfer RNA by rarely used codons in the host cells and thus decreasing the efficiency of the translation (Mayfield & Kindle, 1990). In this respect, successful development of foreign gene expression in green alga *Chlamydomonas reinhardtii* may be an important clue to overcoming the deficiency of the GUS reporter gene in *P. yezoensis* cells. For example, a low expression level of the gene encoding a green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, whose codon usage is rich in AT residues, was dramatically increased by adjustment of the GC content of the coding region corresponding to the codon usage in *C. reinhardtii* nuclear genes (Fuhrmann et al., 1999). In addition, the synthetic luciferase gene whose codon usage was adapted to that in nuclear genes from *C. reinhardtii* was also expressed efficiently, although the original luciferase gene had less efficiency in *C. reinhardtii* cells (Fuhrmann et al., 2004; Ruecker et al., 2008; Shao & Bock, 2008). In fact, EST analysis of *P. yezoensis* reveals that the codons in *P. yezoensis* nuclear genes frequently contain G and C residues especially in their third letters, by which the GC content reaches a high of 65.2% (Nikaido et al., 2000). Since bacterial *GUS* and *lacZ* reporter genes have AT-rich codons, the incompatibility of codon usage might be responsible for the poor translation efficiency of foreign genes in *P. yezoensis* cells. We therefore postulated that modification of codon usage in the *GUS* gene would enable the efficient expression of this gene in *P. yezoensis* cells. This possibility led us to modify the codon usage of the GUS reporter gene to that in the nuclear genes of *P. yezoensis* by introducing silent mutations in the GUS coding region, by which unfavorable or rare codons in the GUS reporter gene were exchanged for favorable ones without affecting amino acid sequences. In the resultant artificially codon-optimized GUS gene, designated *PyGUS*, the GC content was increased from 52.3% to 66.6%.

To transfer the plasmid into cells, we employed particle bombardment, also referred to as micro-projectile bombardment, particle guns, or biolistics, which are among the most popular techniques for achieving nuclear and organelle transformation not only for algae but also for plants, fungi, insects, animals and even bacteria. In this method, DNA-coated heavy-metals, usually gold or tungsten particles, are introduced into cells by helium pressure, which enables the introduction of foreign DNA regardless of whether the cell has a cell wall. This method has been successfully applied in many algal species such as *C. reinhardtii* (Kindle et al., 1989), *Volvox carteri* (Schiedlmeier et al., 1994), *Dunaliella salina* (Tan et al., 2005), and *Phaeodactylum tricornutum* (Apt et al., 1996).

When the *PyGUS* gene directed by the *CaMV 35S* promoter was introduced into *P. yezoensis* gametophytic cells by particle bombardment, very low but significant expression of the *PyGUS* gene was observed by histochemical detection and GUS activity test (Figs. 1 and 2), indicating the enhancement of the expression level of the *GUS* reporter gene. Optimization of the codon usage of the reporter gene is therefore one of the important factors for successful expression in *P. yezoensis* cells (Fukuda et al., 2008; Mikami et al., 2009; Takahashi et al., 2010; Uji et al., 2010; Hirata et al., 2011).

2.3 Employment of an endogenous strong promoter

A low level of *PyGUS* expression under the direction of the *CaMV 35S* promoter was thought to be caused by the low activity of this promoter in *P. yezoensis* cells. Although the *CaMV 35S* promoter is widely used as a heterologous promoter because of its strong, constitutive and non-tissue-specific transcriptional function in dicot plant cells (Louis et al., 2010; Wally & Punja, 2010), it is well known that this promoter has very low activity in cells of green algae such as *Dunaliella salina* (Tan et al., 2005), *Chlorella kessleri* (El-Sheekh, 1999), and *Chlorella vulgaris* (Chow & Tung, 1999) and no activity in *C. reinhardtii* cells (Day et al., 1990; Blakenship & Kindle, 1992; Lumbreras et al., 1998). However, employment of strong endogenous promoters such as the *β-Tub*, *RbcS2* and *Hsp70* promoters has achieved efficient expression of foreign genes in these algae (Davies et al., 1992; Stevens et al., 1996; Schroda et al., 2000; Walker et al., 2004). In addition, many endogenous promoters have been developed and successfully used for foreign gene expression in cells of some microalgae, such as the diatom *Phaeodactylum tricornutum* and the Chlorarachniophyte *Lotharella amoebiformis* (Zaslavskaia et al., 2000; Hirakawa et al., 2008). Therefore, it is possible that recruitment of suitable strong promoters could result in efficient expression of the *PyGUS* reporter gene in *P. yezoensis* cells.

Since we found strong expression of *P. yezoensis* genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin 1 (Act1) by reverse transcription-PCR (RT-PCR) (see Fig. 5), the 5′ upstream regions of these genes were isolated from the *P. yezoensis* genome and fused with the *PyGUS* gene to construct the expression plasmids pPyGAPDH-PyGUS and pPyAct1-PyGUS in addition to the pPyGAPDH-GUS and pPyAct1-GUS plasmids. When pPyGAPDH-GUS and pPyGAPDH-PyGUS were introduced into gametophytic cells by particle bombardment, cells expressing the reporter gene and GUS enzymatic activity were dramatically increased using the *PyGUS* gene as a reporter, although the original *GUS* gene was not efficiently expressed (Figs. 1 and 2). The same results were obtained using pPyAct1-PyGUS and pPyAct1-GUS plasmids (Figs. 1 and 2). Thus, the combination of endogenous strong promoters with codon optimization brings a synergistic effect on the efficiency of the expression of the reporter gene. These results indicate that employment of an endogenous strong promoter is another important factor necessary for high-level expression of the reporter gene in *P. yezoensis* cells (Fukuda et al., 2008).

It is noteworthy that significant expression of PyGUS under the direction of the *PyAct1* and *PyGAPDH* promoters was also observed in sporophytic cells in *P. yezoensis*, when the expression plasmid was introduced using particle bombardment (Fig. 3A). However, the PyGUS enzymatic activity was much lower than those in gametophytic cells (Fig. 3B). This difference was thought to be mainly due to the difference in DNA-transferring efficiency based on the difficulty of targeting the cell for gene transfer in sporophytes of thin filamentous form by particle bombardment.

Our transient gene expression system with the above methodological imorovement is represented schematically in Fig. 4.

2.4 Evaluation of gene transfer methods

Our initial development of transient expression of the *PyGUS* reporter gene in *P. yezoensis* cells depended on the use of particle bombardment, in which the gene transfer rate was usually 10^3 per μg of DNA. However, it is necessary to increase the efficiency of the foreign gene expression to 10^6 to 10^7 per μg of DNA, because the establishment of a genetic

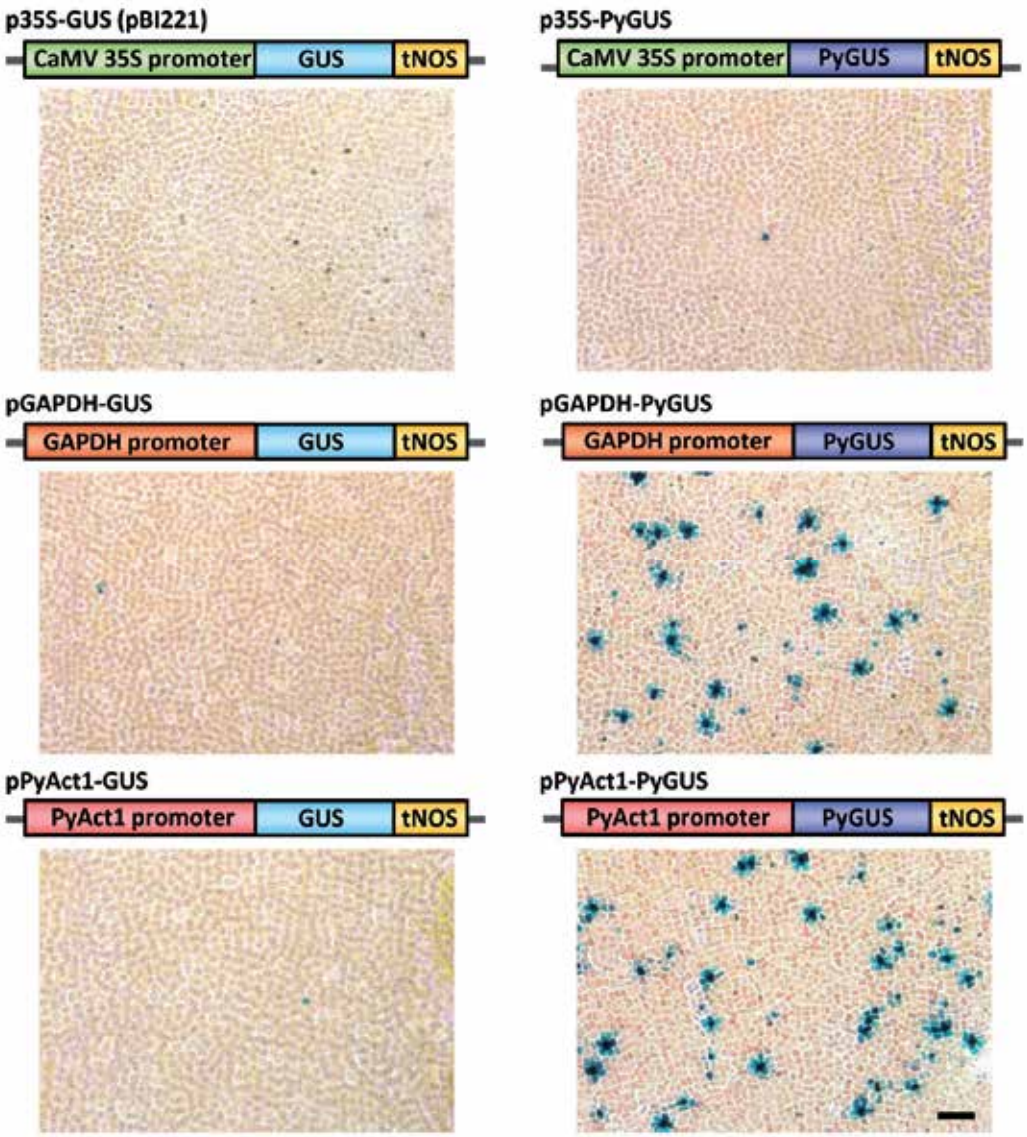

Fig. 1. Effcts of the strength of promoters and codon optimization of the reporter gene on the efficiency of the foreign gene expression in *P. yezoensis* gametophytic cells. The 835 bp *CaMV 35S* (green), 2.3 kb *PyGAPDH* (orange) or 3.0 kb *PyAct1* (red) promoter was fused to the coding region of native (sky blue) or codon-optimized PyGUS (blue), which resulted in the construction of p35S-GUS, p35S-PyGUS, pPyGAPDH-GUS, pPyGAPDH-PyGUS, pPyAct1-GUS and pPyAct1-PyGUS. The tNOS (yellow) indicates the *nos* gene terminator (253 bp). Gametophytic cells of *P. yezoensis* were transiently transformed with each expression plasmid by particle bombardment and then examined by histochemical staining 48 h after transient transformation. Scale bar corresponds to 100 μm.

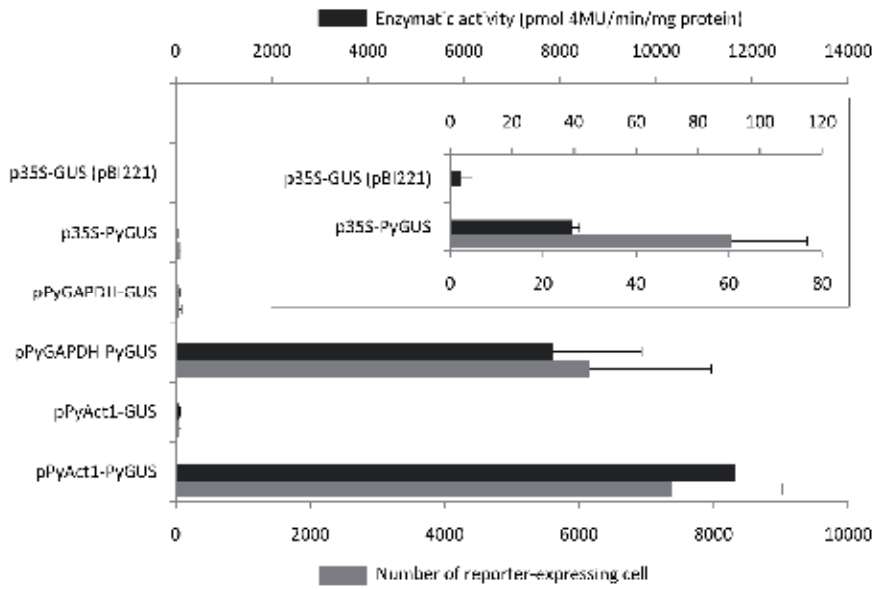

Fig. 2. Confirmation of the synergistic effects of the use of strong promoters and codon optimization on the efficiency of foreign gene expression in *P. yezoensis* gametophytic cells. The GUS or PyGUS enzymatic activity (dark gray) or the number of GUS- or PyGUS-expressing cells (light gray) in gametophytic cells of *P. yezoensis* were compared. The inner box shows a magnified figure of p35S-GUS (pBI221) and p35S-PyGUS.

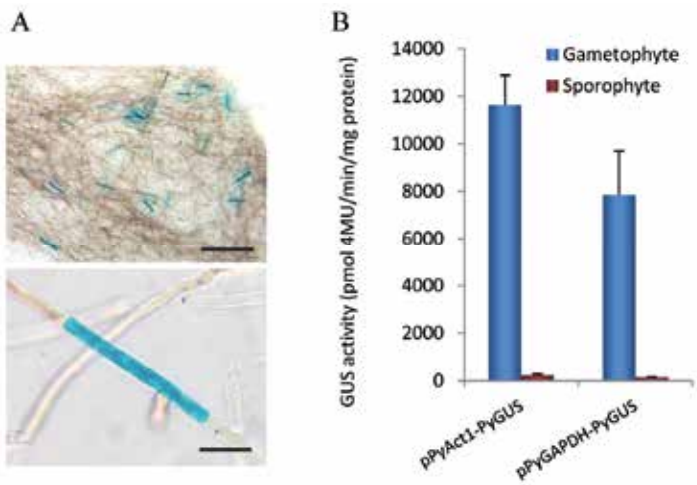

Fig. 3. Availability of PyGUS for transient gene expression in sporophytic cells of *P. yezoensis*. (A) Sporophytic cells expressing PyGUS by transient transformation with pPyAct1–PyGUS and stained with X-gluc solution after 48 h of bombardment. Upper and lower panels show low- and high-magnification images, respectively. Scale bar corresponds to 100 μm (upper) and 10 μm (lower). (B) Comparison of GUS activity between gametophytic and sporophytic cells of *P. yezoensis* when pPyAct1-PyGUS or pPyGAPDH-PyGUS was introduced.

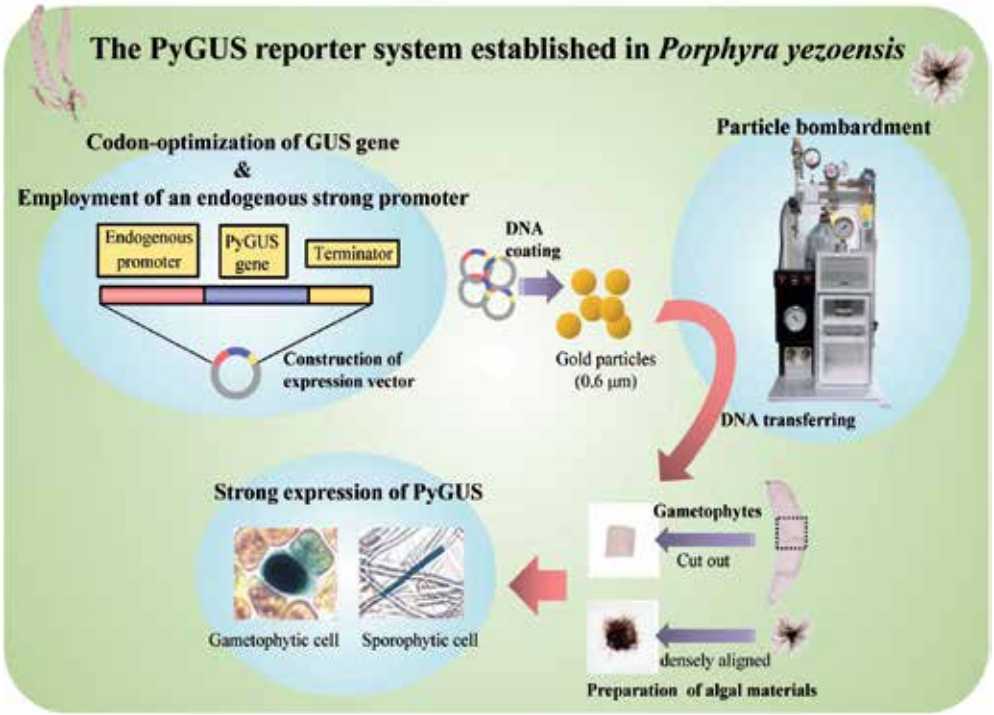

Fig. 4. Representation of the transient gene expression system established in *P. yezoensis*.

transformation system requires a large number of gene-transferred cells, a small portion of which are stably transformed by integration of the foreign gene into the genome. Thus, it is important to develop an efficient method of gene transfer into *P. yezoensis* cells toward the establishment of a stable transformation system via genetic recombination. However, our preliminary experiments with the glass beads method (Kindle, 1990; Feng et al., 2009), PEG method (Ohnuma et al., 2008) and magnetofection (Plank et al., 2003; Svingen et al., 2009) resulted in failure to introduce foreign genes into *P. yezoensis*. Thus, particle bombardment appears to be the only method available for transferring foreign genes into *P. yezoensis* cells at present, and further development of a gene transfer method to acheive very high efficient expression of the *PyGUS* reporter gene is required.

In fact, there have been several reports on DNA transfer into *Porphyra* cells using electroporation (Kübler et al., 1994) and *Agrobacterium*-mediated transformation (Cheney et al., 2001). Although the experiments in these reports had problems in the efficiency of the reporter gene expression based on the use of the *CaMV 35S* promoter and original *GUS* reporter gene, they are valuable as points of comparison for the increase in efficiency of gene transfer and expression by using, for example, the *PyAct1-PyGUS* or *PyGAPDH-PyGUS* reporter gene.

3. Application of the transient gene expression

3.1 Promoter analysis by PyGUS reporter system

Elucidation of the regulatory mechanisms of gene expression is crucial to understanding the molecular mechanisms governing plant stress responses, leading to enhancement of stress tolerance in plants through genetic transformation (Shinozaki & Yamaguchi-Shinozaki,

2007; Bhatnagar-Mathur et al., 2008). The transient gene expression system has been successfully applied to analyze promoters of stress-inducible genes to identify stress-responsible *cis*-regulatory elements in higher plants. For example, cold- and drought-responsible elements have been identified using the GUS reporter system in barley and *Arabidopsis thaliana* (Brown et al., 2001; Sakamoto et al., 2004). These successes are based on the quantitative correlation between promoter strength and reporter gene expression. Confirmation of this point in the transient gene expression system is indispensable for further analysis of stress-inducible genes in *P. yezoensis*.

To check the correlation between the promoter strength and expression level of the PyGUS reporter in *P. yezoensis* cells, mRNA expression levels of the actin 1 (*PyAct1*), GAPDH (*PyGAPDH*), Na^+-ATPase (*PyATP1A*) and transcription elongation factor 1 (*PyElf1*) genes were compared with PyGUS enzymatic activities derived from expression plasmids of the *PyGUS* gene under the direction of these promoters. The result indicated a strong correlation between the mRNA levels of these genes analyzed by RT-PCR and GUS enzymatic activities via expression of the PyGUS reporter gene directed by the corresponding promoter (Fig. 5), demonstrating the applicability of our transient gene expression system with the *PyGUS* gene to promoter analyses of *P. yezoensis* genes. Identification of *cis*-regulatory elements by promoter analysis is now underway for stress-inducible genes using the transient PyGUS reporter expression system.

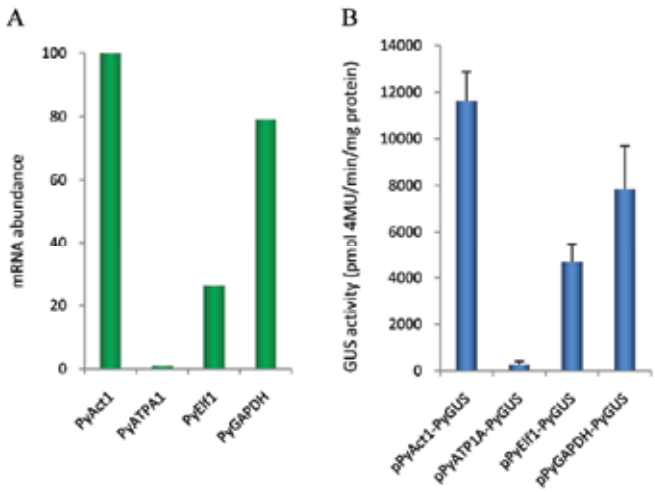

Fig. 5. Correlation of mRNA abundance and the PyGUS enzymatic activity in transient gene expression in *P. yezoensis* cells. (A) Comparison of mRNA abundance for *PyAct1*, *PyATP1A*, *PyElf1*and *PyGAPDH* genes in gametophytic cells. RNA samples were separately prepared from gametophytic cells transiently transformed by these genes and used for quantitative RT-PCR with gene-specific primer sets. The relative abundance of each mRNA is presented, with the abundance of the *PyAct1* mRNA set as 100. (B) Comparison of the GUS enzymatic activities under the direction of different endogenous promoters. Gametophytic cells were transiently transformed with expression plasmids of the *PyGUS* gene under the direction of the *PyAct1*, *PyATP1A, PyElf1*and *PyGAPDH* promoters by particle bombardment. The GUS enzymatic activities were then examined 48 h after transient transformation. Data shown are means ± SD from three independent experiments.

3.2 Development of fluorescent protein reporter system

Since histochemical and quantitative enzymatic analyses in the PyGUS reporter system require cell killing, monitoring of the reporter expression during development or response to environmental stress in living cells is impossible to analyze. However, living cell imaging to monitor gene expression is possible using fluorescent proteins without additional substrates and cofactors (Ehrhardt, 2003). Indeed, fluorescent proteins are usually employed as useful tools to visualize protein localization in living cells in land plants (Buschmann et al., 2010; Kokkirala et al., 2010; Meng et al., 2010; Pribat et al., 2010).

We first examined the usability of fluorescent proteins as reporters in *P. yezoensis* cells, since the expression of fluorescent protein has not yet been demonstrated in red algal cells. Table 1 lists fluorescent protein genes such as green fluorescent protein (GFP) and its variants cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) from various organisms including the jellyfish *Aequorea victoria*, reef coral *Zoanthus* sp. and copepoda *Pontellina plumata*, whose codon usage is biased to be GC-rich by humanization. When these genes were expressed in *P. yezoensis* cells under the direction of the *PyAct1* promoter, a fluorescent signal was clearly observed in every transiently transformed cell. Fig. 6A represents the expression of AmCyan and ZsGreen as examples. Interestingly, the gene encoding plant-adapted GFP(S65T) (Niwa et al., 1999) under the direction of the *PyAct1* promoter also produced the GFP signal (Fig. 6A). Since the GC contents of humanized fluorescent proteins and sGFP(S65T) are as high as 60% (Table 1), a GC content of 60% appears to be sufficient for efficient expression of foreign genes in *P. yezoensis* cells.

Applicability of the fluorescent protein system to visualize protein localization in *P. yezoensis* cells has already been demonstrated. For example, as shown in Fig. 6B, plasma membrane localization of Pleckstrin homology (PH) domains from human phospholipase Cδ1 was demonstrated using AmCyan (Mikami et al., 2009) and nuclear localization of *P. yezoensis* transcription factor PyElf1 was also confirmed using AmCyan and ZsGreen (Uji et al., 2009). Thus, these applications will provide new opportunities for analyzing subcellular localization of various genes from *P. yezoensis*.

Color	Name	Max (Ex[1])	Max (Em[2])	Structure	GC content (%)	Origin	Acquisition
Humanized fluorescent protein							
cyan	AmCyan	458	489	tetramer	63.3	*Anemonia majano*	Clontech
green	mWasabi	493	509	monomer	59.5	*Clavularia* sp.	Allele biotech
	TurboGFP	482	502	monomer	63.8	*Pontellina plumata*	Evrogen
	ZsGreen	493	505	tetramer	63.2	*Zoanthus* sp.	Clontech
yellow	PhiYFP	525	537	monomer	60.9	*Phialidium* sp.	Evrogen
	ZsYellow	529	539	tetramer	62.3	*Zoanthus* sp.	Clontech
Plant-adapted fluorescent protein							
green	sGFP(S65T)	490	510	monomer	61.4	*Aequorea victoria*	Niwa et al. 1999

[1] Excitation. [2] Emission.

Table 1. Properties of humanized and plant-adapted fluorescent proteins whose expression was confirmed in *P. yezoensis*.

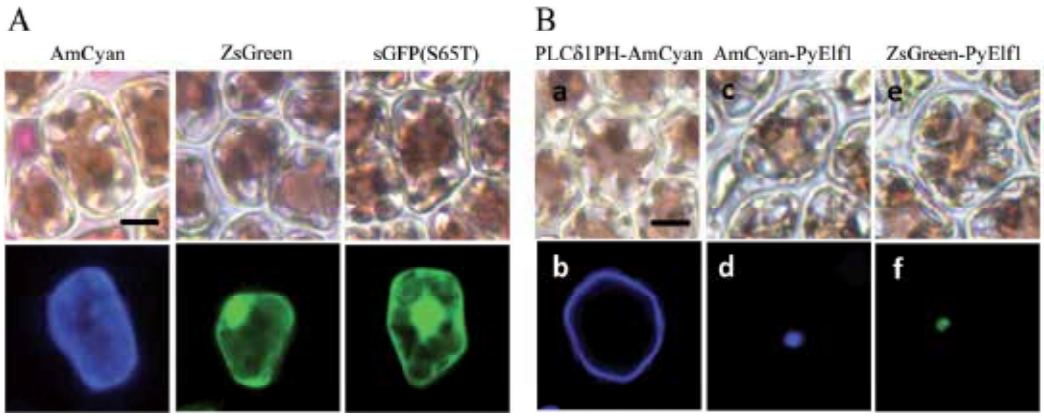

Fig. 6. Successful expression of fluorescent proteins in *P. yezoensis* gametophytic cells. (A) Expression of humanized fluorescent proteins and plant-adapted sGFP(S65T). Gametophytic cells were transiently transformed with expression plasmids containing the *AmCyan*, *ZsGreen* or *sGFP(S65T)* gene under the control of the *PyAct1* promoter by particle bombardment, 48 h after which cells were examined by fluorescent microscopy. Upper and lower panels show bright field and fluorescence images, respectively. The scale bar corresponds to 5 μm. (B) Visualization of subcellular localization with humanized fluorescent proteins in *P. yezoensis* cells. Expression plasmids pPyAct1-PLCδ1PH-AmCyan (a and b), pPyAct1-AmCyan-PyElf1 (c and d) and pPyAct1-ZsGreen-PyElf1 (e and f) were introduced into gametophytic cells of *P. yezoensis* by particle bombardment. Gametophytes were examined by fluorescent microscopy after 48 h of transient transformation. Plasma membrane localization of AmCyan fused with the PH domain from human phospholipase δ1 in gametophytic cells (a, bright field image; b, fluorescent image) and nuclear localization of AmCyan and ZsGreen fused with PyElf1 in gametophytic cells (c and e, bright field images; d and f, fluorescent images) are presented. The scale bar corresponds to 5 μm.

3.3 Application of PyGUS and sGFP(S65T) reporter systems in other red macroalgae

Since red macroalgae include a number of industrially important species such as *Porphyra* as the source of the food known as Nori, *Gracilaria* and *Gelidium* as sources of agar and *Chondrus* and *Kappaphycus* as sources of carrageenan, the establishment of a genetic manipulation system for these algae is eagerly anticipated. However, a transient gene expression system has not yet been developed in these red macroalgae other than *P. yezoensis*. In fact, EST analysis of *P. haitanensis* revealed that the GC content of the ORFs in this alga was as high as that in *P. yezoensis*, and the GC content at the third position of the triplets was significantly higher than those at the other two positions, which is consistent with the result reported in *P. yezoensis* (Fan et al., 2007). Similarly, analysis of the *GAPDH* gene from *Chondrus crispus* showed a high GC content (approximately 60%) in the cording region (Liaud et al., 1993). Thus, it is possible that the *PyGUS* gene could be commonly functional in red algae as a reporter of the gene expression. Indeed, applicability of the *PyGUS* gene in *P. tenera* has recently been confirmed by another laboratory (Son et al., 2011). The applicability of the *P. yezoensis* transient gene expression system was examined using the PyGUS and sGFP(S65T) reporter genes under the direction of the *PyAct1* promoter in the red macroalgae listed in Table 2. Good expression of PyGUS and sGFP(S65T) was observed

in Bangiophyceae including genus *Porphyra* and *Bangia*, whereas no expression of the reporter genes was seen in Florideophyceae (Table 2). Thus, it was concluded that the transient gene expression system developed in *P. yezoensis* is widely applicable in Bangiophycean red algae (Hirata et al., 2011).

The number of reporter-expressing cells varies among species (Table 2; Hirata et al., 2011), suggesting the possibility of the improvement of the gene transfer and expression systems in each species using respective endogenous strong promoters to drive the expression of the PyGUS and the sGFP(S65T) reporter genes. Similarly, expression of these reporter genes in Florideophycean algae seems to be possible if suitable endogenous promoters can be employed. Establishment of an efficient transient gene expression system will stimulate future development of stable transformation systems in various red macroalgae, enabling more effective utilization of them for industrial purposes.

Species	Number of reporter-expressing cells	
	PyGUS	sGFP(S65T)
Bangiophyceae		
Laboratory cultured strain		
Porphyra yezoensis strain TU-1*[1]	2535 ± 506	152 ± 32
Porphyra tenera strain JTW*[1]	2565 ± 600	140 ± 42
Naturally corrected species		
Porphyra yezoensis	721 ± 129	27 ± 8
Porphyra okamurae	290 ± 93	27 ± 9
Porphyra onoi	293 ± 97	20 ± 11
Porphyra variegata	333 ± 76	9 ± 3
Porphyra pseudolinearis (female)	455 ± 124	24 ± 6
Porphyra pseudolinearis (male)	9 ± 4	2 ± 1
Bnagia fuscopurpurea	118 ± 55	3 ± 1
Floredeophyceae		
Naturally corrected species		
Chondrus ocellatus	0	0
Gloiopeltis furcata	0	0
Gracilaria vermiculophylla	0	0
Mazzaella japonica	0	0

*[1] Laboratory-cultured strain

Table 2. Comparison of the number of reporter-expressing cells among red macroalgae after transfection of the pAct1-PyGUS or pAct1-sGFP(S65T) plasmid. The data shown are means ± S.D. from three independent experiments.

4. Conclusion

Genetic transformation is indispensable both to elucidation of gene function and to molecular breeding of crop plants. Our major goal is to establish a stable transformation system to produce *Porphyra* species carrying biotic and abiotic stress tolerances. For this purpose, our recent development of a transient gene expression system for Bangiophycean algae, by which the lack of transfer and expression systems for foreign genes was resolved, is the first breakthrough toward the establishment of a genetic transformation method in red macroalgae. We now know that the construction of an expression plasmid requires both optimization of codon usage in coding regions of the reporter gene and recruitment of endogenous strong promoters; also that particle bombardment is the proven method of gene transfer into red algal cells. The next steps are therefore development of targeted or random integration systems of foreign genes into the genome and selection of marker genes useful for isolation of transformed cells. Resolution of these methodological problems could contribute to accelerating progress in molecular biological research on physiological regulations that will be useful for the industrial production of transgenic red macroalgae.

5. Acknowledgements

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6. References

Apt, K. E., Kroth-Pancic, P. G. & Grossman, A. R. (1996). Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*. *Molecular and General Genetics*, Vol.252, No.5, pp. 572-579.

Asamizu, E., Nakajima, M., Kitade, Y., Saga, N., Nakamura, Y. & Tabata, S. (2003). Comparison of RNA expression profiles between the two generations of *Porphyra yezoensis* (Rhodophyta), based on expressed sequence tag frequency analysis. *Journal of Phycology*, Vol.39, No.5, pp. 923-930.

Bhatnagar-Mathur, P., Vadez, V. & Sharma, K. K. (2008). Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. *Plant Cell Reports*, Vol.27, No.3, pp. 411-424.

Blankenship, J. E. & Kindle, K. L. (1992). Expression of chimeric genes by the light-regulated cabII-1 promoter in *Chlamydomonas reinhardtii*: a cabII-1/nit 1 gene functions as a dominant selectable marker in a nit1-nit2-strain. *Molecular and Cell Biology*, Vol.12, No.11, pp. 5268-5279.

Blouin, N. A., Brodie, J. A., Grossman, A. C., Xu, P. & Brawley, S. H. (2011) *Porphyra*: a marine crop shaped by stress. *Trends in Plant Science*, Vol.16 , No.1 , pp. 29-37. .

Brown, A. P. C., Dunn, M. A., Goddard, N. J. & Hughes, M. A. (2001). Identification of a novel low-temperature-response element in the promoter of the barley (Hordeum vulgare L) gene *blt101.1*. *Planta* Vol.213, No.5, pp. 770-780.

Buschmann, H., Green, P., Sambade, A., Doonan, J. H. & Lloyd, C. W. (2010). Cytoskeletal dynamics in interphase, mitosis and cytokinesis analysed through *Agrobacterium*-mediated transient transformation of tobacco BY-2 cells. *New Phytologist*, doi: 10.1111/j.1469-8137.2010.03587.x.

Cheney, D., Metz, B. & Stiller, J. (2001). *Agrobacterium*-mediated genetic ransformation in the macroscopic marine red alga *Porphyra yezoensis*. *Journal of Phycology*, Vol.37 (Supplement S3), pp.11–12.

Chow, K. C. & Tung, W. L. (1999). Electrotransformation of *Chlorella vulgaris*. *Plant Cell Reports*, Vol.18, No.9, pp. 778-780.

Davies, J. P., Weeks, D. P. & Grossman, A. R. (1992). Expression of the arylsulfatase gene from the beta 2-tubulin promoter in *Chlamydomonas reinhardtii*. *Nucleic Acids Research*, Vol.20, No.12, pp. 2959-2965.

Day, A., Debuchy, R., Dillewijn, J., Purton, S. & Rochaix, J. D. (1990). Studies on the maintenance and expression of cloned DNA fragments in the nuclear genome of the green alga *Chlamydomonas reinhardtii*. *Physiologia Plantarum*, Vol.78, No .2, pp. 254-260.

Ehrhardt, D. (2003). GFP technology for live cell imaging. *Current Opinion in Plant Biology*, Vol.6, No.6, pp. 622-628.

El-Sheekh, M. M. (1999). Stable transformation of the intact cells of *Chlorella kessleri* with high velocity microprojectiles. *Biologia Plantarum*, Vol.42, No.2, pp. 209-216.

Fan, X. L., Fang, Y. J., Hu, S. N. & Wang, G. C. (2007). Generation and analysis of 5318 expressed sequence tags from the filamentous sporophyte of *Porphyra haitanensis* (Rhodophyta). *Journal of Phycology,* Vol.43, No.6, pp. 1287–1294.

Feng, S. Y., Xue, L. X., Liu, H. T. & Lu, P. J. (2009). Improvement of efficiency of genetic transformation for *Dunaliella salina* by glass beads method. *Molecular Biology Reports*, Vol.36, No.6, pp. 1433-1439.

Fuhrmann, M., Oertel, W. & Hegemann, P. (1999). A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii*. *Plant Journal*, Vol.19, No.3, pp. 353–361.

Fuhrmann, M., Hausherr, A., Ferbitz, L., Schödl, T., Heitzer, M. & Hegemann, P. (2004). Monitoring dynamic expression of nuclear genes in *Chlamydomonas reinhardtii* by using a synthetic luciferase reporter gene. *Plant Molecular Biology*, Vol.55, No.6, pp. 869-881.

Fukuda, S., Mikami, K., Uji, T., Park, E. J., Ohba, T., Asada, K., Kitade, Y., Endo, H., Kato, I. & Saga, N. (2008). Factors influencing efficiency of transient gene expression in the red macrophyte *Porphyra yezoensis*. *Plant Science*, Vol.174, No.3, pp. 329-339.

Funabashi, H., Takatsu, M., Saito, M. & Matsuoka, H. (2010). Sox2 regulatory region 2 sequence works as a DNA nuclear targeting sequence enhancing the efficiency of an exogenous gene expression in ES cells. *Biochemical and Biophysical Research Communications*, Vol.400, No.4, pp. 554-558.

Gan, S. Y., Qin, S., Othman, R. Y., Yu, D. & Phang, S. M. (2003). Transient expression of *lacZ* in particle bombarded *Gracilaria changii* (Gracilariales, Rhodophyta). *Journal of Applied Phycology.* Vol.15, No.4, pp. 351–353.

Gong, Q., Yu, W., Dai, J., Liu, H., Xu, R., Guan, H. & Pan, K. (2005). Efficient *gusA* transient expression in *Porphyra yezoensis* protoplasts mediated by endogenous beta-tubulin flanking sequences. *Journal of Ocean University of China*, Vol.6, No.1, pp. 21-25.

Griesbeck, C., Kobl, I., & Heitzer, M. (2006). *Chlamydomonas reinhardtii*. A protein expression system for pharmaceutical and biotechnological proteins *Molecular Biotechnology*, Vol.34, No.2, pp. 213-223.

Hallmann, A. (2007). Algal Transgenics and Biotechnology. *Transgenic Plant Journal*, Vol.1, No.1, pp. 81-98.

Hirakawa, Y., Kofuji, R. & Ishida, K. (2008). Transient transformation of a chlorarachniophyte alga, *Lotharella amoebiformis* (chlorarachniophyceae), with *uidA* and *egfp* reporter genes. *Journal of Phycology*, Vol.44, No.3, pp. 814-820.

Hirata, R., Takahashi, M., Saga, N. & Mikami, K. (2011). Transient gene expression system established in *Porphyra yezoensis* is widely applicable in Bangiophycean algae. *Marine Biotechnology*, in press (doi: 10.1007/s10126-011-9367-6).

Huang, X., Weber, J. C., Hinson, T. K., Mathieson, A. C. & Minocha, S. C. (1996). Transient expression of the GUS reporter gene in the protoplasts and partially digested cells of *Ulva lactuca* L (Chlorophyta). *Botanica Marina*, Vol.39, No.5, pp. 467-474.

Ishihara, K., Oyamada, C., Matsushima, R., Murata, M. & Muraoka, T. (2005). Inhibitory effect of porphyran, prepared from dried "Nori", on contact hypersensitivity in mice. *Bioscience Biotechnology and Biochemistry*, Vol.69, No.10, pp. 1824-1830.

Jiang, P., Qin, S. & Tseng, C. K. (2003). Expression of the *lacZ* reporter gene in sporophytes of the seaweed *Laminaria japonica* (Phaeophyceae) by gametophyte-targeted transformation. *Plant Cell Reports*, Vol.21, No.12, pp. 1211-1216.

Kakinuma, M., Coury, D., Nakamoto, C., Sakaguchi, K. & Amano, H. (2008). Molecular analysis of physiological responses to changes in nitrogen in a marine macroalga, *Porphyra yezoensis* (Rhodophyta). *Cell Biology and Toxicology*, Vol.24, No.6, pp. 629-639.

Kang, H. G. & An, G. H. (2005). Morphological alterations by ectopic expression of the rice *OsMADS4* gene in tobacco plants. *Plant Cell Reports*, Vol.24, No.2, pp. 120-126.

Kindle, K. L., Schnell, R. A., Fernandez, E. & Lefebvre, P. A. (1989). Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. *The Journal of Cell Biology*, Vol.109, No.6, pp. 2589–2601.

Kindle, K. L. (1990). High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.87, No.3, pp. 1228-1232.

Kokkirala, V. R., Yonggang, P., Abbagani, S., Zhu, Z. & Umate, P. (2010). Subcellular localization of proteins of *Oryza sativa* L. in the model tobacco and tomato plants. *Plant Signaling & Behavior*. Vol.5, No.11, pp. 1336-1341.

Kuang, M., Wang, S. J., Li,Y., Shen, D. L. & Zeng, C.K. (1998). Transient expression of exogenous GUS gene in *Porphyra yezoensis* (Rhodophyta). *Chinese Journal of Oceanology and Limnology,* Vol.16 (Supplement), pp. 56–61.

Kübler, J. E., Minocha, S. C. & Mathieson, A.C. (1994). Transient expression of the GUS reporter gene in protoplasts of *Porphyra miniata* (Rhodophyta). *Journal of Marine Biotechnology,* Vol.1, pp. 165–169.

Kurtzman, A. M. & Cheney, D. P. (1991). Direct gene transfer and transient expression in a marine red alga using the biolistic method. *Journal of Phycology*, Vol.27 (Suppliment S3), pp. 42

Liaud, M. F., Valentin, C., Brandt, U., Bouget, F. Y., Kloareg, B. & Cerff, R. (1993). The GAPDH gene system of the red alga *Chondrus crispus*: promoter structures, intron/exon organization, genomic complexity and differential expression of genes. *Plant Molecular Biology*, Vol.23, No.5, pp. 981–994.

Liu, H. Q., Yu, W. G., Dai, J. X., Gong, Q. H., Yang, K. F. & Zhang, Y. P. (2003). Increasing the transient expression of *GUS* gene in *Porphyra yezoensis* by 18S rDNA targeted homologous recombination. *Journal of Applied Phycology*, Vol.15, No.5, pp. 371-377.

Louis, J., Lorenc-Kukula, K., Singh, V., Reese, J., Jander, G. & Shah, J. (2010). Antibiosis against the green peach aphid requires the *Arabidopsis thaliana MYZUS PERSICAE-INDUCED LIPASE1* gene. *Plant Journal*, Vol.64, No.5, pp. 800-811.

Lumbreras, V., Stevens, D. R. & Purton, S. (1998). Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *Plant Journal,* Vol.14, No.4, pp. 441-447.

Mayfield, S. P. & Kindle, K. L. (1990). Stable nuclear transformation of *Chlamydomonas reinhardtii* by using a *C. reinhardtii* gene as the selectable marker. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.87, No.6, pp. 2087-2091.

Meng, Q. T., Du, J. Z., Li, J. J., Lu, X. M., Zeng, X. A., Yuan, M. & Mao, T. L. (2010). Tobacco microtubule-associated protein, MAP65-1c, bundles and stabilizes microtubules. *Plant Molecular Biology*, Vol.74, No.6, pp. 537-547.

Mikami, K., Uji, T., Li, L., Takahashi, M., Yasui, H. & Saga, N. (2009). Visualization of Phosphoinositides via the development of the transient expression system of a cyan fluorescent protein in the red alga *Porphyra yezoensis*. *Marine Biotechnology*, Vol.11, No.5, pp. 563-569.

Nikaido, I., Asamizu, E., Nakajima, M., Nakamura, Y., Saga, N. & Tabata, S. (2000). Generation of 10,154 expressed sequence tags from a leafy gametophyte of a marine red alga, *Porphyra yezoensis*. *DNA Research,* Vol.7, No.3, pp. 223-227.

Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M. & Kobayashi, H. (1999). Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. *Plant Journal*, Vol.18, No.4, pp. 455-463

Noda, H., Amano, H., Arashima, K. & Nisizawa, K. (1990). Antitumor activity of marine algae. *Hydrobiologia*, Vol.204-205, No.1, pp. 577-584.

Ohnuma, M., Yokoyama, T., Inouye, T., Sekine, Y. & Tanaka, K. (2008). Polyethylene glycol (PEG)-mediated transient gene expression in a red alga, *Cyanidioschyzon merolae* 10D. *Plant and Cell Physiology*, Vol.49, No.1, pp. 117-120.

Okauchi, M. & Mizukami, Y. (1999). Transient β-Glucuronidase (GUS) gene expression under control of *CaMV 35S* promoter in *Porphyra tenera* (Rhodophyta). *Bulletin of National Research Institute of Aquaculture*, Vol.1, pp. 13-18.

Park, C. S., Sakaguchi, K., Kakinuma, M. & Amano, H. (2000). Comparison of the morphological and physiological features of the red rot disease fungus *Pythium* sp isolated from *Porphyra yezoensis* from Korea and Japan. *Fisheries Science*, Vol.66, No.2, pp. 261-269.

Plank, C., Schillinger, U., Scherer, F., Bergemann, C., Remy, J. S., Krotz, F., Anton, M., Lausier, J. & Rosenecker, J. (2003). The magnetofection method: Using magnetic force to enhance gene delivery. *Biological Chemistry*, Vol.384, No.5, pp. 737-747.

Pribat, A., Noiriel, A., Morse, A. M., Davis, J. M., Fouquet, R., Loizeau, K., Ravanel, S., Frank, W., Haas, R., Reski, R., Bedair, M., Sumner, L. W. & Hanson, A. D. (2010). Nonflowering plants possess a unique folate-dependent phenylalanine hydroxylase that is localized in chloroplasts. *Plant Cell*, Vol.22, No.10, pp. 3410-3422.

Ruecker, O., Zillner, K., Groebner-Ferreira, R. & Heitzer, M. (2008). Gaussia-luciferase as a sensitive reporter gene for monitoring promoter activity in the nucleus of the green alga *Chlamydomonas reinhardtii*. *Molecular Genetics and Genomics*, Vol.280, No.2, pp. 153-162.

Sakamoto, H., Maruyama, K., Sakuma, Y., Meshi, T., Iwabuchi, M., Shinozaki, K. & Yamaguchi-Shinozaki, K. (2004). Arabidopsis Cys2/His2-type zinc-finger proteins function as transcription repressors under drought, cold, and high-salinity stress conditions. *Plant Physiology*, Vol.136, No.1, pp. 2734-2746.

Schiedlmeier, B., Schmitt, R., Muller, W., Kirk, M. M., Gruber, H., Mages, W. & Kirk, D. L. (1994). Nuclear transformation of *Volvox carteri*. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.91, No.11, pp. 5080-5084.

Schroda, M., Blöcker, D. & Beck, C.F. (2000). The *HSP70A* promoter as a tool for the improved expression of transgenes in *Chlamydomonas*. *Plant Journal*, Vol.21, No.2, pp. 121-131.

Shao, N. & Bock, R. 2008. (2008). A codon-optimized luciferase from *Gaussia princeps* facilitates the in vivo monitoring of gene expression in the model alga *Chlamydomonas reinhardtii*. *Current Genetics*, Vol.53, No.6, pp. 381-388.

Shinozaki, K. & Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany*, Vol.58, No.2, pp. 221-227.

Son, S. H., Ahn, J.-W., Uji, T., Choi, D.-W., Park, E.-J., Hwang, M. S., Liu, J. R., Choi, D., Mikami, K. & Jeong, W.-J. (2011) Development of an expression system using the heat shock protein 70 promoter in the red macroalga, *Porphyra tenera*. *Journal of Applied Phycology*, in press (doi: 10.1007/s10811-011-9652-9).

Specht, E., Miyake-Stoner, S. & Mayfesqaxeld, S. (2010). Micro-algae come of age as a platform for recombinant protein production. *Biotechnology Letters*, Vol.32 No.10, pp. 1373-1383.

Stevens, D. R., Rochaix, J. D. & Purton, S. (1996). The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*. *Molecular & General Genetics*, Vol.251, No.1, pp. 23-30.

Sugawara, T., Sakai, S. & Hirata, T. (2011). Nutritional and physiological functions of *Porphyra*. In: Porphyra yezoensis: *Frontiers in Physiological and Molecular Biological Research*, K. Mikami, (Ed.), in press, Nova Science Publishers, New York, U.S.A.

Sun, M., Qian, K. X., Su, N., Chang, H. Y., Liu, J. X. & Chen, G. F. (2003). Foot-and-mouth disease virus VP1 protein fused with cholera toxin B subunit expressed in *Chlamydomonas reinhardtii* chloroplast. *Biotechnology Letters*, Vol.25, No.13, pp. 1087-1092.

Svingen, T., Wilhelm, D., Combes, A. N., Hosking, B., Harley, V. R., Sinclair, A. H. & Koopman, P. (2009). Ex vivo magnetofection: A novel strategy for the study of gene function in mouse organogenesis. *Developmental Dynamics*, Vol.238, No.4, pp. 956-964.

Takahashi, M., Uji, T., Saga, N. & Mikami K (2010). Isolation and regeneration of transiently transformed protoplasts from gametophytic blades of the marine red alga *Porphyra yezoensis*. *Electronic Journal of Biotechnology*, Vol.13, No.2, http://www.ejbiotechnology.cl/content/vol13/issue2/full/7/index.html.

Tan, D., Qin, S., Zhang, Q., Jiang, P. & Zhao, F. (2005). Establishment of a micro-particle bombardment transformation system for *Dunaliella salina*. *The Journal of Microbiology*, Vol.43, No.4, pp. 361-365.

Torney, F., Moeller, L., Scarpa, A. & Wang, K. (2007). Genetic engineering approaches to improve bioethanol production from maize. *Current Opinion in Biotechnology*, Vol.18, No.3, pp. 193-199.

Uji, T., Takahashi, M., Saga, N. & Mikami, K. (2010). Visualization of nuclear localization of transcription factors with cyan and green fluorescent proteins in the red alga *Porphyra yezoensis*. *Marine Biotechnology*, Vol.12, No.2, pp. 150-159.

Walker, T. L., Becker, D. K. & Collet, C. C. (2004). Characterisation of the Dunaliella tertiolecta RbcS genes snd their promoter activity in *Chlamydomonas reinhardtii*. *Plant Cell Reports*, Vol.23, No.10-11, pp. 727-735.

Walker, T. L., Collet, C. & Purton, S. (2005). Algal transgenics in the genomic ERA. *Journal of Phycology*, Vol.41, No.6, pp. 1077-1093.

Wally, O. & Punja, Z. K. (2010). Enhanced disease resistance in transgenic carrot (*Daucus carota* L.) plants over-expressing a rice cationic peroxidase. *Planta*, Vol.232, No.5, pp. 1229-1239.

Wu, S. X., Huang, R., Xu, L. L., Yan, G. Y. & Wang, Q. X. (2010). Improved hydrogen production with expression of *hemH* and *lba* genes in chloroplast of *Chlamydomonas reinhardtii*. *Journal of Biotechnology*, Vol.146, No.3, pp. 120-125.

Zaslavskaia, L. A., Lippmeier, J. C., Kroth, P. G., Grossman, A. R. & Apt, K. E. (2000). Transformation of the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) with a variety of selectable marker and reporter genes. *Journal of Phycology*, Vol.36, No.2, pp. 379-386.

Zemke-White, W. L. & Ohno, M. (1999). World seaweed utilisation: An end-of-century summary. *Journal of Applied Phycology*, Vol.11, No.4, pp. 369-376.

Zorin, B., Lu, Y. H., Sizova, I. & Hegemann, P. (2009). Nuclear gene targeting in *Chlamydomonas* as exemplified by disruption of the *PHOT* gene. *Gene*, Vol.432, No.1-2, pp. 91-96.

Part 3

Plant Transformation as a Tool for Regulating Secondary Metabolism

13

Application of Agrobacterium *Rol* Genes in Plant Biotechnology: A Natural Phenomenon of Secondary Metabolism Regulation

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1. Introduction

The *rolA*, *rolB* and *rolC* genes are plant oncogenes that are carried in plasmids of the plant pathogen *Agrobacterium rhizogenes*. Following agrobacterial infection, these genes are transferred into the plant genome and cause tumor formation and hairy root disease. The *rolB* and *rolC* genes of *Agrobacterium rhizogenes* were studied extensively for the past two decades as regulators of cell growth and differentiation. A new function for the *rol* genes in plant-Agrobacterium interactions became apparent with the discovery that these genes are also potential activators of secondary metabolism in transformed cells in different plant families (reviewed by Bulgakov, 2008).

Classically, *rolB* and *rolC* have been considered closely related genes, possessing similar biological functions. However, they demonstrated different, or even opposite, effects on cell death processes (Schmulling et al., 1988), calcium balance in transformed cells (Bulgakov et al., 2003), sensitivity to auxin (Maurel et al., 1991), growth of transformed tissues (Capone et al., 1989) and secondary metabolism (Shkryl et al., 2008).

Plant-microbe interactions often lead to the development of defense mechanisms in plant cells. Since reactive oxygen species (ROS) play a pivotal role in the regulation of plant defense mechanisms, extensive experiments were performed to study the relationship between secondary metabolism (phytoalexin production) and the production of ROS in cells transformed with *rol* genes. Here, we summarize these results. Surprisingly, the *rolB* and *rolC* genes not only activated phytoalexin production but also suppressed intracellular ROS levels. This combination of defense responses, coupled with the effect of ROS suppression, represents a unique case in plant-microbe interactions. These findings suggest that bypassing upstream cell control mechanisms may be useful in the construction of plant cells possessing stable production of secondary metabolites. This chapter describes the new findings relating to secondary metabolism and ROS production under the individual and combined expression of the *rol* genes in plant cells.

2. *Agrobacterium rhizogenes rol* genes as activators of secondary metabolism

The interest in *rol* genes stems from the well-known fact that hairy-root cultures, derived from various plants species, stably produce high amounts of secondary metabolites. Among T-DNA genes, three *rol* genes (*rolA*, *rolB* and *rolC*) expressed individually or in a combination (*rolABC*) seem to be most efficient at inducing the production of secondary metabolites (Shkryl et al., 2008). Although it is known that the *rol* genes activate the transcription of defense genes, the mechanism of activation is unclear. Evidence indicates that the *rol* genes mediate uncommon signal transduction pathways in plants. They act on phytoalexin production independently of plant defense hormones and the calcium-dependent NADPH oxidase pathway (Bulgakov, 2008). The extent of secondary metabolism activation varies between plant species, from 2- to 300-fold depending on the group of secondary metabolites and the plant species (Bulgakov, 2008). Transformation with the *rol* genes is especially useful in those cases where different methods commonly used to increase secondary metabolite production (cell selection, elicitor treatments and addition of a biosynthetic precursor) only slightly enhance cell productivity. In some cases, transformation with the *rol* genes provokes a biphasic effect with an initial suppression and the subsequent activation of biosynthesis for particular groups of secondary metabolites (Bulgakov et al., 2005; Bulgakov, 2008; Inyushkina et al., 2009). The information about the effect of *rol* genes on secondary metabolism is still limiting and transformation in some cases causes unpredictable results. For example, *rolB* has been shown to cause a significant stimulatory effect on resveratrol production by cultured cells of *Vitis amurensis* (Kiselev et al., 2007). However, the resveratrol content was lowered after a 2-year cultivation of transformed cells. The reason for this is unknown because only one *rolB*-transformed cell line was analyzed.

The *rolB* and *rolC* genes are the most interesting candidates for plant biochemical engineering. High expression of the *rolB* gene in transformed plant cells dramatically increased the biosynthesis of secondary metabolites (Shkryl et al., 2008); however, excessive expression of the gene inhibited cell growth. Compared to the *rolB* gene, the *rolC* gene activated the biosynthesis of secondary metabolites to a lesser extent. However, the *rolC* gene possesses an important and interesting ability to increase cell growth. Evidence indicates that each of the *rol* genes has its own role in plant metabolic processes (Bulgakov, 2008).

3. Agrobacterium and ROS

Reactive oxygen species play an important role during plant-pathogen interactions. Avirulent and virulent pathogens elicit ROS accumulation in plant cells with different dynamics, and elicitors of defense responses, often referred to as microbe-associated molecular patterns (MAMPs), also trigger oxidative bursts (Torres et al., 2006). ROS act as executioners of pathogens and host cells by causing a hypersensitive response; they also act as signaling molecules that activate defense mechanisms. To ensure their own survival, pathogens commonly inactivate ROS produced during plant-pathogen interactions. The plant pathogen *Pseudomonas syringae* is a well-studied pathogen model that has been used to demonstrate this effect. The effector HopAO1 (HopPtoD2) protein of the *P. syringae* pv. *tomato* strain DC300 is injected from the bacterial cell into the plant cell to promote bacterial growth by suppressing the innate immunity of the host cell. It was shown that HopAO1

suppresses ROS induction in plants (Bretz et al., 2003) as well as several defense mechanisms associated with MAMP-triggered innate immunity (Underwood et al., 2007).
Agrobacterium tumefaciens also employs this strategy to combat the plant cells' defense mechanisms (reviewed by Escobar & Dandekar 2003). This pathogen can detoxify hydrogen peroxide, a primary component of plant ROS, using an agrobacterial catalase KatA (Xu & Pan 2000). *A. tumefaciens* can also suppress the induction of the hypersensitive response in plants elicited by *P. syringae* pv. *phaseolicola* (Robinette & Matthysse 1990). *A. rhizogenes* is a plant pathogen closely related to *A. tumefaciens.* Many laboratories have studied this pathogen extensively, so it is surprising that the effects of *A. rhizogenes* on ROS metabolism in host cells have never been investigated.

4. ROS and secondary metabolism

In some plant cell cultures, ROS are shown to be sufficient for the induction of plant secondary metabolite accumulation, whereas they are not involved in regulation of secondary metabolism in some other plants (reviewed by Zhao et al., 2005). It has been shown that ROS mediate the elicitor-induced accumulation of isoflavonoids in soybean and alfalfa, indole alkaloids in *Catharanthus roseus*, ginsenosides in ginseng, thujaplicin in Mexican cypress, momilactones in rice cell cultures, furanocoumarin in parsley cell cultures, diterpene rishitin and acridone alkaloid *p*-coumaroyloctopamine in potato and capsidiol in tobacco (Zhao et al., 2005).
The involvement of the oxidative burst generated by NADPH oxidase in the process of phytoalexin stimulation is well known (Guo et al., 1998; Jabs et al., 1997). There are, however, several examples of Ca^{2+}-dependent regulation of defense genes, where the NADPH oxidase pathway is not involved (Romeis et al., 2000; Sasabe et al., 2000). It is clear that several different mechanisms regulate secondary metabolism in plants. Although the details of these mechanisms in different regulatory situations are poorly investigated, the general rule postulates that ROS are important inductors of secondary metabolism.

5. Unexpected complicity of related genes: *rolC* inhibits ROS production and *rolB* activates ROS degradation

5.1 ROS levels in transformed cells

Fig. 1 presents results indicating inverse relationship between the production of secondary metabolites (anthraquinones) and ROS levels in callus cultures of *R. cordifolia* transformed with *rol* genes (Bulgakov et al., 2008; Bulgakov et al., submitted). The use of confocal microscopy and fluorogenic dyes revealed the strong inhibitory effect of *rolC* on ROS levels in transformed plant cells. The constitutive expression of the gene led to decreased steady-state levels of ROS in the cells and prevented the ROS accumulation that was induced by different treatments. The ROS inhibition was dose-dependent: the highest levels of the *rolC* expression caused maximal ROS suppression. The maximal inhibition was 46% of the basal level of ROS. This result was confirmed with an independent method employing luminol-based fluorimetric detection of ROS (Bulgakov et al., 2008).
The effect of the *rolB* gene on ROS metabolism in transformed cells has also been studied. One might expect that the gene would act in concert with the *rolC* gene to decrease ROS levels. However, the involvement of *rolB* in the induction of cellular death (necrosis) in callus and leaves of transformed plants (Schmülling et al., 1988) and in the activation of

secondary metabolism (Bulgakov 2008), i.e., in the processes which are often associated with increased production of ROS, would indicate that *rolB* expression is associated with increased ROS levels in transformed tissues. The investigation performed to discriminate between these possibilities showed a high variation of ROS levels in *rolB*-transformed cells. Extensive studies revealed that *rolB* decreased steady-state ROS levels in transformed cells up to 81-85% of control cells. It should be noted that the *rolC* gene was much more active as a ROS suppressor. However, *rolB* was more active in suppressing induced levels of ROS. Expression of *rolB* was sufficient to inhibit excessive elevations of ROS induced by paraquat, menadione and light stress and prevented cell death induced by chronic oxidative stress.

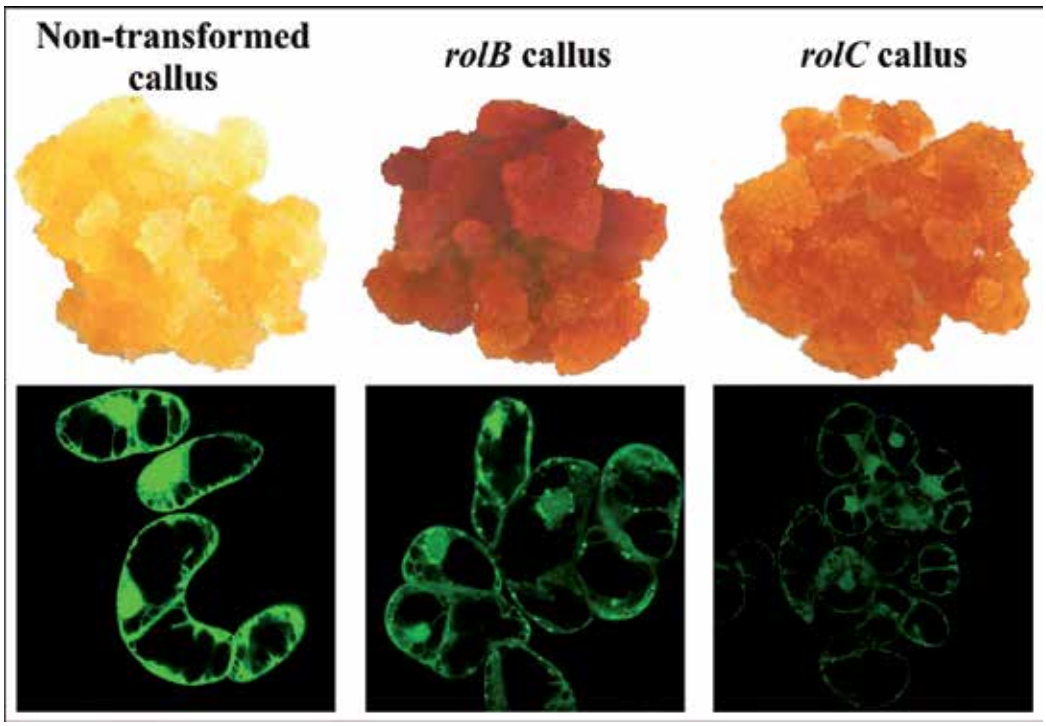

Fig. 1. Relation between secondary metabolism and intracellular ROS level in *R. cordifolia* cells. The upper panel presents phenotypes of *R. cordifolia* calli transformed with the *rol* genes. The *rolB*-calli and *rolC*-calli contained ten times and six times more anthraquinones, respectively, compared to the non-transformed calli. At the same time, ROS levels in cells of these transformed calli were low (see the bottom panel). Green fluorescence inside cells reflects summarized ROS (such as hydrogen peroxide, peroxyl radicals and peroxynitrite) levels measured by laser-scanning confocal microscopy and visualized by dichlorofluorescein diacetate.

5.2 A model in which *rolC* inhibits NADPH oxidase via CDPK

It is known that particular calcium-dependent protein kinase (CDPK) isoforms could activate stress-induced NADPH oxidases of plants by phosphorylation. For example, potato StCDPK5 induces the phosphorylation of StRBOHB (*Solanum tuberosum* NADPH oxidase)

and regulates the oxidative burst (Kobayashi et al., 2007). An investigation of *R. cordifolia* NADPH oxidase genes led to the identification of the *RcRboh1*, *RcRboh2* and *RcRboh3* genes. The alignment of deduced amino acid sequences and comparison with known RBOH proteins showed that RcRBOH1 is most homologous to NADPH oxidases that are responsible for stress-induced oxidative burst, whereas RcRBOH3 is a constitutively active oxidase, supporting ROS homeostasis under normal conditions. Real-time PCR measurements showed dose-dependent inhibition of *RcRboh3* in *rolC*-transformed cultured cells.

By investigating CDPK genes of *R. cordifolia*, 20 CDPK gene isoforms were identified. The closest analog of *StCDPK5* was found to be the *RcCDPK3* gene. We expected that *RcCDPK3* expression in *rolC*-transformed cells is inhibited. In this scenario, the deficient NADPH oxidase phosphorylation should prevent the generation of ROS. This hypothesis was confirmed by experimental data, which showed that *RcCDPK3* expression was inhibited in *rolC*-transformed cells in a dose-dependent manner (the extent of repression was dependent on the strength of *rolC* expression). Interestingly, the expression of most of the other 19 CDPK genes was unchanged in *rolC*-transformed cells, or even upregulated (Shkryl & Veremeichik, unpublished result).

Additional experiments showed that *rolC* does not affect the cellular ROS-detoxifying system. Expression of genes encoding extracellular class III peroxidases, ascorbate peroxidases, catalases and superoxide dismutases was not changed in *rolC*-cells compared to normal cells (Veremeichik et al., submitted; our unpublished result).

These results led us to postulate that *rolC* is involved in the regulation of plant NADPH oxidases. It is likely that *rolC* inhibits the CDPK-mediated NADPH oxidase pathway. This leads to lowered ROS production and ultimately decreases basal intracellular ROS levels. Because ROS levels in transformed cells are low, antioxidant genes are not activated.

In a new research, an Arabidopsis CDPK gene was expressed in *R. cordifolia* cells. Constitutive expression of the gene caused significant activation of anthraquinone biosynthesis (Shkryl et al., 2011), thus confirming our expectation that CDPKs are directly involved in secondary metabolism regulation.

5.3 A model for *rolB*: NADPH oxidase activation leads to the induction of antioxidant defense system

In contrast to *rolC*, the *rolB* gene caused significant activation of both the inducible and constitutive forms of *R. cordifolia* NADPH oxidase genes, *RcRboh1* and *RcRboh3*. This effect is reproducible and does not depend on different external stimuli, such as cold, heat and high salt conditions (Shkryl & Veremeichik, unpublished observation). Thus, *rolB* constitutively activates ROS generation via the activation of the NADPH oxidase ROS-generating system. In this scenario, the cells should defend themselves against the toxicity of excessive ROS. The experimental data showed a massive induction of ROS-detoxifying systems, such as extracellular class III peroxidases (Veremeichik et al., submitted), ascorbate peroxidases and some isoforms of catalases and superoxide dismutases in transformed cells (Shkryl et al., 2010). Thus, *rolB*-transformed cells generate ROS and simultaneously destroy excessive ROS. Equilibrium between these processes determines the resulting ROS level in *rolB*-transformed cells. One can speculate that the slow growth of *rolB*-transformed cells is a consequence of high consumption of cell metabolites and energy in these opposed processes.

5.4 How do the *rol* genes stabilize the biosynthesis of secondary metabolites?

In some cases, the effect of secondary metabolism activation mediated by the *rolB* and *rolC* genes is remarkably stable over time. For example, *R. cordifolia* transformed cells produce large amounts of anthraquinones over a long period of time (over 10 years) without any selection.

It is evident that the *rol* genes somehow avoid the regulatory controls of host cells. The data indicating suppression of basic and induced levels of ROS, together with earlier reported results indicating that the *rol* genes modulate phytoalexin production independently of ethylene, salicylic acid and methyl jasmonate-mediated pathways as well as the NADPH oxidase pathway (Bulgakov et al., 2002, 2003, 2004), point toward a signaling sequence through which the *rol*-gene signals bypass the control mechanisms of host cells. The resulting output is the development of defense reactions that are independent of cellular control mechanisms. In this scenario, the *rol* genes directly activate key genes of secondary metabolism, probably via transcription factors.

Investigation of such complex processes is a subject of systems biology. Comprehensive study of the regulatory networks involved in the biosynthesis of secondary metabolites by proteomics methods is an exciting and new field of knowledge (Bulgakov et al., 2011). This methodology will be used to unravel the complex mechanisms of the *rol* genes.

6. Combined effect of the *rolA*, *B* and *C* genes

The combined action of the *rol* genes on intracellular ROS levels was studied using pRiA4 and *rolABC* constructs. Because the *rolA* gene only slightly affected ROS levels, the main players affecting ROS metabolism were found to be the *rolB* and *rolC* genes. When expressed together, *rolC* and *rolB* balanced the effects of each other. Consequently, ROS levels in pRiA4- and *rolABC*-transformed cells were 83% and 90% of the basal levels, respectively.

It is clear that the combined actions of the *rolA*, *B* and *C* genes do not cause significant ROS suppression. If it were otherwise, the combined effect of the *rol* genes could cause a disruption in ROS homeostasis and cell death. However, the strategy of the phytopathogen *A. rhizogenes* is not to kill cells. Instead, the bacteria, acting via the transferred genes, render cells more tolerant of environmental stresses and increase their defense potential. In many cases, the *rol* genes ensure a high growth rate of transformed cells and their hormonal independence. In this context, the *rol* genes appear to be in tune with each other, providing physiological conditions for better cell fitness in the face of changing environmental conditions (Fig. 2). Perhaps, this is the main effect of the *rol* genes as members of the RolB (*plast*) gene family.

Let's consider the situations in which the *rol* genes would have a favorable effect on cell survival. If *A. rhizogenes*-transformed plant cells were subjected to signals causing ROS elevation (cold, heat, high-intensity light stress, necrotrophic pathogens, etc.), *rolC* would prevent excessive ROS by the suppression of the NADPH oxidase gene. This effect would be strengthened by *rolB*, which activates the antioxidant system. If plant cells were subjected to a signal causing ROS suppression (this is a strategy utilized by many plant pathogens), *rolB* would prevent the decrease of ROS levels by the activation of NADPH oxidase genes. Currently, it is known that *rolC* increases the cold, heat, salt and light resistance of transformed cells. The *rolB* gene exerts the same effects. Additionally, *rolB*-transformed cells are resistant to the ROS-generating herbicide paraquat and external hydrogen peroxide. In contrast, *rolC*-transformed cells are not resistant to these treatments because they do not

possess sustained antioxidant defenses. Simultaneous expression of *rolC* and *rolB* in transformed cells confers resistance to all of these treatments.
In addition, these results reveal an interesting analogy between *rol*-transformed plant cells and mammalian tumor cells, which are also highly viable and resistant to different therapeutic treatments.

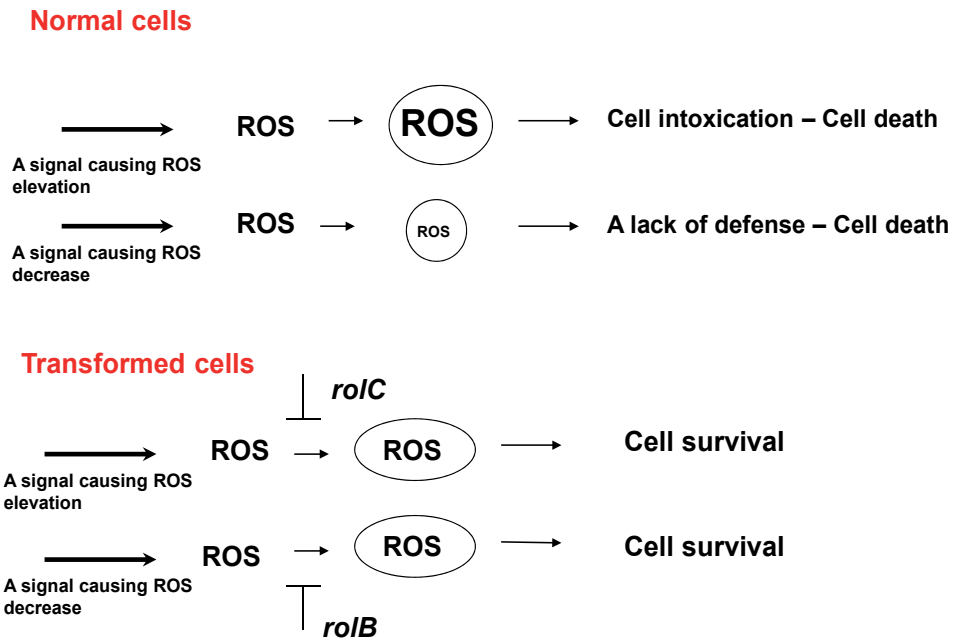

Fig. 2. A scheme illustrating the possible role of *rol* genes in cell survival. The *rolC* and *rolB* genes mitigate ROS changes caused by environmental stimuli. The signals causing acute ROS elevations are high temperature, cold, high salt conditions, excessive light and others. Signals causing decreased intracellular ROS levels are provoked by many pathogens.

7. Conclusion

The combination of defense mechanisms, coupled with the effect of ROS suppression described in this chapter, represents a unique case of plant-microbe interactions. *A. rhizogenes* is closely related to *A. tumefaciens,* which also suppresses the host immune response by ROS inhibition. There is, however, a fundamental difference between these pathogens. *A. tumefaciens* expresses a chromosome catalase gene and suppresses ROS during contact with plant cells. *A. rhizogenes*, acting via T-DNA genes, suppresses ROS in many generations of transformed cells, thereby ensuring a long-term effect on ROS homeostasis. In the studies discussed here, many questions dealing with the relationship between secondary metabolism and ROS metabolism remain to be answered. Nevertheless, the mechanism underlying the action of the *rol* genes is currently emerging. Unraveling this mechanism would allow the engineering of plant cells with improved characteristics and free of shortcomings inherent to the *rol* genes.

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9. References

Bretz, JR Mock NM, Charity JC, Zeyad S, Baker CJ, Hutcheson SW. (2003). A translocated protein tyrosine phosphatase of *Pseudomonas syringae* pv. *tomato* DC3000 modulates plant defence response to infection. *Mol. Microbiol* 49: 389-400.

Bulgakov VP, Tchernoded GK, Mischenko NP, Khodakovskaya MV, Glazunov VP, Radchenko SV, Zvereva EV, Fedoreyev SA, Zhuravlev YN. (2002). Effect of salicylic acid, methyl jasmonate, ethephon and cantharidin on anthraquinone production by *Rubia cordifolia* callus cultures transformed with the *rolB* and *rolC* genes. *J Biotechnol* 97: 213-21.

Bulgakov VP, Tchernoded GK, Mischenko NP, Shkryl YN, Glazunov VP, Fedoreyev SA, Zhuravlev YN. (2003). Effects of Ca^{2+} channel blockers and protein kinase/phosphatase inhibitors on growth and anthraquinone production in *Rubia cordifolia* cultures transformed by the *rolB* and *rolC* genes. *Planta* 217: 349-55.

Bulgakov VP, Tchernoded GK, Mischenko NP, Shkryl Y N, Fedoreyev SA, Zhuravlev YN. (2004). The *rolB* and *rolC* genes activate synthesis of anthraquinones in *Rubia cordifolia* cells by mechanism independent of octadecanoid signaling pathway. *Plant Sci* 166: 1069-75.

Bulgakov VP, Veselova MV, Tchernoded GK, Kiselev KV, Fedoreyev SA, Zhuravlev YN. (2005). Inhibitory effect of the *Agrobacterium rhizogenes rolC* gene on rabdosiin and rosmarinic acid production in *Eritrichium sericeum* and *Lithospermum erythrorhizon* transformed cell cultures. *Planta* 221: 471-8.

Bulgakov VP. (2008). Functions of *rol* genes in plant secondary metabolism. *Biotechnol Adv* 26: 318-24.

Bulgakov VP, Aminin DL, Shkryl YN, Gorpenchenko TY, Veremeichik GN, Dmitrenok PS, Zhuravlev YN. (2008). Suppression of reactive oxygen species and enhanced stress tolerance in *Rubia cordifolia* cells expressing the *rolC* oncogene. *Mol Plant Microbe Interact* 21: 1561-70.

Bulgakov VP, Inyushkina YV, Fedoreyev SA. (2011). Rosmarinic acid and its derivatives: biotechnology and applications. *Crit Rev Biotechnol* (in press).

Capone I, Spano L, Cardarelli M, Bellincampi D, Petit A, Costantino P. (1989). Induction and growth properties of carrot roots with different complements of *Agrobacterium rhizogenes* T-DNA. *Plant Mol Biol* 13: 43-52.

Escobar MA, Dandekar AM. (2003). *Agrobacterium tumefaciens* as an agent of disease. *Trends Plant Sci* 8: 380-386.

Guo ZJ, Lamb C, Dixon RA. (1998). Potentiation of the oxidative burst and isoflavonoid phytoalexin accumulation by serine protease inhibitors. *Plant Physiol* 118: 1487-94.

Inyushkina YV, Kiselev KV, Bulgakov VP, Zhuravlev YN. (2009). Specific genes of cytochrome P450 monooxygenases are implicated in biosynthesis of caffeic acid

metabolites in *rolC*-transgenic culture of *Eritrichium sericeum*. *Biochemistry (Moscow)* 74: 917-24.

Jabs T, Tschöpe M, Colling C, Hahlbrock K, Scheel D. (1997). Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc Natl Acad Sci USA* 94: 4800-05.

Kiselev KV, Dubrovina AS, Veselova MV, Bulgakov VP, Fedoreyev SA, Zhuravlev YN. (2007). The *rolB* gene-induced overproduction of resveratrol in *Vitis amurensis* transformed cells. *J Biotechnol* 128: 681-92.

Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, Shimamoto K, Doke N, Yoshioka H. (2007). Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. *Plant Cell* 19: 1065-80.

Maurel C, Barbier-Bryqoo H, Spena A, Tempe J, Guern J. (1991). Single *rol* genes from the *Agrobacterium rhizogenes* T(L)-DNA alter some of the cellular responses to auxin in *Nicotiana tabacum*. *Plant Physiol* 97: 212-216.

Robinette D, Matthysse AG. (1990). Inhibition by *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* of development of the hypersensitive response elicited by *Pseudomonas syringae* pv. *phaseolicola*. *J Bacteriol* 172: 5742-49.

Romeis T, Piedras P, Jones JDG. (2000). Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defense response. *Plant Cell* 12: 803-15.

Sasabe M, Takeuchi K, Kamoun S, Ichinose Y, Govers F, Toyoda K, Shiraishi T, Yamada T. (2000). Independent pathways leading to apoptotic cell death, oxidative burst and defense gene expression in response to elicitin in tobacco cell suspension culture. *Eur J Biochem* 267: 5005-13.

Schmulling T, Shell J, Spena A. (1988). Single genes from *Agrobacterium rhizogenes* influence plant development. *EMBO J* 7: 2621-29.

Shkryl YN, Veremeichik GN, Bulgakov VP, Tchernoded GK, Mischenko NP, Fedoreyev SA, Zhuravlev YN. (2008). Individual and combined effects of the *rolA*, *B*, and *C* genes on anthraquinone production in *Rubia cordifolia* transformed calli. *Biotechnol Bioeng* 100: 118-25.

Shkryl YN, Veremeichik GN, Bulgakov VP, Gorpenchenko TY, Aminin DL, Zhuravlev YN. (2010). Decreased ROS level and activation of antioxidant gene expression in *Agrobacterium rhizogenes* pRiA4-transformed calli of *Rubia cordifolia*. *Planta* 232: 1023-32.

Shkryl YN, Veremeichik GN, Bulgakov VP, Zhuravlev YN. (2011). Induction of anthraquinone biosynthesis in *Rubia cordifolia* cells by heterologous expression of a calcium-dependent protein kinase gene. *Biotechnol Bioeng* 108: 1734-38.

Torres MA, Jones JDG, Dangl JL. (2006). Reactive oxygen species signaling in response to pathogens. *Plant Physiol* 141: 373-8.

Underwood W, Zhang S, He SY. (2007). The *Pseudomonas syringae* type III effector tyrosine phosphatase HopAO1 suppresses innate immunity in *Arabidopsis thaliana*. *Plant J* 52: 658-72.

Veremeichik GN, Shkryl YN, Bulgakov VP, Zhuravlev YN. Effect of agrobacterial *rolB* and *rolC* genes on expression of class III peroxidase genes in *Rubia cordifolia* transgenic callus cultures. Planta (submitted).

Xu XQ, Pan SQ. 2000). An *Agrobacterium* catalase is a virulence factor involved in tumorogenesis. *Mol Microbiol* 35: 407-14.

Zhao J, Davis L, Verpoorte R. (2005). Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol Adv* 23: 283-333.

14

Transformed Root Cultures of *Solanum dulcamara* L.: A Model for Studying Production of Secondary Metabolites

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1. Introduction

Solanum dulcamara L. (dogwood or bitter sweet), Solanaceae, is one of the recommended species for growing in the temperate regions as a source of steroidal alkaloids. These alkaloids are suggested to be alternatives for diosgenin in the commercial production of steroidal pharmaceuticals (Mathé et al., 1986). Steroidal alkaloids like solasodine and its C-25 epimer tomatidenol can be easily converted to pregdienolone which is an important intermediate in the synthesis of steroids (Sato et al., 1951). *Solanum dulcamara* L. exists in three chemovarieties that contain either solasodine, soladulcidine or tomatidenol glycosides (Willuhn, 1966). These *Solanum* alkaloids are always accompanied by varying quantities of their corresponding oxygen analogues, i.e. the neutral saponins. So, these chemovarities can be relisted as tomatidenol/yamogenin, solasodine/diosgenin and soladulcidine/tigogenin types (Hegnauer, 1989). The tomatidenol-producing taxa are found in the humid Atlantic climate of Western Europe, the soladulcidine type occurs in the drier continental climates while the solasodine variety is comparatively rare (J.R Mathé & I. Mathé, 1979). Only the solasodine and tomatidenol-producing varieties are of interest, but their productivity would not be comparable to that of other tropical or subtropical species as *S. laciniatum*. So, if this steroidal alkaloids content could be boosted by manipulation, *S. dulcamara* could be of interest for commercial growing due to its other qualities like fair cold hardiness, good growth on poor soils and perennial life cycle. An alternative approach was to produce these alkaloids intensively *in vitro*. Several *Solanum* species including *S. dulcamara* were the subject of many *in vitro* manipulations, but attempts, which involved techniques like cell suspension and callus cultures failed to achieve the target. Secondary metabolites production, in general, needs a certain degree of tissue differentiation, something that is obviously lacking in those *in vitro* systems (Ehmke & Eilert, 1993; Rhodes et al., 1987). A more promising technique has been introduced as an alternative to the classical cell

suspension and callus cultures, the transformed or hairy roots cultures. These roots are obtained by a natural genetic transformation mechanism *via* the soil pathogen *Agrobacterium rhizogenes* (Chilton et al., 1982). *A. rhizogenes* is the genetic engineer which inserts a part of its root-inducing plasmid (transfer or T-DNA) into the plant genome, inducing the proliferation of the amazing hairy roots. Among the many advantages of hairy roots are rapid biomass accumulation, typically accompanied with a high production of secondary metabolites, high stability in growth characteristics and metabolic profile and the possibility for upscaling in specialized bioreactors (Georgiev et al., 2007; Hamill & Lidgett, 1997). Few hairy root cultures which, deal with production of steroidal alkaloids are reported for *Solanum* species. Only those of *S. elaeagnifolium, S. muritianum* and *S. aviculare* could be traced (Alvarez et al., 1994; Argolo et al., 2000; Drewes & Van Staden, 1995; Kittipongapatana et al., 1998; Subroto & Doran, 1994; Yu et al., 1996). Others have also investigated the production of neutral steroidal saponins from hairy roots of *S. aculeatissimum* (Ikenga et al., 1995).

Another group of alkaloids has been detected in *Solanum dulcamara* L., fairly recently, by Asano et al., 2001. These are the calystegines. Calystegines are a class of polyhydroxy nortropane alkaloids, which are characterized by a bicyclic nortropane ring structure and unique aminoketal functionality at the bridgehead. Tepfer et al. (1988) reported, for the first time, the presence of this group of alkaloids in the underground organs and the root exudates of *Calystegia sepium*, *Convolvulus arvensis* (Convolvulaceae) and *Atropa belladonna* (Solanaceae**).** Since then, several compounds belonging to this class have been identified in plants of the families Solanaceae, Convolvulaceae and Moraceae (Asano et al., 1994, 2001; Nash et al., 1993; Schimming et al., 1998). Interest rose in studying the potential biological activities and therapeutic uses of calystegines due to their structural similarities to another class of polyhydroxylated alkaloids, which are collectively known as the sugar mimic glycosidase inhibitors. This class comprises four structural types, piperidine, pyrrolidine, pyrrolizidine and indolizidine derivatives, which are involved in a wide range of biological activities such as intestinal digestion, post-translational processing of glycoproteins, the lysosomal catabolism of glycoconjugates and have enormous therapeutic potential in many diseases such as viral infection including HIV, cancer and diabetes (Asano et al., 2000). Several calystegines were proved to have potent glycosidase inhibitory activity (Asano, et al., 1997, Watson et al., 2001). In this work we describe, for the first time, the production of steroidal and the polyhydroxy-alkaloids, calystegines, from hairy root cultures of *S. dulcamara* L. The growth characteristics of cultures and their morphology by scanning electron microscopy were also investigated.

2. Materials and methods

2.1 General experimental procedures

Plant materials were purchased from local nurseries (UK); salts for media preparation were obtained from Sigma (UK); PCR reagents were obtained from Perkin Elmer (UK); primers from VH Bio Ltd (UK); Perkin Elmer GeneAmp 480 thermal cycler (UK) was used for DNA amplification; Column chromatography was performed on silica gel (Merck, 70-230 mesh) and Sephadex LH-20 (Sigma, UK); TLC was performed on precoated TLC plates with silica gel 60 F_{254} (Merck, 0.25 mm, USA); solvents for chromatography were reagent grade; ^{1}H and ^{13}C-NMR spectra (400 and 100 MHz, respectively) were acquired on Bruker dpx 400 spectrometer using pyridine-d_5 or D_2O as solvents; samples for SEM were gold coated in Polaron E5350; SE micrographs were taken using a Joel JSM T220 scanning electron microscope; standard solasodine (Koch-Light laboratories, UK) and diosgenin (Sigma, UK).

2.2 Establishment of transformed root cultures

Transformed roots were obtained by infecting surface sterilised leaf and stem segments with *Agrobacterium rhizogenes* strain A4 (a kind gift from Dr A. Petit, Laboratoire De Biologie De La rhizosphere, Versailles Cedex, France in 1998). Strain A4 harbours the root inducing plasmid pRiA4 and was engineered to contain in addition plasmid Bin 19, which harbours a kanamycin-resistant gene as a selectable marker. An overnight bacterial suspension in yeast mannitol broth (YMB) (Hooykaas et al., 1977) supplemented with 50μM acetosyringone (as virulence inducer) was used for inoculation into freshly wounded explants. Infected samples were transferred to one tenth Murashige and Skoog (MS) agar solidified media (Murashige & Skoog, 1962), kept in the dark for 48 h then incubated under 16 h photoperiod at light intensity of approximately 1.8 wm^{-2} at 20 ± 2 °C. The putative hairy roots, were transferred to hormone-free MS liquid media supplemented with 30 g l^{-1} sucrose. Ampicillin sodium salt 500 mg l^{-1} was added until cultures were free from the residual bacteria. The putative transgenic roots were maintained on the same liquid media (50 ml in 250 ml flasks) on gyratory shakers (90 rpm), at 20 ± 2 °C in the dark or under illumination for 16 h day^{-1} or under continuous light. The roots were sub-cultured every two weeks. For isolation of compounds, roots were inoculated into 5 l flasks containing 2 l of Gamborg's B5 liquid media, aerated with a bubble-type sparger and incubated for a period of four weeks in the dark. Some root samples were subcultured and maintained in other liquid media, *viz*. B5 and SH (Gamborg's B5 and Schenk and Hildebrandt) (Gamborg, et al. 1968; Schenk & Hildebrandt, 1972) for purpose of comparison.

2.3 Confirmation of transformation by Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from 100 mg (fresh wt.) of putative hairy roots and normal non-transformed roots (as controls) using commercially available plant DNA extraction kit (Nucleon-Phytopure, UK). These DNAs were used as templates for the reaction. PCR was performed with *rol* B gene specific primers: 5`-ATG GAT CCC AAA TTG CTA TTC CTT CAA CGA-3`and 5`-TAA GGC TTC TTT CTT CAG GTT TAC TGC AGC-3` (VH bio Ltd, UK). Amplification was carried out according to Hamill et al. (1990). Products of the reaction were run on 1.5 % agarose electrophoretic gel stained with ethidium bromide, along with a standard DNA marker.

2.4 Growth rate analysis

Growth rate analysis, for some root lines which showed good growth characteristics in liquid media, was determined by both dry weight and the dissimilation methods according to Schripsema et al., 1990.

2.5 Scanning electron microscopy

Fresh hairy root material was fixed with 1.5% glutaraldehyde (GA) in 0.05 M sodium cacodylate buffer, pH 7.0, for 45 min. After 1 - 2 min in vacuum (26 mm Hg, 3.46 kPa) the fixative was substituted by 3% GA in 0.1 M cacodylate buffer, pH 7.0 for 2 h. The material was then post-fixed in 1% aqueous solution of osmium tetroxide for 2 h. All treatments were carried out at room temperature. The fixed material was dehydrated in graded ethanol series, dried by the critical point drying method and sputter coated with gold before observation in the electron microscope (Ascenão et al., 1998).

2.6 Extraction and isolation of steroidal compounds

Fresh root material (400 g), grown in B5 liquid media and incubated in dark, was extracted with cold methanol (MeOH) overnight (1 l x 3). The combined methanolic extracts were concentrated under vacuum at 40 °C, partitioned between distilled water and petroleum ether, $CHCl_3$ and finally with *n*-butanol. The *n*-butanol fraction (3.1 g) was column chromatographed on silica gel using vacuum liquid chromatography. Fractions eluted with 15% MeOH in $CHCl_3$ (220 mg) were further chromatographed on Sephadex LH-20 using mixtures of $CHCl_3$/MeOH of increasing polarity. Fractions eluted with 8% MeOH (54 mg) were further purified on preparative TLC plates using $CHCl_3$-MeOH-H_2O (70:30:0.5) for development to yield compound **1** (22 mg, R_f 0.46, $CHCl_3$-MeOH-H_2O, 70:30:3) and compound **2** (6 mg in a mixture, R_f 0.43, $CHCl_3$-MeOH-H_2O, 70:30:3).

2.7 Determination of total steroids

2.7.1 Determination of steroidal bases

A spectrophotometric assay adopted from a method described by Briner, 1969 and modified by Crabbe & Fryer, 1982, was followed. The method depends on formation of a coloured complex of the steroidal bases with methyl orange, after acid hydrolysis of the glycosides in the extract and its spectrophotometric measurement at 425 nm. Determination of the concentration of test samples was achieved by constructing a calibration curve using standard solasodine base.

2.7.2 Determination of neutral sapogenins

A specific spectrophotometric analysis method for the determination of the total steroidal sapogenins, after acid hydrolysis, was followed. This method is based on chromophore formation with a reagent composed of anisaldehyde and sulphuric acid. The produced colour has one absorbance peak at 430 nm. The method is capable of determining different sapogenin types of different structures, irrespective of differences in stereochemistry at rings E and F, rings A/B conformation, presence or absence of unsaturation at C5-C6 or presence of keto or hydroxyl groups at C-3. Other compounds like sterols, triterpenoid sapogenins and/or sugars from glycosides, do not interfere with the determination. The reaction with the chromogen is believed to be restricted to rings E and F (Baccou et al., 1977). Determination of the concentration of test samples was achieved by constructing a calibration curve using standard diosgenin.

2.8 Investigation of calystegines content

2.8.1 Sample preparation

Oven dried samples (70 °C), 10 g each, of roots grown under light and in dark were extracted with 50% aqueous methanol (100 ml x 3) for 12hr. The hydro-methanolic extract in each case was filtered and concentrated to a syrupy consistency under vacuum at 40°C. The extracts were partially purified by ion exchange chromatography on a strong acidic resin, Dowex-50W in the H^+ form. Each extract was loaded onto glass columns packed with the resin and unbound material (sugars and phenolic compounds) was washed through with distilled water. The bound fraction (basic compounds and calystegines) was eluted with 2M ammonium hydroxide solution. Ammonia was removed from the samples by evaporation under vacuum at 40°C. Each sample was then processed for GC-MS analysis as follows.

2.8.2 Sample derivatisation for GC-MS

Small aliquots of the above-purified bound fraction (1ml each) were freeze-dried. The freeze-dried samples were silylated using 100µl mg^{-1} of Sigma-Sil A reagent (Sigma, UK), which consisted of a mixture of trimethylchlorosilane [$(CH_3)_3$ Si Cl], hexamethyldisilizane [$(CH_3)_3$ Si NH Si $(CH_3)_3$] and pyridine in the ratio of 1:3:9. Samples were mixed using a vortex mixer and then heated at 55°C for 15 minutes. The reaction mixture was cooled down to room temperature for at least one hour and then centrifuged at 2 x 10^3 rcf for 20 minutes to remove any precipitate formed during the reaction. The supernatants were then transferred to GC vials and analysed by GC-MS as follows: a BPX5 25m capillary column, 0.22mm ID, film thickness 0.25µm (SGE Ltd., UK) was used, helium at a pressure of 10psi. Temperature programme started with an isothermal hold at 180 °C for 5 minutes, followed by a linear temperature rise to 300 °C at 10 °C/min. The final temperature was held for 10 minutes and the total length of the program was 27 minutes. Samples were introduced at 1.0 µl per injection. EI-MS of the column effluent was carried out on Perkin-Elmer Q-Mass 910 Benchtop Mass Spectrometer with quadruple mass filter system. The system was set to a constant temperature of 280°C. The effluent from the gas chromatograph was transferred to the mass spectrometer via a temperature controlled line set at 250 °C.

2.8.3 Large scale fractionation

The calystegine-containing fraction from 500g fresh hairy roots of *S. dulcamara* was prepared as above. The bound material eluted from Dowex-50W was fractionated on weakly acidic cation exchanger (Amberlite CG-50, H^+) using water as an eluant. The column was finally washed with dilute ammonium hydroxide solution to elute the strongly bound bases. Fractions collected from the CG-50 column were further purified by extensive ion exchange chromatography on either weakly acidic (CG-50, H^+) or weakly basic (Dowex-2x8, Cl^-) resins using water for elution and a final rinsing with dilute ammonium hydroxide solution. Small aliquots of each fraction were freeze-dried and derivatized for GC-MS analysis as described above.

2.8.4 GC-MS data for calystegines

Calystegine A_3: R_t (4.95 minutes), EI/MS (positive mode) at m/z (rel. intensity) 286 (8), 244 (20), 170 (12), 156 (100), 147 (9).
Calystegine A_5: R_t (4.54 minutes), EI/MS (positive mode) at m/z (rel. intensity) 286 (15.4), 244 (17), 169 (15), 156 (100), 147 (17).
Calystegine B_1: R_t (7.28 minutes), EI/MS (positive mode) at m/z (rel. intensity) 373 (19.5), 332 (29), 285 (6), 258 (23), 244 (100), 168 (17), 147 (26), 129 (63).
Calystegine B_2: R_t (8.70 minutes), EI/MS (positive mode) at m/z (rel. intensity) 284 (11), 259 (14), 229 (8), 217 (100), 204 (8), 156 (20), 147 (21).
Calystegine B_1 glucoside: R_t (14.65 minutes), EI/MS (positive mode) at m/z (rel. intensity) 430 (38), 316 (100), 287 (38), 217 (76), 205 (38), 156 (25), 147 (79).
Calystegine A_3 (A_5) glycoside: R_t (14.74 minutes), EI/MS (positive mode) at m/z (rel. intensity) 286 (100), 244 (6), 217 (12), 204 (12), 169 (8), 156 (65), 147 (19), 129 (8).

2.8.5 ^{13}C data for Calystegine B_2

(100 MHz, D_2O and drops of acetone-D_6 for signal calibration, δ values in ppm): δ 90.0 (C1), 74.5 (C2), 74.6 (C3), 77.3 (4), 55.5 (C5), 21.3 (C6), 28.4 (C7).

3. Results and discussion

3.1 Transformation

Secondary metabolites production, in general, needs a certain degree of tissue differentiation, something that is obviously lacking in dedifferentiated *in vitro* systems, like callus and cell suspension cultures. For example, Ehmke and Eilert (1993) did not detect any alkaloids in cell suspension cultures of *S. dulcamara* L. grown in dark. These alkaloids started to be synthesized when the cultures were grown in light and began to show some differentiation and rooting, but their level was not comparable to the original intact plant. A more promising technique has been introduced as an alternative to the classical cell suspension culture that is transformed or hairy root cultures. These roots are obtained by a natural genetic engineering mechanism *via* the soil pathogen *Agrobacterium rhizogenes*. The produced hairy roots are genetically and chemically stable and are characterized by high productivity of secondary metabolites, which in most cases mirrors that of the original non-transformed plant (Hu & Du, 2006). Hence, a transformed root culture was established for *S. dulcamara*. The putative hairy roots appeared on the infected stem segments as early as one week after infection, in some of the samples, and were complete within two weeks in the rest of them. Some roots appeared directly from or near the wounding sites and some differentiated from small calli, which formed at the wounding sites (Fig. 1, A). No hairy roots appeared on the infected leaf segments; instead, green normal roots were developed from the cut ends of the midribs and from the basal parts of the infected as well as the control samples of the leaves and the stems. These normal roots failed to grow when transferred to liquid media lacking growth hormones, which confirmed their untransformed nature. Although solanaceous plants are reported to be highly susceptible to infection with *A. rhizogenes* (Porter, 1991), the transformation frequency obtained by us with *S. dulcamara* (>93%) far exceeds transformation rates reported for other *Solanum* species. For example, Drewes & Van Staden (1995) obtained only one transformation event out of 80 infected samples of *S. mauritianum* Scop., whether acetosyringone was used or not, while Argolo et al. (2000) managed to obtain 20-90 % transformation frequency for *S. aviculare* Forst. depending on the bacterial strain used for infection.

A. B. C.

Fig. 1 A. Initiation of hairy roots on infected stem segments with *A. rhizogenes*; B. Hairy roots in liquid culture; C. Regenerated shoots from roots grown in continuous light

Roots incubated under photoperiod showed greening and those incubated under continuous light regenerated small shoots within four weeks (Fig. 1, C). Roots grown in different liquid media, MS, B5 and SH (Fig.1, B) displayed typical properties of transformed roots, i.e. fast growth, high degree of branching, abundant root hairs and lack of positive geotropism. Insertion of the root inducing plasmid was confirmed by PCR using primers specific to *rol B* gene (root-inducing *locus*). Results showed amplification band expected for that gene (0.78 kb), while normal non-transformed roots used as controls did not show any amplification bands (Fig. 2).

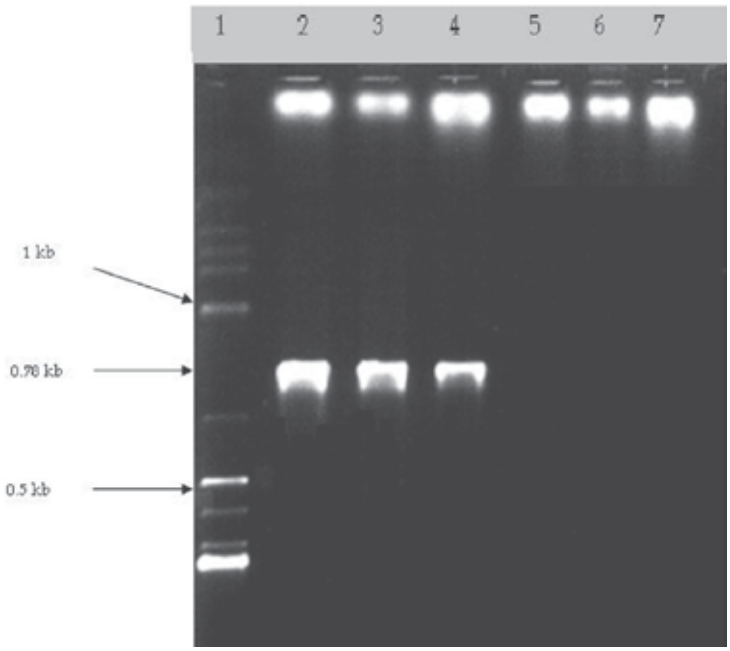
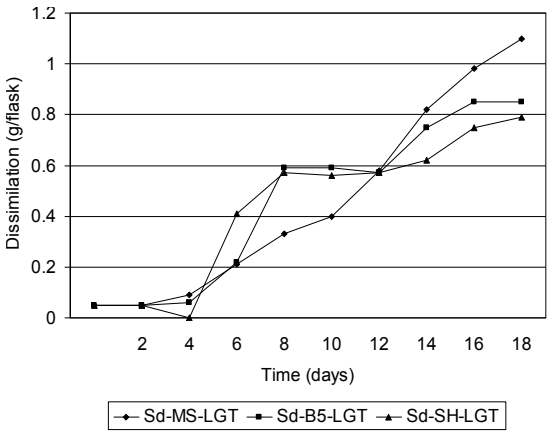


Fig. 2. PCR products of *S. dulcamara*. Lane1, DNA marker; lanes 2-4, DNA from hairy roots (1-3 μL); lanes, 5- 7, DNA from normal roots (1-3 μL).

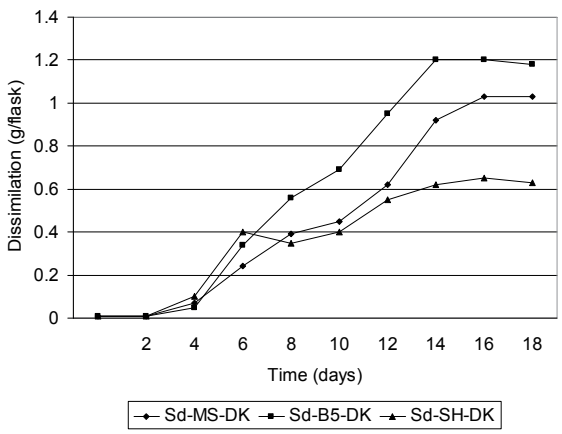
3.2 Growth characteristics of the cultures

Growth rate analysis of the transformed root cultures was performed using two methods. The first, depended on determination of the dry mass accumulation over a period of four weeks and the second, involved determination of the growth characteristics by the dissimilation method i.e. determination of the loss of carbon dioxide due to carbohydrate consumption. Growth rate determination by the dissimilation method was carried out in three different liquid media (MS, B5 and SH) either under dark or light conditions. None of the cultivation media was inhibitory to the growth of the transformed roots, but differences were observed in the growth rate among these media. Roots showed best growth characteristics in MS media incubated under light conditions and in Gamborg's B5 media under dark conditions (Fig. 3, A & B). The cultures showed a very short lag phase of less than two days and continued an active exponential phase for 17 days before entering a stationary phase. Generally, roots grown under dark conditions grew more actively, except for roots grown in SH media (Fig. 3, A & B). Growth rate determination by dry mass

accumulation method (Fig.4) showed a similar pattern of growth with a 45-fold increase in root mass per flask, with an inoculum size of 200 mg fresh roots. The results demonstrate the importance of selection of the hairy root clones and also the possibility to manipulate the cultures through media selection and incubation conditions. The different clones are derived from different transformation events as a result of the insertion of the T-region of the root inducing plasmid, which is known to be a random process. This variability makes the hairy root system as amenable and flexible to manipulation as the dedifferentiated cell cultures, but having the advantage of still being an organised system with certain degree of maturation that allows expression of the enzymatic systems leading to the different secondary metabolic pathways.



A



B

Fig. 3. Growth rate analysis of hairy roots in different media; A, under light; B in dark (dissimilation method).

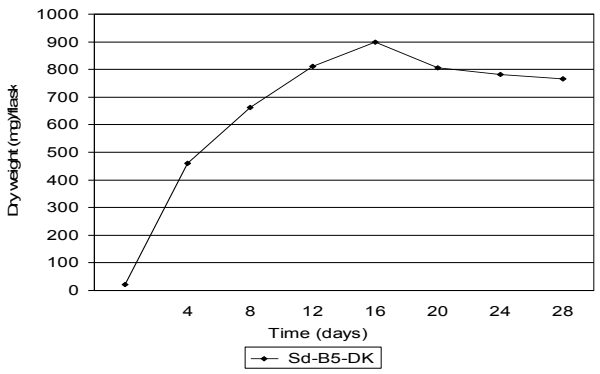
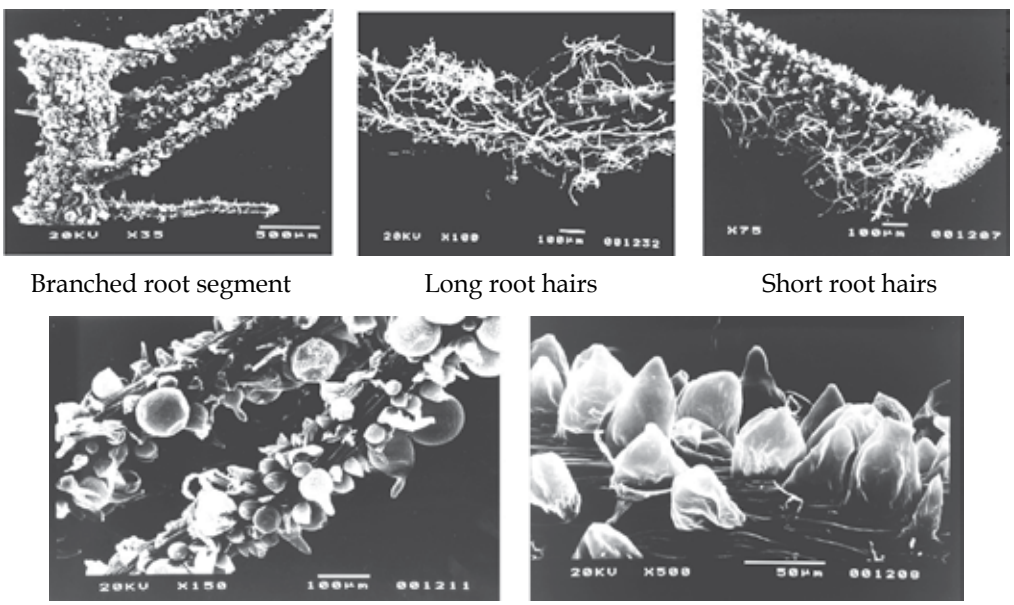


Fig. 4. Growth rate analysis of hairy roots in B5 media in dark (Dry weight method).

3.3 Scanning Electron Microscopy (SEM)

SEM of the transformed roots (Fig. 5) showed high degree of branching and large number of root hairs. Some of the hairs appeared as long unicellular tubes, while others like short papillae covering the majority of the roots surfaces, especially those growing near and out of the surface of the media. Cut surfaces of the roots, revealed the structure of young dicot root with abundant starch granules and microcrystals of calcium oxalates in the cortical parenchyma (not shown). These cell inclusions which are characteristic features of plants belonging to the family *Solanaceae* (Metcalf & Chalk, 1957) reflect genetic stability of the transformed cultures as they retain the histological fingerprints of the mother plant. The high degree of branching and the abundant root hairs are typical characteristics of transformed roots.



Branched root segment | Long root hairs | Short root hairs

Magnification of short root hairs

Fig. 5. Scanning electron micrographs of different root hairs.

3.4 Investigation of the produced secondary metabolites

3.4.1 Steroidal compounds

Chromatographic investigation (TLC) of the methanolic extracts of the transformed roots and different parts of the non-transformed plant (roots, aerial parts and fruits) showed almost similar pattern of secondary metabolites with one major alkaloidal spot and several minor ones. Analytical HPLC (data not shown) revealed much more complex profile of the hairy root extracts. No qualitative differences were observed for transformed roots grown in different liquid media or for roots grown in the dark and those grown in light, except for the formation of higher amounts of less polar compounds in the roots grown in dark (higher percentage of chloroform extractives, data not shown). Chromatographic separation of the *n*-butanol extract of roots grown in dark in B5 liquid media lead to the isolation of two compounds, **1** and **2**. Compound **1** was obtained as amorphous solid, yield 0.0055%, (w/w, on fresh wt. basis), positive Dragendorff's and was identified as (25*S*)-3-β-{[*O*-α-L-rhamnopyranosyl-(1``→2`)-*O*-α-L-rhamnopyranosyl-(1```→4`)]-β-D-glucopyranosyloxy} -22-β-N-spirosol-5-ene, (β-solamarine or tomatidenol-3-*O*-β-chacotrioside), based on spectroscopic data. Positive FAB MS exhibited a base peak at m/z 868 $[M+1]^+$, corresponding to the molecular formula $C_{45}H_{73}O_{15}N$. EI mass spectrum displayed fragment ions at m/z 114 (90%) and m/z 138 (65%) characteristic of spirosolane-type alkaloids. The first fragment arises from the cleavage of the cyclic ether ring (ring E) at C20, C22 and C22-O sites. The latter results from cleavage of ring D (Atta-ur-rahman & Choudhary, 1993).Thus the mass spectral data suggested **1** to be a glycoalkaloid of the spirosolane-type.^{1}H-NMR spectrum (Table 2) displayed proton resonances characteristic of a steroid aglycone of the spirosolenol type, in addition to sugar protons. Some of the diagnostic proton resonances associated with the aglycone was observed as two tertiary methyl singlets at δ 0.80 and δ1.06 attributable to the methyl groups at positions C18 and C19 of the steroid nucleus, respectively. Two secondary methyl signals at δ 1.10 (d, *J* = 8.2 Hz) and δ 0.81 (d, *J* = 7.2 Hz) were assigned to methyls at C21 and C27, respectively. A broad singlet at δ 5.34 was assigned to the olefinic proton at C6 and a multiplet at δ 3.90 to proton at C3. The ^{1}H-NMR spectrum showed further resonances associated with sugar protons. Two broad singlets at δ 5.86 and δ 6.40 are characteristic of the anomeric protons of α-linked sugars. The observation of another two upfield secondary methyl signals at δ 1.64 (d, *J* = 6.1 Hz) and δ 1.78 (d, *J* = 6.1 Hz) suggested two molecules of 6-deoxy sugars. A third anomeric proton observed at δ 4.94 (d, *J* = 8.0 Hz), typical of β-linked glucose, and showed a direct correlation to an anomeric sugar carbon at δ 100.8 in HMQC experiment. This indicated a glycoalkaloid with a trisaccharide moiety. The ^{13}C spectrum supported by APT measurements (Table 3) showed 45 carbon resonances, among them 27 carbons were assigned to the aglycone part. Those included signals due to a spiro carbon at δ 98.9, assigned to C22 and olefinic carbons at δ 122.3 and 141.3, assigned to C6 and C5, respectively. The remaining carbon signals in the spectrum could be assigned to three sugar moieties, a hexose and two 6-deoxy sugars. The direct connectivity of the protons and the carbon atoms were revealed through HMQC experiment. The identity of the aglycone part as well as the that of the building blocks of the glycosidic moiety and the interglycosidic linkages were confirmed through HMBC experiment (Fig. 6) as follows: many parts of ring A were defined by observation of cross peaks between a complex signal centred at δ 2.75 assigned to the two protons at C4 and carbon signals at δ 38.0 (C10), 78.6 (C3) and 122.3 (C6) and a further 2*J* coupling with a quaternary carbon signal at δ 141.3 (C5), the latter two correlations confirmed unsaturation

between C5 and C6 and indicated a derivative of either solasodine or its 25-epimer tomatidenol. Another cross peak between one of the methyl singlets at δ 1.06 (Me-19) and a carbon resonance at δ 37.7 confirmed the latter position as C1. The position of C17 was also confirmed by the observation of another 3*J* coupling with the methyl doublet at δ 1.10 (proton at C21). This doublet showed a further 2*J* coupling to the Spiro carbon at δ 98.8 assigned to C22. The positions of the carbons of ring F were assigned also through HMBC. 3*J* cross peaks were observed between the doublet at δ 0.81 (methyl at C27) and carbon signals at δ 29.8 (C24) and 51.1 (C26) and another 2*J* coupling with C25 (δ 32.1). Comparison of the spectral data for the aglycone part with those for standard solasodine and tomatine performed in the same solvent (pyridine-d_5) revealed close resemblance to those of the aglycone part of tomatine except for the presence of a double bond between C5 and C6. Furthermore, the NMR spectral data for the aglycone part of **1** were found to be in good agreement with those published for tomatidenol tetraoside (Usubillaga et al., 1997). The HMBC spectrum in the sugar region (Fig. 6) showed that the anomeric proton at δ 4.94 (d, *J* = 8.0 Hz), which was showed by the HMQC experiment to be connected to a sugar anomeric

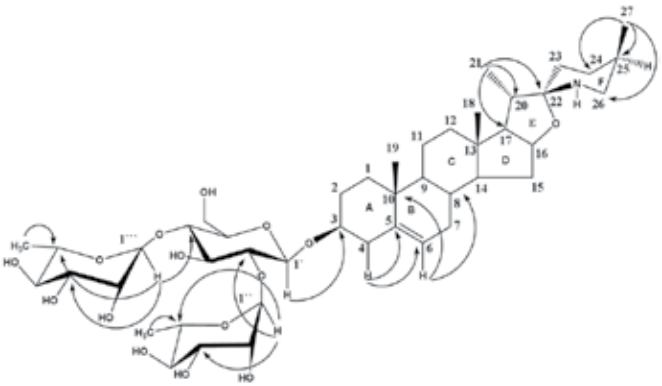


Fig. 6. HMBC correlations observed for β-solamarine, **1**

carbon at δ 100.8, correlated to a carbon resonance at δ 78.6 (C3). The downfield shift observed for C3 (about 8.0 ppm) from that published for free aglycones (70.5 - 71.3 ppm) confirmed glycosylation at this carbon (Toshinori et al., 1993; Ripperger, 1996). Mutually the proton at C3 of the steroid nucleus (multiplet at δ 3.90) correlated to the sugar anomeric carbon at δ 100.8. The proton and carbon shifts of the other two sugars (Tables 1 and 2) suggested that they are rhamnoses. Cross peaks in the HMBC spectrum between the anomeric protons of the two rhamnoses (δ 6.40 and 5.86, br s) and carbon signals at δ 78.4 ppm and δ 79.1 confirmed that those sugars were attached to glucose through (1``→2`) and (1```→ 4`) linkages. Other important correlations in the sugar region were observed between the anomeric protons of rhamnoses and C5 and C3 of both sugars and also between protons at C6 of the same sugars and the corresponding C4 and 2*J* couplings to C5. Protons at C4 of the rhamnose molecules showed similar 3*J* couplings to the corresponding C6. Another correlation between H4` of glucose (δ 4.33 - 4.39, m) and C1 of the same molecule (carbon signal at 100.8 ppm) was observed. The evidence derived from these spectral data led to the identification of 1 as (25 S)-3-β-{[O-α-L-rhamnopyranosyl-(1``→2`)-O-α-L-rhamnopyranosyl-(1```→4`]-β-D-glucopyranosyloxy}-22β-N-spirosol-5-ene (β-Solamarine or tomatidenol-3-β-

D-chacotrioside). This compound has been identified before in the tomatidenol variety by Rönsch and Schreiber (1966). Compound **2** could be the oxygen analogue of **1** based on giving negative reaction with Dragendorff's reagent and the detection of a carbon resonance at δ 108.0 in its ^{13}C-NMR spectrum (C22 of neutral saponins) and close NMR data to those of **1**, but it needs further purification.

These results demonstrate the metabolic stability of the cultures, where preliminary investigations showed no major differences between hairy roots and the original plant. Other work on *S. dulcamara* shooty teratoma cultures obtained by transformation *via A. tumefaciens* revealed alteration of soladulcidine to solasodine-type glycosides (Atta-ur-rahman & Choudhary, 1993). The total yield of steroids in our cultures, which ranged from approximately 2 to 7 mg g-1 (Table 1), is still below the requirement for commercial exploitation, so other methods of manipulating the cultures should be tried out.

Sample	Alkaloids	Sapogenins
B5 media in dark	4.29[a]	2.52[a]
B5 media under light	0.60	1.24
SH media in dark	2.90	1.19
SH media under light	0.96	0.59
MS media in dark	0.57	1.89
MS media under light	0.52	1.50
Normal roots	4.14	1.37
Aerial parts	3.28	2.11

Table 1. Alkaloids and neutral sapogenins in hairy roots and normal plant organs. [a] mg g-1 (Dry wt.).

H	δ ppm	H	δppm
1	1.01,*dd* (14.3, 3.4), 1.77[a]	24	1.57-1.59 [a], *m*
2	1.86 [a], 2.11 [a]	25	1.89, *m*
3	3.90, *m*	26	2.83 [a], *m*, 2.95 [a], *m*
4	2.71, *m*, 2.80 [a], *m*	27	0.81, *d* (8.2)
5	-	1`	4.94, *d*, (8.0)
6	5.34 *br s*	2`	4.37 [a], *m*
7	1.46 [a], *m*, 1.89 [a], *m*	3`	4.33-4.39 [a], *m*
8	1.52-1.54 [a], *m*	4`	4.33-4.39 [a], *m*
9	0.92, *m*	5`	3.66 [a]
10	-	6`	4.10, 4.23, *dd* (3.6, 13.2)
11	1.46-1.47 [a], *m*	1``	6.40 *s*
12	1.12, *m*, 1.68-1.76 [a], *m*	2``	4.69, *br s*
13	-	3``	4.55, *dd* (3.2, 13.4)

14	1.10 [a], *m*	4``	4.33, *dd* (9.4, 9.4)
15	1.56 [a], *m* , 1.94 [a], *m*	5``	4.93-4.99 [a], *m*
16	4.18 [a], *m* , 4.23 [a], *m*	6``	1.78 *d* (6.1)
17	1.82-1.84 [a], *m*	1```	5.86 *br s*
18	0.83 *s*	2```	4.84, *dd* (1.5, 4.0)
19	1.06 *s*	3```	4.64, *dd* (3.2, 13.2)
20	1.96-2.02 *dq* (8.6, 7.4)	4```	4.39, *dd* (9.4, 9.4)
21	1.10, *d* (7.24)	5```	4.93-4.99 [a], *m*
22	-	6```	1.64, *d* (6.1)
23	1.75 [a], *m*		

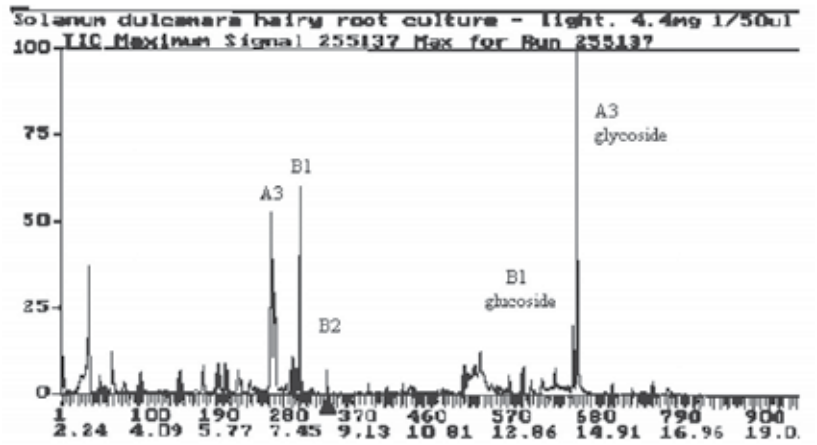
Table 2. ^{1}H-NMR data of β-Solamarine (400 MHz, Pyridine-d_5).
[a]Overlapped signals assigned by 2D NMR. [b]*J* values between parentheses in Hz.

C	δ ppm	C	δppm
1	37.7	24	29.8
2	30.7	25	32.1
3	78.6	26	51.1
4	39.5	27	20.2
5	141.3	1`	100.8
6	122.3	2`	78.4
7	32.9	3`	79.0
8	32.9	4`	79.1
9	50.9	5`	77.4
10	38.0	6`	61.8
11	21.7	1``	102.5
12	40.6	2``	73.0
13	46.0	3``	73.0
14	56.4	4``	74.4
15	33.1	5``	68.6
16	78.3	6``	18.9
17	64.0	1```	103.4
18	17.0	2```	73.0
19	19.9	3```	73.2
20	42.1	4```	74.6
21	16.2	5```	70.0
22	98.9	6```	19.1
23	35.2		

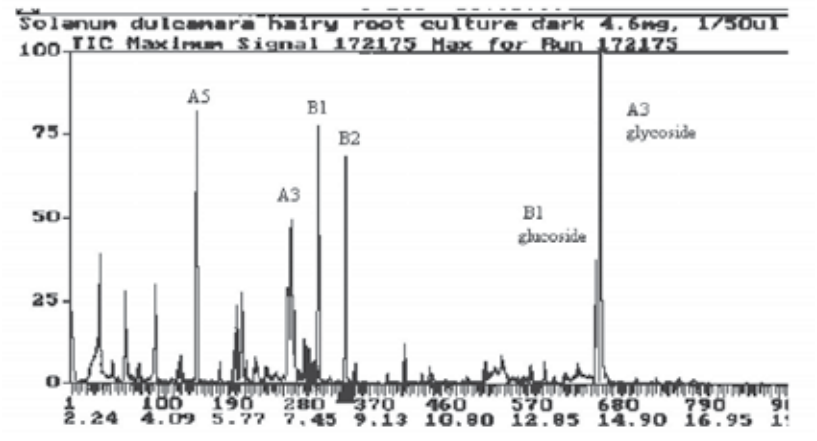
Table 3. ^{13} CNMR data of β-Solamarine (100 MHz, Pyridine-d_5).

3.4.2 Calystegines

A mixture of calystegines could be identified in hairy root cultures of *S. dulcamara* L. The compounds were identified in semi-pure fractions. Identification based on comparison of their retention times and mass fragmentation patterns with those of standard calystegines as well as comparing to published data (Schimming et al., 1998). The identified compounds included calystegines A_3, B_1 and B_2 as major components. Calystegine A_5 could be detected only in cultures grown under dark conditions (Fig. 7, B). Further chromatography of the bound fraction on different ion exchange resins lead to the isolation of calystegine B_2 in pure form. Its identity was further confirmed by comparison of ^{13}C NMR data (section 2.8.5) with those published for calystegine B_2 (Goldman et al., 1990). Other minor components were concentrated in some fractions and calystegine B_1-glucoside could also be identified by analysis of its mass spectral data (section 2.8.4). The detection of another major compound, which had a longer retention time than the other calystegines, 14.7 minutes,



A



B

Fig. 7. GC traces of calystegines content of hairy roots grown under light (A) and in dark (B).

which is very close to the retention time of B_1-glucoside (14.4 minutes), suggested that it might also be a glycoside. The mass fragmentation pattern (experimental, section 2.8.4) was typical of trihydroxylated nortropane derivatives (calystegine-A group). Hence, this compound is suggested to be calystegine A_3 or/A_5 glycoside. It would be impossible to distinguish A_3 and A_5 glycosides without purification and NMR analysis. However this A-glycoside would be a novel compound.

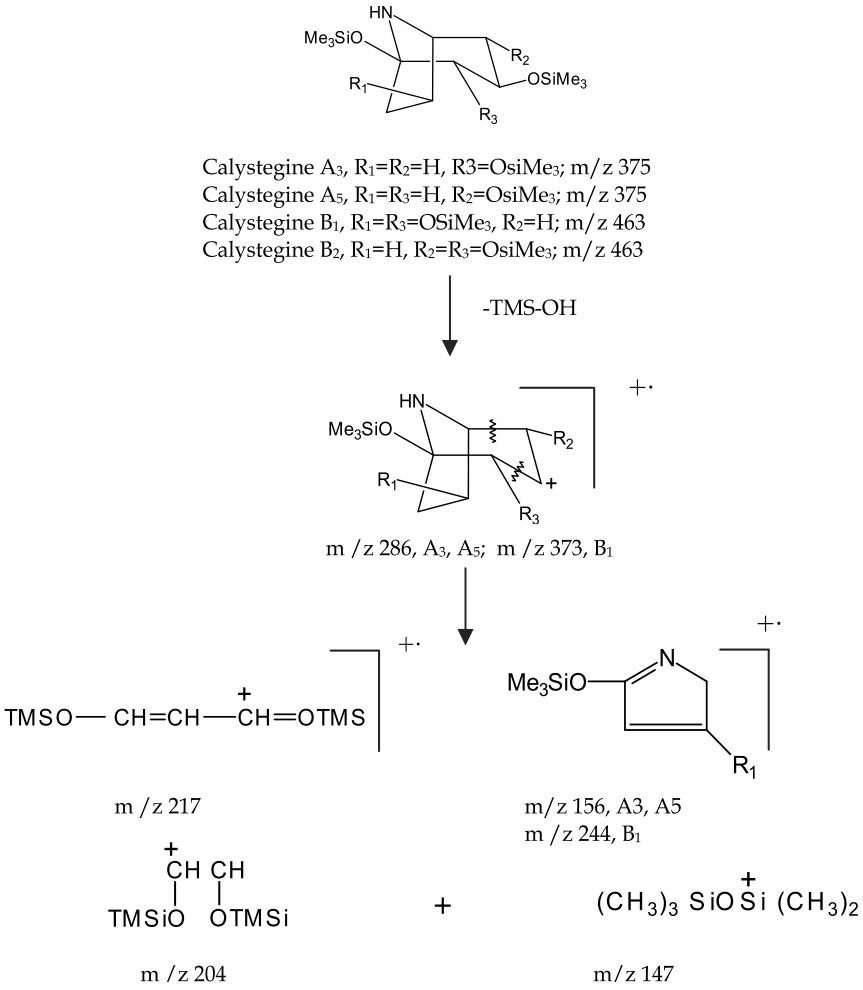


Fig. 8. Major fragment ions in the Mass spectra of calystegines.

Other major unidentified compounds were also detected in the GC-MS trace of the bound fraction of the hairy root cultures. They are likely to be also novel polyhydroxy alkaloids according to their chromatographic behaviour on ion exchange resins and mass fragmentation patterns and are not accumulated in the whole plant (data not shown). It is worth noting that calystegines A_3, A_5, B_1 and B_2 have been identified before in non-transformed plants (Asano et al., 2001). The same research group also identified calystegine N_1 that had not been detected in our root cultures. The glycosylated derivatives of

calystegines B_1 and A-type are reported here for the first time from transformed root cultures of *S. dulcamara* L. They have not been reported before in the original plant. The A-type glycoside would be a novel natural product.

It is evident, from the data given above (experimental section 2.8.4), that the mass fragmentation pattern of the TMS derivatives of the different calystegines, mainly followed that described for the O-TMS derivatives (Molyneaux et al., 1996). Major fragment ions due to the loss of TMS-OH (-90 mu), were observed (ions at m/z: 286, for calystegines A_3 and A_5; 373, for calystegine B_1 and 284, for calystegine B_2).

In the case of A-type calystegines, the base peak was due to the 2-substituted pyrrolinium ion formed via cleavage of the six-member ring (fragment ion at m/z 156). For the B-type

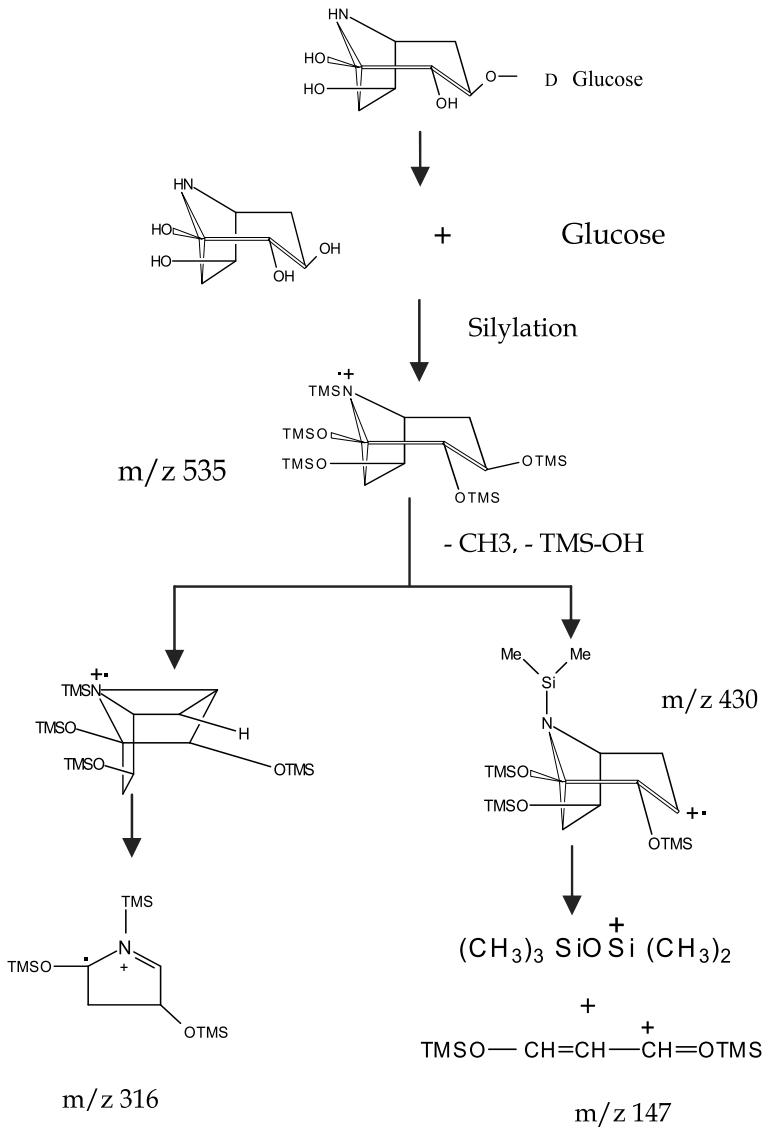


Fig. 9. Mass fragmentation pattern of calystegine B_1 glucoside

calystegines, the base peak was at m/z 244, which was also due to the formation of the 2-substituted pyrrolinium ion in the case of calystegine B_1, while in the case of calystegine B_2 this fragment ion was detected at a lower abundance (see experimental, section 2.8.4). Calystegine B_2 showed a base peak ion at m/z 217. This is still a common ion fragment for TMS derivatives of polyhydroxylated compounds and sugars (DeJongh et al., 1969). Other fragments, which are characteristic of sugars, were also observed at m/z 204,147 and 129. The structures of these fragments are illustrated in Fig. (8). Surprisingly, the mass fragmentation pattern of calystegine B_1-glucoside seems to have mainly proceeded *via* the route described for the persilylated TMS derivatives. Major fragments were observed due to loss of CH_3 followed by loss of TMS-OH (ion at m/z 430). The base peak (at m/z 316) was due to the dihydropyrrolinium ion formed *via* the cleavage of a tricyclic ion. The fragment ions at m/z 217, 205 and 147 occurred at higher abundance than in the other calystegines, probably because they were also coming from the sugar part. A possible explanation is that the reaction under heating condition, has caused the hydrolysis of the glycoside and the silylation of the fourth hydroxyl group took place. The fragmentation pattern of this compound is illustrated in Fig. (9).

4. Conclusion

Hairy root cultures could be successfully established for *S. dulcamara* L. High transformation rates (> 90%) could be obtained and were attributable to a number of factors including selection of the bacterial strain; *A. rhizogenes* is known to be host specific with a narrower spectrum of susceptibility than *A. tumefaciens*. Another factor is the addition of acetosyringone as virulence gene inducer of *Agrobacterium*. Moreover, two compounds identified in the cultures by LC/MS (data not shown) as vanillic and ferulic acids may have contributed to the ease of transformation. Those molecules have the same structural features of the monocyclic polyphenolics known as the virulence-gene inducers which include acetophenones, syringaldehyde, vanillin, synapinic and syringic acids as well as their glycosylated derivatives (Stachel et al., 1985, Delmotte et al., 1991). Phytochemical investigation of *S. dulcamara* cultures revealed a complex pattern of secondary metabolites. Several compounds belonging to different chemical classes have been identified. Those included the triterpene betulinic acid, the steroid daucosterol (data not shown), the glycoalkaloid β-solamarine and a group of nortropane alkaloids, recently identified in the family Solanaceae (calystegines) in addition to the aforementioned phenolic derivatives. In general the profile of secondary metabolite production by the cultures was much similar to that of the original plants from which they were derived. Some metabolic shifts were observed as follows: the major compound in *S. dulcamara* hairy roots was a calystegine glycoside (A_3/A_5), which was not synthesized by the original plant; betulinic acid which is isolated from the cultures could not be traced in the literature for other *Solanum* species or in the family Solanaceae. These differences could be attributed to the transformation process itself. The insertion of the transfer DNA from *Agrobacterium* is known to be a random process that is accompanied by addition or rearrangement deletions of sequences in the transformed plant cell DNA (Gheysen et al., 1989). Thus the insertion of the transfer DNA could have caused an interruption of certain genes that encode for certain steps in the secondary metabolic pathways. In brief, the use of transformed root cultures for production of secondary metabolites is very promising. The cultures were able to synthesize the target compounds at levels comparable to the original plants and showed both structural and metabolic stability over a long period (over two years). The shifts that were noticed in the

metabolic pathways are not deleterious. Moreover, they can be used to produce novel compounds from those cultures. The cultures are amenable to techniques applied for product enhancing, like selection. Each root line is derived from individual transformation event that could give rise to different production capacity. The only limitation to the commercialization of hairy root cultures of this species is the complexity of the secondary metabolic profile. On the other hand they would be an excellent system for studying biosynthetic pathways for steroidal compounds and the calystegines.

5. References

Alvarez, M., Talou, J., Paniego, N. & Giulietti, A. (1994). Solasodine production in transformed organ cultures (roots and shoots) of *Solanum eleagnifolium* Cav. *Biotechnology Letters*, 16, 393-396.

Argolo, A., Charlwood, B. & Pletsch, M. (2000). The regulation of solasodine production by *Agrobacterium rhizogenes* transformed roots of *Solanum aviculare*. *Planta Medica*, 66, 448-451.

Asano, N. Yokoyama, K., Sakurai, M., Ikeda, K., Kizu, H., Kato, A., Arisawa, M., Hoke, D., Drager, B., Watson, A. A. & Nash, R. J. (2001). Dihydroxynortropane alkaloids from calystegine-producing plants. *Phytochemistry*, 57, 721-726.

Asano, N., Kato, A. Miauchi, M., Kizu, H., Tomimori, T., matsui, K., Nash, R. & Molyneux, R. (1997). Specific α-galactosidase inhibitors, N-metyl calystegines: structure-activity relationships of calystegines from *Lycium chinense*. *European Journal of Biochemistry*, 248, 296-303.

Asano, N., Nash, R. J., Molyneaux, R. & Fleet, G. (2000). Sugar-mimic glycosidase inhibitors: Natural occurrence, biological activity and prospects for therapeutic application. *Tetrahedron Asymmetry*, 11, 1645-1680.

Asano, N., Oseki, K., Tomioka, A., Kizu, H.& Matsui, K. (1994). N-containing sugars from *Morus alba* and their glycosidase inhibitory activities. *Carbohydrate Research*, 259, 243-255.

Ascenão, L., Figueiredo, A.C., Barroso, J., Pedro, L., Schripsema, J., Deans, S. & Scheffer, J. (1998). *Plectranthus madagascariensis*: Morphology of the glandular trichomes, essential oil composition and its biological activity. *International Journal of Plant Science*, 159, 31-38.

Atta-ur-rahman, N., choudhary, M. (1993). Steroidal Alkaloids, In: *Methods in Plant Biochemistry*, *Alkaloids and Sulphur Compounds*, Waterman, P. (ed.), Vol. VIII, chapter 13, pp. 473-510, Academic Press, London.

Baccou, J., Lambert, F. & Sauvaire, Y. (1977). A spectrophotometric method for determination of total sapogenins. *Analyst* , 102, 458-465.

Briner, J. (1969). Determination of total steroid bases in *Solanum* species. *Journal of Pharmaceutical Science*, 58, 258-259.

Chilton, M., Tepfer, D., Petit, A, David, C. & Tempè, J. (1982.) *A. rhizogenes* inserts T-DNA in the genome of the host plant root cells. *Nature*, 295, 432-434.

Crabbe, P., Fryer, C. (1982). Evaluation of chemical analysis for the determination of solasodine in *Solanum laciniatum*. *Journal of Pharmaceutical Sciences*, 71, 1356-1362.

DeJongh, D., Radford, T., Hribar, J., Hanessian, S., Bieber , M., Dawson, G. & Sweeley, C. (1969). Analysis of trimethylsilyl derivatives of carbohydrates by gas chromatography and mass spectrometry. *Journal of the American Chemical Society*, 91, 1728-1740.

Delmotte, F, Delay, D., Cizeau, J., Guerin, B. & Leple, J. (1991). *Agrobacterium vir*-inducing activities of glycosylated acetosyringone, acetovanillone, syringaldehyde and syringic acid derivatives. *Phytochemistry*, 30, 3549-3552.

Drewes, F., Van Staden, J. (1995). Initiation of and solasodine production in hairy root cultures of *Solanum mauritianum* Scop. *Plant Growth Regulation*, 17, 27-31.

Ehmke, A., Eilert, E. (1993). *Solanum dulcamara* (bittersweet): Accumulation of steroidal alkaloids in the plant and different *in vitro* systems, In: *Biotechnology in Agriculture and Forestry 21, Medicinal and Aromatic Plants IV*, Bajaj, Y. (ed.), pp. 339-352, Springer-Verlag, Berlin.

Gamborg, O., miller, R. & Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, 50, 151-158.

Georgiev, M., Pavlov, A. & Bley, T. (2007). Hairy root type plant *in vitro* systems as sources of bioactive substances. *Applied Microbiology and Biotechnology*, 74:1175–1185.

Ghyesen, G., Herman, L., Breyne, P., Van Montagu, M. & Depicker, A. (1989). *Agrobacteriium tumifaciens* as a tool for the genetic transformation of plants, In: *Genetic transformation and expression*, Butler, L., Harwood, C. & Moseley, B. (eds.), pp.151-174, Intercept Ltd., England.

Goldman, A., Milat, M., Ducort, L., Lallemand, J., Maille, M., Lepingle, A., Charpin, I. & Tepfer, D. Tropane derivatives from *Calystegia sepium*. *Phytochemistry*, 29, 2125-2127.

Hamill, J., Lidgett, A. (1997). Hairy root cultures: Opportunities and key protocols for studies in metabolic engineering. In: *Hairy Roots: Culture and Applications*, Doran, P. (ed.), pp 1-29, Harwood Academic Publishers, Australia.

Hamill, J., Rounsley, S., Spencer, A., Todd, G., Rhodes, M.J.C. (1991). The use of the polymerase chain reaction in plant transformation studies. *Plant Cell Reports*, 10, 221-224.

Hegnauer, R. (1989). Solanaceae, In: *Chemo-taxonomie Der Pflanzen, Birkhauser*, Vol. VI, pp. 403-452, Verlag, Switzerland.

Hooykaas, P., Klapwijk, M., Nuti, M., Schilperoort, P. & Rorsch, A. (1977). Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent *Agrobacteria* and *Rhizobium* ex *planta*". *Journal of General Microbiology*, 98, 477-484.

Ikenaga, T., Oyama, T. & Muranaka, T. (1995). Growth and steroidal saponin production in hairy root cultures of *Solanum aculeatissimum*. *Plant Cell Reports*, 14, 413-417.

Kittipongapatana, N., Hock, R. & Porter, J. (1998). Production of solasodine by hairy root cultures of *Solanum aviculare* Forst. *Plant Cell, Tissue and Organ Culture*, 52, 133-143.

Mathé, JR., Mathé, I, Botz, L. & Koch, L. (1986). Possibilities of *Solanum* alkaloids production in European temperate zone. *Acta Horticultura*, 188, 193-201.

Mathé, JR., Mathé, I. (1979). Variation in Alkaloids in *Solanum dulcamara* L., In: *The Biology and Taxonomy of the Solanaceae*, Hawkes, J., Lester, R. & Skelding, A. (eds), pp. 211-222, Academic press, London.

Metcalf, C., Chalk, L. (1957). Solanaceae, In: *Anatomy of the Dicotyledons*. Vol. II., pp. 965-978, Clarendon Press, UK.

Molyneaux, R., Nash, R. & Asano, N. (1996). The chemistry and biological activity of calystegines and related nortropane alkaloids, In: *Alkaloids*: *Chemical and Biological Perspectives*, Pelletier, S. (ed.), volume II, chapter 4, pp. 303-343, Elsvier Science Ltd., UK.

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15, 473-497.

Nash, R., Rotschild, M., Porter, A., Watson, A., Waigh, R. & Waterman, P. (1993). Calystegines in *Solanum* and *Datura* species and the death's head hawk-moth (*Acherontia atropus*). *Phytochemistry*, 34, 1281-1283.

Porter, J. (1991). Host Range and implication of plant infection by *Agrobacterium rhizogenes*. *Critical Reviews in Plant Sciences*, 387-421.

Rhodes, M., Robins, R., Hamill, J., Parr, A. & Walton, N. (1987). Secondary product formation using *Agrobacterium rhizogenes*-transformed "hairy root" cultures. *IAPTC Newsletters*, 53, 2-15.

Ripperger, H. (1996). 22, 26-epiminocholestane alkaloids with unusual (20R) configurations from *Solanum* species. *Phytochemistry*, 41, 1629-1631.

Rönsch, H., Schreiber, K. (1966). *Solanum* Alakloide-LXXII. Uber δ_1- und δ-solamarin, zwei neue tomatidenol glycoside aus *Solanum dulcamara* L. *Phytochemistry*, 5, 1227-1233.

Sato, Y., Miller, H. & Mosettig, E. (1951). Degradation of solasodine. *Journal of the American Chemical Society*, 73, 5009-5011.

Schenk, R., Hildebrandt, A. (1972). Medium and techniques for induction of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany*, 50, 199.

Schimming, T., Tofern, B., Mann, P., Richter, A., Jennett-Siems, K., Drager, B., Asano, N., Gupta, M., Correra, M. & Eich, E. (1998). Distribution and taxonomix significance of calystegines in the Convolvulaceae. *Phytochemistry*, 49, 1989-1995.

Schripsema, J., Meijer, A., Van Iren, F., Hoopen, H. & Verpoorte, R. (1990). Dissimilation curves as a simple method for the characterisation of growth of plant cell suspension cultures. *Plant Cell, Tissue and Organ Culture*, 22, 55-64.

Stachel, S., Messens, E, Van Montagu, M. & Zambryski, P. (1985). Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature*, 318, 624-629.

Subroto, M., Doran, P. (1994). Production of steroidal alkaloids by hairy roots of *Solanum aviculare* and the effect of gibberelic acid. *Plant Cell, Tissue and Organ Culture*, 38, 93-102.

Tepfer, D., Goldman, A., Pamboukdjian, N., Maille, M., Lepingle, A., Chevalier, D., Denarie, J. & Rosenberg, C. (1988). A plasmid of *Rhizobium meliloti* 41 encodes catabolism of two compounds from root exudates of *Calystegia sepium*. *Journal of Bacteriology*, 170, 1153-1161.

Toshinori, N., Teruhiko, Y., Junko, O., Sadao, S. (1993). Steroidal alkaloids from tomato stock. *Phytochemistry*, 34, 1153-1157.

Usubillaga, A., Aziz, I., Tettamanizi, M., Waibel, R. & Achenbach, H. (1997). Steroidal alkaloids from *Solanum sycophanta*. *Phytochemistry*, 44, 537-543.

Watson, A., Fleet, G., Asano, N., Molyneaux, R. & Nash, R. (2001). Polyhydroxylated alkaloids, natural occurrence and therapeutic applications. *Phytochemistry*, 56, 265-295.

Willuhn, G. (1966). Undersuchungenzur chemi-schen differenzierung bei *S. dulcamara* L. I. Genetische fixierung der unter-schiedlichen steroidalkaloid fÜhrung. *Planta Medica*, 14, 408-420.

Yu, S., Kwok, K. & Doran, P. (1996). Effect of sucrose, exogenous product concentration and other culture conditions on growth and steroidal alkaloid production by *Solanum aviculare* hairy roots. *Enzyme and Microbial Technology*, 18, 238-243.

15

Genetic Transformation for Metabolic Engineering of Tropane Alkaloids

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1. Introduction

1.1 Tropane alkaloids: history and relevance

Historically, plants have been the major source of active compounds for the pharmaceutical industry. Today, plant extracts and its active principles represent 25% of the annual commercialized drugs in the United States (Kinghorn and Seo, 1996, Buttler 2004, Prakash Rout et al., 2009, Qurishi et al., 2010). Moreover, the World Health Organization has estimated that more than the 80% of the population in developing countries relies for their health care on traditional medicines mostly from plant origin (Canter et al., 2005). The plant active principles are, in general, a product of the plant secondary metabolism; they are low-molecular-weight compounds that in general participate in defence mechanisms against diseases (phytoalexins) or as attractants for pollinator insects (pigments and fragrances) (Canter et al., 2005). The production of each group of secondary metabolites, as counterpart of primary metabolites, is in general restricted to a limited number of species, and they usually are organ-specific and furthermore, tissue-specific.

According to their chemical properties, secondary metabolites could be classified in three main groups: a) terpenes, b) phenols and c) alkaloids. Terpenes and terpenoids are the primary constituents of the essential oils, widely used as natural flavour additives for food and fragrances. Phenols are a class of chemical compounds consisting of a hydroxyl group directly bonded to an aromatic hydrocarbon group that seem to be universally distributed in plants. They are essential for the growth and reproduction of plants, and are produced as a response for defending injured plants against pathogens. Finally, alkaloids are characterized for being alkaline nitrogenated organic compounds, derived from aminoacids, generally insoluble in water and soluble in alcohol, ether, chloroform, etc. Among the alkaloids, those derived from tropane have received particular attention for their properties.

1.2 Tropane alkaloids

Tropane alkaloids are distributed among the plant families *Orchidaceae, Dioscoreaceae, Proteaceae, Rhizophoraceae, Cruciferae, Eritroxilaceae, Euphorbiaceae, Convolvulaceae, Brassicaceae, Olacaceae* and *Solanaceae*. Their principal characteristic is the presence of a pyrrolic ring derived from the ornintine and arginine aminoacids by a chemical reaction catalyzed by ornitin decarboxilase (ODC) and arginin decarboxilase (ADC) respectively (Figure 1). Ornithine and arginine also are the precursors of the polyamines putrescine, espermidine and espermine, which play a critical role in plant development by the regulation of the cell

division in plants (Theiss et al., 2002). On the other hand, putrescine, by the action of the enzyme putrescine N- methyltransferase (PMT; EC 2.1.1.53), is sequestered to the secondary metabolism being the first step of the tropane alkaloid biosynthetic pathway.

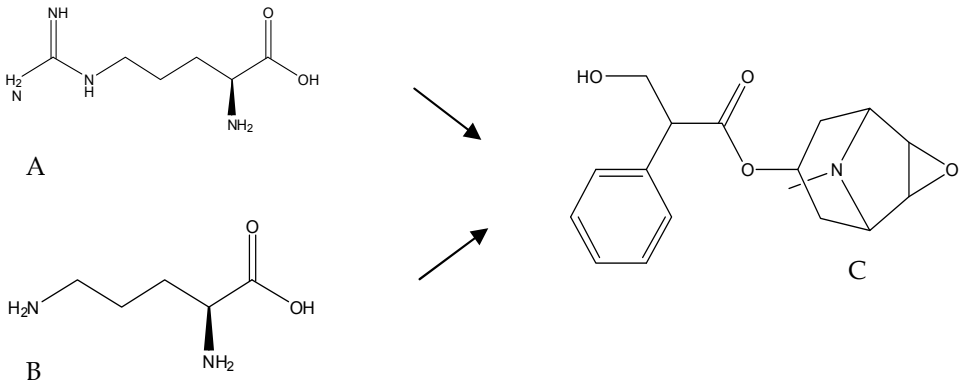

Fig. 1. Primary metabolism precursors: arginine (A) and ornitine (B) and the tropane alkaloid final product scopolamine (C).

Hyoscyamine and scopolamine are the end products of the tropane alkaloid biosynthetic pathway having both pharmacological applications for its action in the parasympathetic nervous system. Scopolamine, a 6,7-β-epoxide of hyoscyamine, is derived from hyoscyamine by the intermediate form 6β-hydroxyhyoscyamine (Fig. 2). The pharmaceutical industry employs hyosciamine, and its racemic form atropine, to obtain hyoscine N-butyl bromide that is used for its antispasmodic action since 1951. This semisynthetic derivative has pharmacological proprieties similar to those from scopolamine but with less activity and more adverse effects (Tytgat, 2007). Figure 2 compares the industrial process for producing the atropine racemic form of hyosciamine, and the scopolamine biosynthetic pathway in plants.

1.2.1 Scopolamine

Scopolamine is one of the earlier alkaloids purified from plant sources, described by Albert Ladenburg in 1880. It is the most valuable of the tropane alkaloids being its worldwide demand 10-fold the demand of the sum of hyosciamine (its precursor) and atropine (the semisynthetic drug) (Moyano et al., 2003). As atropine, scopolamine is an anticholinergic drug, besides it also has anti-muscarinic activity. Anticholinergic drugs are, in general, competitive and reversible inhibitors from acetylcholine with effects at parasympathetic level. Because of that pharmacological activity scopolamine has a number of uses in medicine. Its primary use is for the therapy of nausea (transdermal patchs), motion sickness and intestinal cramping. Also, it is used for ophtalmic purposes (to induce mydriasis and cycloplegia) and as a general depressant in combination with narcotic painkillers. It also has secondary uses are a preanesthetic agent, a drying agent for sinuses, lungs and related areas and to reduce motility and secretions in the gastrointestinal tract (tinctures). Uncommonly, it is also used for some forms of Parkinsonism, combined with opioids (e.g.: with morphine). The production and purification of scopolamine is extremely expensive which have made that several groups have focused in finding scopolamine analogs or synthetic forms.

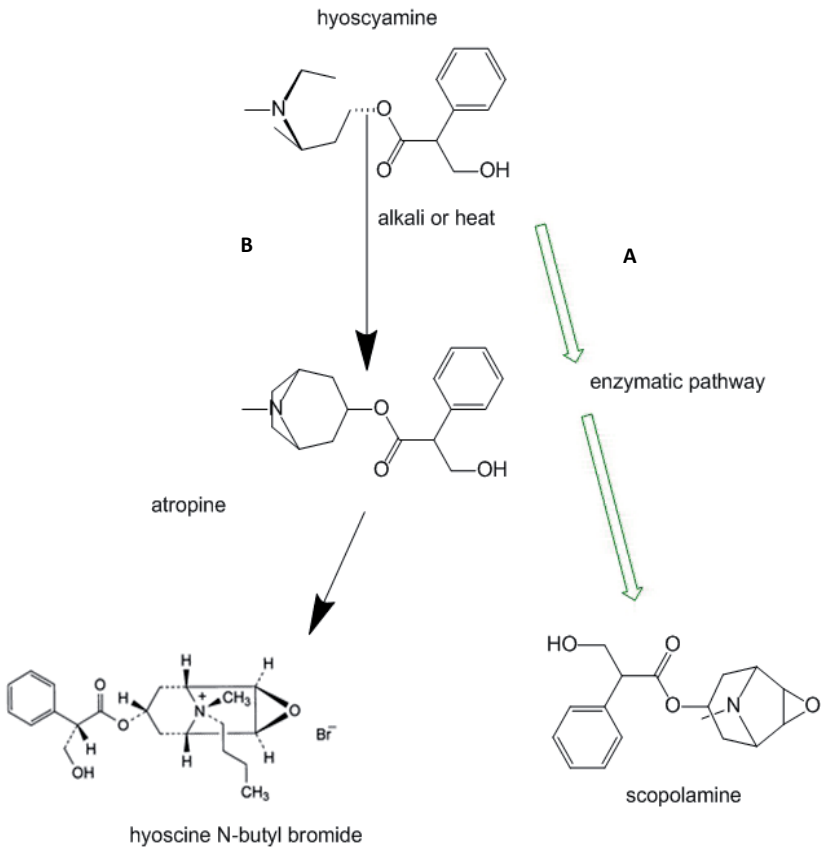

Fig. 2. The racemic hyoscine N-butyl bromide is obtained from natural sources as hyoscyamine (A) or by semi-synthesis from atropine (B). Scopolamine is the final product in the tropane alkaloid pathway being hyoscyamine its precursor.

1.2.3 Biosynthetic pathway, key points: PMT and H6H

As was mentioned above, scopolamine and hyoscyamine are the most relevant tropane alkaloids widely in use. Their metabolic pathway starts in putrescine, a polyamine that is shared by several metabolic pathways (e.g.: pyridinic alkaloids). The role of putrescine methyl transferase (PMT; EC 2.1.1.53) is to sequester putrescine from the polyamine pool, which is the cross-point between the primary and secondary metabolism towards the tropane alkaloid pathway. The enzyme PMT catalyses the reaction from putrescine to N-methyl putrescine with S-adenosyl methionine as methyl donor (Zhang et al., 2004, Stenzel et al., 2006). It was described by Mizusaki et al. (1973) in tobacco for the first time, afterwards it has also been described in *Hyoscyamus* sp. (Hashimoto et al., 1989, Suzuki et al., 1999). The last part of the tropane alkaloid biosynthetic pathway is the enzymatic reaction catalyzed by hyoscyamine-6β-hidroxylase (H6H), a 2-oxoglutarate dependent dioxygenase (EC 1.14.11.11, MW: 41.000 ± 1.0, optimal pH 7.8) that catalyses the hydroxylation of hyoscyamine to scopolamine in two steps. The first step is the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine, a reaction that requires 2-oxoglutarate, Fe^{2+}, molecular oxygen, and ascorbate. The second step is the epoxidation of the intermediate

6β-hydroxyhyoscyamine producing the 6,7-β- hyoscyamine epoxide (scopolamine) (Hashimoto and Yamada, 1986 and 1987).

2. Strategies for scopolamine production

The chemical complexity of tropane alkaloids turns almost impossible its substitution by other compounds or the *de novo* synthesis. To date, alkaloids are extracted from the macerated leaves of wild plants growing in greenhouses. The whole process, performed under Good Manufacture Practices (GMP), makes the final product very expensive thus the search of an alternative production strategy is a priority. The alternative methods comprise biotechnology approaches as the genetic transformation for metabolic engineering to overexpress the key proteins in the biosynthetic pathway and the establishment of *in vitro* organ, tissue and plant cultures from scopolamine producer species.

2.1 Metabolic engineering

The in-depth understanding of biosynthetic pathways, along with the increasing number of cloned genes involved in biosynthesis, enable the exploration of metabolic engineering as a potential effective approach to increase the yield of specific metabolites. It could be achieved by enhancing rate-limiting steps or by blocking competitive pathways. Metabolic engineering is a multidisciplinary powerful tool that uses from bioinformatics, genetics, biochemistry, systems biology, molecular biology, biochemical engineering, etc, for re-direct the metabolic flux generally in order to increase secondary metabolite production yields (e.g.: alkaloids). It also could be useful for a rational and directed modification of metabolic pathways to understand their design and regulation and to study the impact of intermediates and end products in a specific organism.

When the improvement of a productive process is the main goal, the *a priori* knowledge of the metabolic pathway is fundamental. The next step is to know the kinetic of growth of the biomass that produces the product of interest, the parameters that could reflect that growth, and the election of the parameters that will led to the optimization of the process. Metabolic engineering uses different strategies such as improving the precursors production, re-directing one or more enzymatic reactions in a metabolic pathway, widening the metabolic capacities by stress or increasing the enzymatic activities, etc. Also a reliable strategy is to direct the carbon flux towards the biosynthetic pathway by the overexpression of the genes codifying the limiting enzymes, blocking the feedback inhibition mechanism or the competitive pathways, diminishing catabolism, etc..

2.1.1 Hairy root culture initiation and maintenance

Hairy roots are obtained by infection of the plant with different *Agrobacterium rhizogenes* strains (e.g.: LBA9402 and LBA15834) that harbor the Ri vector (In: *Agrobacterium-mediated genetic transformation: history and progress*, present book). They are characterized by their indefinite and fast growth and its higher chromosomal stability than *in vitro* clonal multiplication. The first hairy root cultures were established at the end of the 1980's (Oksman-Caldentey and Strauss, 1986, Hamill et al. 1987, Oksman-Caldentey et al., 1987). Since then, it has been established hairy roots from numerous species, among them scopolamine-producing species (*Hyoscyamus, Brugmansia, Duboisia, Atropa*, and *Scopolia,* etc). In general, hairy roots are initiated from axenic plants by infection with *A. rhizogenes* the hairy root tips usually appear in the infected areas approximately after one week of

infection, being a chimera between the infected and non-infected cells. Once initiated, they are maintained by transfer to selective media without the addition of plant growth regulators. The hairy root cultures are subcultured in the same medium approximately every 20 days. When the process is performed in liquid medium it is carried on under agitation in an orbital shaker (100 rpm) with 16 h- photoperiod using cool white fluorescent lamps.

As we have said before, the synthesis of tropane alkaloids is produced in the pericycle of roots (Hashimoto et al., 1991) being the final products translocated to the aerial part of the plant. It is not surprising then that the establishment of hairy roots was considered as an alternative strategy for scopolamine production (Oksman-Caldentey et al., 1991, Oksman-Caldentey and Arroo, 2000, Palazón et al., 2003 and 2008). It was reported that *Datura stramonium* hairy roots de-differentiated and loose their ability to synthesize scopolamine when treated with auxines (Palazón et al., 1995). On the other hand, undifferentiated cultures (calli) do not produce scopolamine proving the need of some degree of tissue specialization (Moyano et al., 1999; Jouhikainen et al., 1999). Evidently, the tropane alkaloid synthesis requires root organization when scopolamine is the end product.

Even though hairy roots are scopolamine producers the yields achieved are low, which has fostered the quest of an alternative production strategy. In the 1990's the first studies about the influence of plant growth regulators on scopolamine yields were performed. The effect of the addition of GA_7 on kinetics of growth and alkaloid accumulation in two different *Brugmansia candida* hairy root clones have demonstrated that GA_7 (10^{-4} to 10^{-1} $mg.l^{-1}$) increased the scopolamine/hyoscyamine ratios in the early phases of growth, the sum of scopolamine plus hyoscyamine per flask decreased during the same growth period. Indeed the addition of GA_3 produced higher growth rates but lower alkaloid concentration (Pitta Alvarez & Giulietti, 1997).

2.1.2 Elicitation

The term elicitor was first introduced to describe the action of biomolecules able to induce phytoalexin production. In general, elicitors induce defence systems and increase the resistance to pathogens in plants. Also, pathogens biomolecules derived from the pathogen cell wall (exogenous elicitors), and compounds released from plants by the action of the pathogen, triggers that defence response (Angelova et al., 2006).

At the end of the 1990's several groups started the study of the effect of elicitors on secondary metabolism. Hence, it was evident that biotic elicitors (produced by pathogenic microorganisms or released from their cell walls by plant enzymes) were able to induce changes in secondary metabolites patterns. Also, the release of certain compounds, such as cellulose or pectinase and molecules active in the signal transduction pathway (salicylic acid, jasmonic acid), could also promote the plant defence response (Benhamou, 1996; Guo et al., 1997; Lawrence et al., 2000). That response includes the production of phytoalexins (pathogeneses related proteins), protease inhibitors and a variety of other defence compounds (among them alkaloids). *In vitro* plant cell cultures have been used for studying the influence of elicitors on secondary metabolism (Zabetakis et al., 1998, Jung et al., 2003, Marconi et al., 2008), and also their influence on tropane alkaloid metabolism particularly in hairy roots (Pitta Alvarez et al., 2000, 2001, Ajungla et al., 2009). Non-conventional elicitors (e.g.: acetate buffer, pectinase, *Hormonema* sp. homogenate), increased intracellular hyoscyamine content (200-300%), and the release of scopolamine (up to 1500%) and hyosciamine (up to 1100%) in *B. candida* hairy roots (Pitta-Alvarez et al., 2003). Thus, elicitors

represent a promising and inexpensive alternative to increase production of hyoscyamine and scopolamine by *in vitro* culture.

3. Metabolic and genetic engineering

Molecular biology, as a tool for DNA manipulation, could be used for engineering metabolic pathways. Figure 3 shows the usual strategies employed for overproducing a specific metabolite (C).

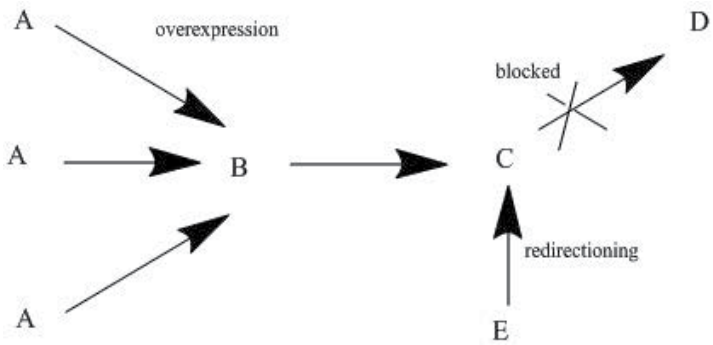

Fig. 3. Strategies for increasing secondary metabolite production by engineering metabolism.

The strategies metabolic engineering uses to increase secondary metabolite production are a) the overexpression of secondary metabolite precursors (A); b) the overexpression of genes whose products are rate-limiting (B), c) the creation of new branches in the biosynthetic pathway (E to C), d) the blocking of reactions (C to D) redirecting them to the C pathway, among others. Also, there are other strategies for overproducing C such as a) manipulation of regulatory genes, b) a positive or negative regulation of gene expression, c) selection of mutants with increased expression of metabolites, d) use of specific promoters for tissue/organ specific expression, e) iRNA technology and gene silencing. Each of the strategies above mentioned has unpredictable consequences in the cell homeostasis.

3.1 Overexpression of key enzymes

Overexpression of a key enzyme in the biosynthetic pathway is one of the strategies used to increase secondary metabolite production. In general, the overexpressed enzyme catalyzes a bottleneck step. In the tropane alkaloid pathway the key enzyme is H6H. As was mentioned above, H6H catalyzes two reactions, the hydroxylation at position 6, rendering an intermediate 6β-hydroxyhyoscyamine; and the epoxidation that leads to the end product scopolamine. Therefore, overexpression of H6H is an attractive strategy for poor scopolamine producing species with hyoscyamin-rich accumulation. *In vitro* shoot and callus cultures shown very low concentration of H6H and, hence, scopolamine production. On the contrary, hairy root cultures from scopolamine-producing species have high concentration of H6H. The difference in the expression of this enzyme could be attributed to the specialization of root pericycle for producing H6H. Several attempts were made in order to isolate and overexpress the H6H cDNA in hairy roots for scopolamine production. The *h6h* cDNA was isolated from *B. candida, H. muticus, A. belladonna, Duboisia* hybrid with high producing scopolamine capacity (Matsuda et al., 1991, Jouhikainen et al., 1999, Cardillo et

al., 2005; Canter et al., 2005, Oksman-Caldentey et al., 2007, Palazón et al., 2008). All the isolated sequences reveled high homology (>80%). A strategy used is to introduce in low-producing scopolamine species numerous copies of their own H6H gene. All the protocols have several common steps. First, the *h6h* cDNA is cloned into the binary vector and amplified in *Escherichia coli*. Then, *A. rhizogenes* is transformed with the binary vector obtained and used for re-introducing, by agrotransformation, that *h6h* cDNA into the genome of the same species from which it was isolated. However, the results were not successful because the H6H overexpressing clones obtained did not have a significant increase on alkaloid production. Those results could be attributed to an upstream regulation of alkaloid biosynthesis, to a rate limiting speed of H6H or to a deficiency of precursors.

Another strategy focus on the lack of substrates in the tropane alkaloid pathway. Thus, the carbon flux is redirect from the primary to the secondary metabolism, being PMT the key and pivot enzyme between both metabolisms. There is a strong expression of the *pmt* gene in the pericycle of *Atropa belladona* roots that is suppressed by the addition of exogenous auxins (Suzuki et al., 1999, Hibi et al., 1994, Palazón et al., 1995). There are several protocols describing the establishment of PMT overexpressing hairy roots. Nevertheless the results obtained are as disappointing as those obtained overexpressing H6H. The first report of PMT overexpression in hairy roots was with *Hyoscyamus albus* (Hashimoto et al., 1989). Tracer-feeding studies with radioactive aminoacids demonstrated that putrescine is the precursor of tropane alkaloids. Sato et al. (2000) have reported the overexpression of PMT in *Atropa belladonna* hairy roots revealing an increase in polyamines pools. However, the alkaloid profile remained unchangeable. On the other hand, the overexpression of PMT from *Nicotiana tabacum* in *Duboisia hybrid* hairy roots (yielding scopolamine) produced an increase of *pmt* gene expression that is not reflected in the alkaloid production (Moyano et al. 2000). Also, there are reports of heterologous tests with non-tropane alkaloid producing species such as *Solanum tuberosum* (Stenzel et al., 2006) and *Nicotiana sylvestris* (Sato et al., 2001). The overexpression of PMT in *N. sylvestris* increasead the nicotine concentration, probably for a higher metabolic flux towards the tropane and pyridinic alkaloid pathways. However, when the *pmt* gene was overexpressed in the tropane alkaloid producer *A. belladonna* (Sato el al., 2001) and the *Duboisia* hybrid (Moyano et al., 2002), no significant increase in scopolamine was observed suggesting that methylputrescine is not the rate limiting substrate in the tropane alkaloid pathway.

The overexpression of only one enzyme in a complex metabolic net could not be sufficient to increase some secondary metabolite expression. Particularly, there are several works about the overexpression of more than one of the enzymes involved in the alkaloid tropane pathway (Zhang et al., 2004, Liu et al., 2010, Chunxian et al., 2011). However, not significant scopolamine yields were attained. The unsuccessful results could be attributed to the transgenic transformation processes itself. The mechanism of integration of transgenes into plant DNA is poorly understood, the integration of many genes at one or a few loci could not happen by chance. Also, multiple copies of one or more transgenes can result in postranscriptional gene silencing and turn unstable and reduce gene expression (Matzke & Matzke; 1998; Palazón et al., 1998; Bulgakov et al., 2004; Kutty et al., 2011). Evidently, *h6h* played a more important role in stimulating scopolamine accumulation than *pmt*. Nevertheless, when *pmt* redirects the carbon flux to the tropane pathway and *h6h* is overexpressed there is an accumulation of scopolamine (Zhang et al. 2004).

The genetic transformation with numerous in-tandem genes could be troublesome. For hairy root induction and *pmt* and *h6h* cDNA insertion, at least three tandem transformations

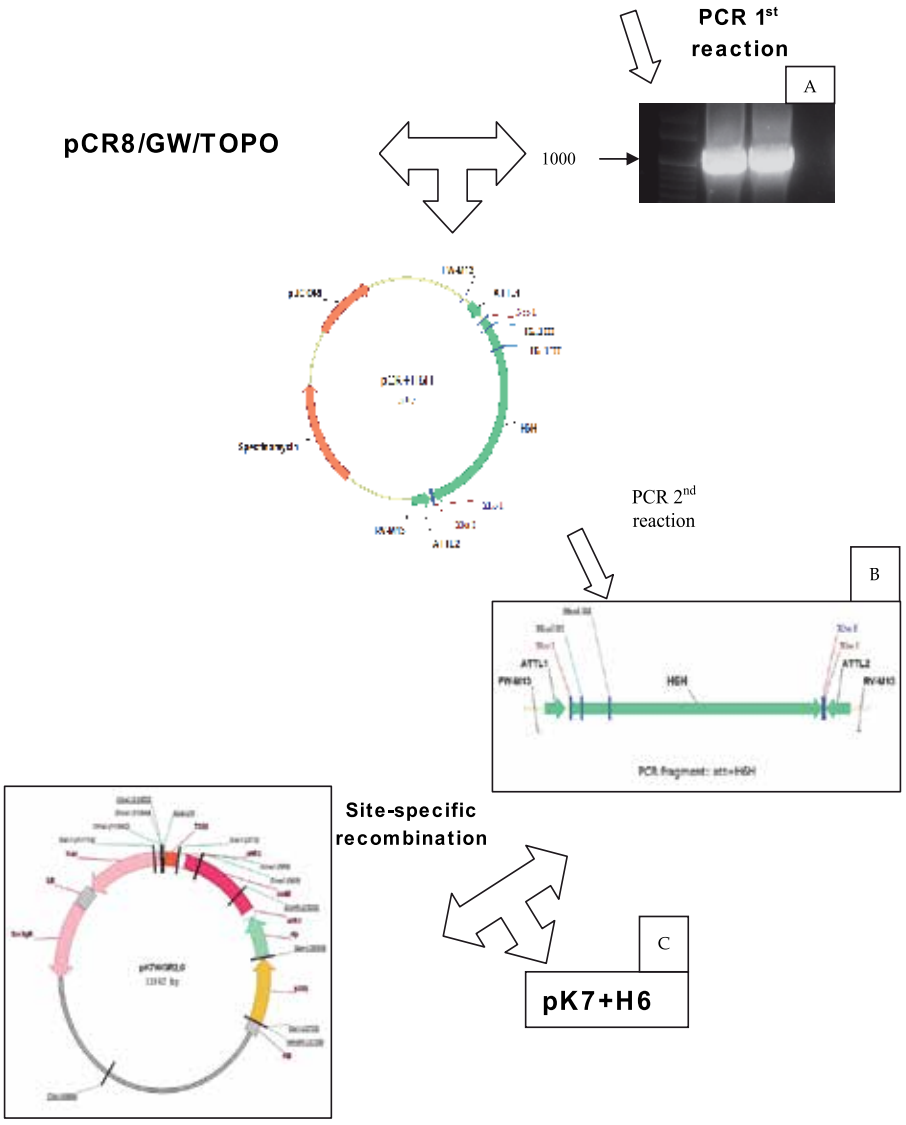

Fig. 4. Scheme of the site-specific recombination between the PCR product harboring *pmt* or *h6h* cDNA and the binary vector. During the first PCR round (PCR 1st Reaction), the H6H (or PMT) cDNA is isolated and purified from an agarose gel electrophoresis (A). The PCR fragment is inserted between the specific attachment sites sequences (att sites) into the Entry pCR8/FW/TOPO vector producing the plasmid pCR+H6H (B). The second round of PCR (PCR 2nd Reaction) is carried out with the M13 forward and reverse primers in order to amplify a cDNA fragment encoding the sequence of interest flanked by the att left and right recombination sequences. These sequences permit the site-specific recombination between the extended PCR fragments and a Destination binary plasmid (pK7WG2.00) following the Gateway protocol. LR Clonase II enzyme MIX is used to catalyze the site specific recombination reaction which is performed according to the manufacturer's protocol (Invitrogen). The final product is the binary destination vector pK7+H6H (C).

are needed. New and simple DNA transfer methods simplify the process as, for example, the Gateway system®, based on bacteriophague Lambda site-specific recombination system (Karimi et al., 2002; Attanasov et al., 2009; Dubin et al., 2008, Xu & Quinn, 2008). Figure 4 shows a protocol designed in our lab for the production of a PCR fragment for *h6h* cDNA ready to recombine into the destination vector independently of the antibiotic gene resistance.

4. New approaches

In this chapter, we have reviewed some of the most relevant strategies for improving tropane alkaloid biosynthesis such as the establishment of scopolamine overproducing organ cultures, the elicitation and the genetic transformation with homologous genes. Nevertheless, the knowledge generated and the strategies in use have demonstrated that the tropane alkaloid metabolism is immersed into a complex net of metabolic pathways with a delicate equilibrium quite difficult to be manipulated. Modern system biology tools, like elicitation and overexpression, allow the carbon flux redirection with some limitations. These margins cannot be overcome and decelerate the development of a competitive and sustainable production platform. Those troubles and limitations have fostered new strategies based on functional genomics (Goossens et al., 2002; Goossens & Rischer, 2007, Oksman-Caldentey & Inzé, 2004) such as biotransformation.

Biotransformation is one of those new strategies. The production of scopolamine and other alkaloids was studied in engineered *N. tabacum* hairy roots overexpressing the *h6h* cDNA after feeding the cultures with exogenous hyoscyamine (Hakkinen et al., 2005). The results obtained have shown an efficient uptake of hyoscyamine from the culture medium and a higher rate of bioconversion of hyoscyamine to scopolamine (up to 85% of the total scopolamine being released to the culture medium). Moreover, it was also evident an enhanced production of various nicotine alkaloids suggesting that the regulation of the alkaloid production is probably more complex than presently known.

Another approach was the bioconversion of hyoscyamine to scopolamine using recombinant *Saccharomyces cerevisiae* that expresses the *h6h* cDNA isolated from *B. candida* as a biocatalyzer. Transformed *S. cerevisiae* CEM PK2 expressing H6H as heterologous protein was able to grow into yeast base medium supplemented with hyoscyamine. However, the results have shown a low ability of hyoscyamine conversion to scopolamine (Cardillo et al., 2005 and 2008).

5. Conclusions

In the last two decades plant biotechnology has made considerable advances in the quest of a scopolamine and other tropane alkaloids productive process. Several groups have explored a wide spectrum of strategies that have led to the exhaustive knowledge of the tropane alkaloid pathway, its limiting steps and some of the regulation pathways. It is evident that genetic transformation is a promissory tool for engineering tropane alkaloid biosynthetic metabolism in order to produce high amounts of scopolamine. Combining genetic transformation and metabolic engineering would be a powerful strategy to re-direct the metabolic flux towards that biosynthetic pathway. It became clear, from the results already published, that the overexpression of *pmt* gene and/or *h6h* gene not only stimulated the conversion of hyoscyamine to scopolamine, but also the capacity of these hairy root lines

to synthesize both tropane alkaloids. However, the yields obtained up to now did not reach those of the current scopolamine productive process. Future research could be done considering the higher structural diversity of tropane alkaloids that could be functional to create new metabolic pathways and biological active products. A possible strategy would be cloning and engineering those new metabolic pathways in heterologous organism to produce a chemical structure diversity for generating new compounds with scopolamine-like activity (Harvey, 2000, Butler, 2004, Potterat & Hamburger, 2008).

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7. References

Ajungla, L., Patil, P.P., Barmukh, R.B. Nikam, T D (2009) Influence of biotic and abiotic elicitors on accumulation of hyoscyamine and scopolamine in root cultures of *Datura metel* L. *Indian J Biotechnol* 9: 317-322.

Angelova, Z., Georgiev, S. & Ross, W. (2006) Elicitation in plants. *Biotechnol Eq J* 20: 72-83.

Attanassov, I.I., Etchells, J.P. & Turner, S.R. (2009) A simple, flexible & efficient PCR-fusion/Gateway cloning procedure for gene fusion, site-direct mutagenesis, short sequence insertion & domain deletions and swaps. *Plant Methods* 4: 1-11.

Benhamou, N. (1996) Elicitor-induced plant defence pathways. Trends in Plant Sc 1:233-240.

Bulgakov, V.P., Tchernoded, G.K., Mischenko, N.P., Shkryl, Y.N., Fedoreyev, S.A. & Zhuravlev, Y.N. (2004). The rolB and rolC genes activate synthesis of anthraquinones in *Rubia cordifolia* cells by mechanism independent of octadecanoid signaling pathway. *Plant Sc* 166:1069- 1075.

Butler, M.S. (2004) The role of natural product chemistry in drug discovery. *J Nat Prod* 67: 2141-2153

Canter, P.; Howard, T. & Edzard, E. (2005) Bringing medicinal plants into cultivation: opportunities and challenges for biotechnology. *Trends in Biotechnol* 23:180-185.

Cardillo, A.B., Silberstein, S., Giulietti, A.M. & Marconi, P.L. (2005) Biotransformation of hyoscyamine by a recombinant *Saccharomyces cereviciae* strain. 2nd Mercosur Congress on Chemical Engineering 4th Mercosur Congress on Process Systems Engineering, Brasil.

Cardillo, A.B., Rodriguez Talou, J. & Giulietti, A.M. (2008) Expression of Brugmansia candida Hyoscyamine 6beta-Hydroxylase gene in Saccharomyces cerevisiae and its potential use as biocatalyst. Microbial Cell Factories 7: 1-7.

Chunxian, Y., Min, Ch, Lingjiang, Z., Lei, Z., Xiaoqiang, L., Xiaozhong, L., Kexuan, T. & Zhihua, L. (2011) Improvement of tropane alkaloids production in hairy root cultures of *Atropa belladonna* by overexpressing *pmt* and *h6h* genes. *Plant Omics* J 4: 29-33.

Dubin, M.J., Bowler, C. & Benvenuto, G. (2008) A modified gateway cloning strategy for overexpressing tagged proteins in plants. *Plant Methods* 4: 1-11.

Hamill J.D., Parr A.J., Rhodes H.J.C., Robbins R.J., Walton N.J. (1987) New routes to plant secondary products. *Biotechnology*, p. 800-804.

Guo, Z. J., Lamb, C. & Dixon, R. (1997) Release and biological activity of diffusible signal compounds from elicited plant cells. *J Plant Physiol* 151: 699-710.

Goossens, A., Ha, S.T., Into, L. K. , Seppa, T., nen-Laakso, S.B., De Sutter, V., Lammertyn, F., Nuutila, A.M., So derlund, S., Zabeau, M., Inze, D. & Oksman-Caldentey, K-M (2002) A functional genomics approach toward the understanding of secondary metabolism in plant cells. *Proc Natl Acad Sc* 14: 8595-8600.

Goossens, A. & Rischer, H. (2007) Implementation of functional genomics for gene discovery in alkaloid producing plants. *Phytochem Reviews* 6: 35-49.

Hakkinen, S.T., Moyano, E., Cusido, M.R., Palazon, J., Teresa Pinol, M. & Oksman-Caldentey, K-M (2005) Enhanced secretion of tropane alkaloids in *Nicotiana tabacum* hairy roots expressing heterologous hyoscyamine-6b-hydroxylase. *J Experimental Botany* 56: 2611–2618.

Harvey, A. (2000) Strategies for discovering drugs from previously unexplored natural products. *Drug Discov Technol* 5: 294-300.

Hashimoto, T. & Yamada, Y. (1986) Hyoscyamnine6-β hydroxylase, a 2 oxaglutarate – dependent oxygenase, in alkaloid production root cultures. *Plan Physiol* 81: 619-625.

Hashimoto, T. & Yamada, Y. (1987) Purification and characterization of hyoscyamine 6 β-hydroxylase from root culture of *Hyoscyamus niger* L. *Eur J Biochem* 194: 277 – 285.

Hashimoto, T, Yukumine, Y. & Yamda, Y (1989) Putrescine and putrescine N-methylransferase in the biosynthesis of tropane alkaloids in culture roots of *Hyoscyamus albus*. *Planta* 178: 131-137

Hashimoto, T., Hayashi, A., Amano, Y., Kohno, J., Iwanari, H., Usuda, S. &, Yamada, Y. (1991) Hyoscyamine 6 beta-hydroxylase, an enzyme involved in tropane alkaloid biosynthesis, is localized at the pericycle of the root. *J Biol Chem* 5: 4648-53.

Hashimoto, T., Yun, D.J. & Yamada, Y. (1993) Production of tropane alkaloids in genetically engineered root cultures. *Phytochem* 32 713–718.

Hashimoto, T., Matsuda, J. & Yamada, Y. (1993) Two-step epoxidation of hyoscyamine to scopolamine is catalyzed by bifunctional hyoscyamine 6-hydroxylase. *FEBS* Lett 329: 35–39.

Hibi, N., Higashiguchi, S., Hashimoto, T. &, Yamada, Y. (1994) Gene expression in tobacco low-nicotine mutants. *Plant Cell* 6: 723-735.

Jouhikainen, K., Lindgren, L., Jokelainen, T., Hiltunen, R., Teeri, T.H. & Oksman-Caldentey, K-M. (1999) Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures by genetic engineering. *Planta* 208, 545-551.

Jung, H, Kang, S, Kang, M., Bahn, J, Yang, J. & Choi M. (2003) Enhanced production of scopolamine by bacterial elicitors in adventitious hairy root cultures of *Scopolia parviflora*. *Enzyme Microb Technol* 33: 987-900.

Karimi, M., Inzé, D. & Depicker, A. (2002) GATEWAY-vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sc* 7: 193-195.

Kinghorn, A. D. & Seo, E. K. (1996) Plants as sources of drugs. *Agricultural Materials as Renewable Resources* 647: 179−193.

Kutty, P.C., Parveez, G.K.A. & Huyop, F. (2011) *Agrobacterium tumefaciens* infection strategies for greater transgenic recovery in *Nicotiana tabacum* cv. TAPM26. I. *J Agricultural Research* 6: 119-133.

Lawrence, C.B., Singh, N.P., Giu, J.S., Gardener, R.G. & Tuzun, S. (2000) Constitutive hydrolytic enzymes are associated with polygenic resistance of tomato to *Alternaria*

solani and may function as an elicitor release mechanism. *Physiol Molec Plant Pathol* 57: 211-220.

Liu, X., Yang, C., Chen, M., Li, M., Liao, Z. & Tang, K. (2010) Promoting scopolamine accumulation in transgenic plants of *Atropa belladonna* generated from hairy roots with over expression of *pmt* and *h6h* gene. *J Medicinal Plants Res* 4: 1708–1713.

Marconi, P.L., Alvarez, M.A., & Pitta-Alvarez, S.I. (2006) How polyamine synthesis inhibitors and cinnamic acid affect tropane alkaloid production. *Applied Biochem Biotechnol* 136: 63-76.

Marconi, P.L., Setten, L., Cálcena, E., Alvarez, M.A. & Pitta Alvarez, S.I. (2008) Changes in growth and tropane alkaloid production in long-term culture of hairy roots of *Brugmansia candida*. *J Integrative Biosc* 3: 38-44.

Matsuda, J., Okabe, S., Hashimoto, T., & Yamada, Y. (1991) Molecular cloning of hyoscyamine 6B-hydroxylase, a 2-Oxoglutarate-dependent dioxygenase, from Cultured Roots of *Hyoscyamus niger*. *J Biol Chem* 266: 9460-9464.

Matzke, A.J.M. & Matzke, M.A. (1998) Position effects and epigenetic silencing of plant transgenes. *Current Opinion in Plant Biol* 1 :142-l 48

Mizusaki, S., Tanabe, Y., Noguchi, M. & Tamaki, E. (1973) Changes in the activities of ornithine decarboxylase, putrescine N-methyltransferase and N-methylputrescine oxidase in tobacco roots in relation to nicotine biosynthesis. *Plant Cell Physiol* 14: 103-110.

Moyano E, Fornale A.S., Palazon J., Cusido R.M., Bonoll M, Morales C. & Piñol M.T. (1999) Effect of *Agrobacterium rhizogenes* T-DNA on alkaloid production in Solanaceae plants. *Phytochem* 52, 1287-1292.

Moyano, E, Fornale, A.S., Palazón, J., Cusido, R.M., Bagni, N. & Piñol, M.T. (2002) Alkaloid production in *Duboisia hybrid* hairy root cultures overexpressing the *pmt* gene.*Phytochem* 59, 697-702.

Moyano, E., Jouhikainen, K., Tammela, P., Palazón, J., Cusido, R.M., Piñol, M.T., Teeri H.T. & Oksman-Caldentey K-M. (2003) Effect of *pmt* gene overexpression on tropane alkaloid production in transformed root cultures of *Datura metel* and *Hyoscyamus muticus*. *J Exp Botany* 54: 203–211.

Oksman-Caldentey, K-M. & Strauss, A. (1986) Somaclonal variation of scopolamine content in protoplast-derived cell culture clones of *Hyoscyamus muticus*. *Planta Medica* 52, 6-12.

Oksman-Caldentey, K-M., Vuorela, H, Strauss, A. & Hiltunen, R. (1987) Variation in the tropane alkaloid content of *Hyoscyamus muticus* plants and cell culture clones. *Planta Medica* 53, 349-354.

Oksman-Caldentey, K-M., KivelaÈ, O. & Hiltunen, R. (1991) Spontaneous shoot organogenesis and plant regeneration from hairy root cultures of *Hyoscyamus muticus*. *Plant Science* 78,129-136.

Oksman-Caldentey, K-.M. & Arroo, R. (2000) Regulation of tropane alkaloid metabolism in plants and plant cell cultures, in: R. Verpoorte, A.W. Alfermann (Eds.), Metabolic Engineering of Plant Secondary Metabolism, Kluwer Academic Publishers, Dordrecht, 2000, pp. 253–281.

Oksman-Caldentey, K.M. & Inze, D. (2004) Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. *Trends Plant Sc* 9: 433-440.

Oksman-Caldentey, K.M., Häkkinen, S.T. & Rischer, H. (2007) Chapter 4: Metabolic Engineering of the Alkaloid Biosynthesis in Plants: Functional Genomics Approaches in: Applications of Plant Metabolic Engineering. Veerporte R., Alfermann, A.W., Johnson, T.S. Eds. Springer Netherlands, pp. 109-124.

Palazón, J., Altabella, T., Cusido, R.M., Ribo, M. & Piñol, M.T. (1995) Growth and tropane alkaloid production in *Agrobacterium* transformed roots and derived callus of *Datura*. *Biologia Plantarum* 37, 161-168.

Palazón, J., Cusidó, R.M., Roig, C. & Piñol, M.T. (1998) Expression of the *rolC* gene and nicotine production in transgenic roots and their regenerated plants. *Plant Cell Rep* 17: 384-390.

Palazón, J., Moyano, E, Cusidó, R.M., Bonfill, M., Oksman-Caldentey, K-M & Piñol, M.T. (2003) Alkaloid production in *Duboisia* hybrid hairy roots and plants overexpressing the *h6h* gene. *Plant Sc* 165: 1289–1295.

Palazón, J, Navarro-Ocaña, A., Hernandez-Vazquez, L. & Hossein Mirjalili, M. (2008) Application of metabolic engineering to the production of scopolamine. *Molecules* 13: 1722-1742.

Pitta Alvarez, S.I. & Giulietti, A.M. (1997) Effect of gibberellin GA7 on kinetics of growth and tropane alkaloid accumulation in hairy roots of *Brugmansia candida*. *In Vitro-Plant*. , 33:147 – 153.

Pitta Alvarez, S.I., Spollansky, T.C. & Giulietti, A.M. (2000) Scpolamine and hyoscyamine production by hairy root cultures of *Brugmansia candida*: influence of calcium chloride, hemicelullase and theophylline. *Biotechnol Lett* 22: 1653-1656.

Pitta Alvarez, S.I., Marconi, P.L. & Giulietti, A. (2003) Comparison of the influence of different elicitors of hyosciamine and scopolamine content in hairy roots cultures of *Brugmansia candida*. *In Vitro-Plant*, 36: 640-644.

Potterat, O. & Hamburger, M. (2008) Drug discovery and development with plant-derived compounds. In: progress in drug research, Vol. 65. Eds: F. Petersen & R. Amstutz. Birkhauser Verlag, Basel, Switzerland. pp. 45-118.

Prakash Rout, S., Choudary, K.A., Kar, D.M., Das. L. & Jain, A. (2009) Plants in traditional medical system- future source of new drugs. *I J Pharmacy Pharmac Sc* 1: 1-23.

Qurishi, Y., Hamid, A., Zargar, M.A., Singh, S.K. & Saxena, A.K. (2010) Potential role of natural molecules in health and disease: Importance of boswellic acid. *J Medicinal Plants Res* 4: 2778-2785.

Sato, F., Hashimoto, T., Hachiya, A., Tamura, K., Choi, K., Morishige, T., Fujimoto, H. & Yamada, Y. (2001) Metabolic engineering of plant alkaloid biosynthesis. *Proc Natl Acad Sci* 98: 367-372.

Shinjiro, O.; Uefuji, H., Masayki, M. & Hiroshi, S. (2005) Metabolic engineering of caffeine production. *Plant Biotechnol* 22: 461-468.

Stenzel, O., Teuber, M. & Drager, B. (2006) Putrescine N-methyltransferase in *Solanum tuberosum* L., a calystegine-forming plant. *Planta* 223: 200-212.

Suzuki, K.; Yun, D.-J.; Chen, X.Y.; Yamada, Y. & Hashimoto, T. (1999) An *Atropa belladona* hyoscyamine 6 β-hydroxylase gene is differentially expressed in the root pericycle and anthers. *Plant Mol Biol* 40: 141-152.

Theiss, C., Bohley, P. & Voigt, J. (2002) Regulation by polyamines of ornithine decarboxylase activity and cell division in the unicellular green alga *Chlamydomonas reinhardtii*. *Plant Physiol* 128: 1470.

Tytgat, G.L. (2007) Hyoscine Butylbromide: A Review of its Use in the Treatment of Abdominal Cramping and Pain. *Drugs* 67: 1343-1357

Xu, R. & Quinn, Q.Q. (2008) Protocol: Streamline cloning of genes into binary vectors in Agrobacterium via the Gateway® TOPO vector system. *Plant Methods* 8: 1-7.

Zabetakis, I., Edwards, R. & O´Hagan, D. (1998) Elicitation of tropane alkaloid biosynthesis in transformed root cultures of *Datura stramonium*. *Phytochem* 50:53-56.

Zhang, L., Ding, R., Chai, Y., Bonfill, M., Moyano, E., Oksman-Caldentey, K-M., Xu, T., Pi, Y., Wang, Z., Zhang, H., Kai, G., Liao, Z., Sun, X., & Tang, K. (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. *Proc Natl Acad Sci* 101: 6786–6791.

16

Transgenic Plants for Enhanced Phytoremediation – Physiological Studies

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1. Introduction

Natural processes such as volcanic eruptions, continental dusts and anthropogenic activities like mining, combustion of fossil fuel phosphate fertilizers, military activities and metal working industries lead to emission of heavy metals and accumulation of these chemicals in ecosystem. So, the metals found in our environment come from natural weathering process of earth's crust, soil erosion, mining, industrial discharge, urban runoff, sewage effluents, air pollution fall out, pest or disease control agents. The concentrations of the contaminants can vary from highly toxic concentrations from an accidental spill to barely detectable concentrations that, after long-term exposure, can be detrimental to human health (Alexander, 1999; Doty, 2008). Particularly, heavy metals are toxic because they cause DNA damage and their carcinogenic effects in animals and humans are probably caused by their mutagenic ability (Knasmuller et al., 1998; Baudouin et al., 2002; Hooda, 2007). Potential threat is that heavy metals are not degradable and without intervention stay in soil for centuries. The cleanup of most of the contaminated sites is mandatory in order to reclaim the area and to minimize the entry of toxic elements into the food chain. Various engineering – based methods such as soil excavation, soil washing or burning or pump and treat systems are already being used to remediate metal contaminated soils ((Hooda, 2007). As a result over recent decades an annual worldwide release of heavy metals reached 22,000 t (metric ton) for cadmium, 939,000 t for copper, 783,000 t for lead and 1,350,000 t for zinc (Singh et al., 2003).

The cost of cleaning up contaminated sites is extremely high. In the USA alone, U$ 6–8 billion is annually spent in remediation efforts, with global costs in the range of U$ 25–50 billion (Glass, 1999; Tsao, 2003). Engineering methods for the remediation of contaminated sites include excavation, transport, soil washing, extraction, pumping and treating of contaminated water, addition of reactants such as hydrogen peroxide or potassium permanganate, and incineration. A serious consequence of the high cost of remediation technologies is that polluted commercial properties are often abandoned rather than cleaned up. There are over 500,000 of these so-called brownfields in the USA.

Elemental pollutants are particularly difficult to remediate from soil, water, and air because, unlike organic pollutants that can be degraded to harmless small molecules, toxic elements

such as mercury, arsenic, cadmium, lead, copper, and zinc, are immutable by all biochemical reactions and hence remain in the ecosystem (Kramer & Chardonnens, 2001). The heavy metals remainings in various ecosystems would seep into surface water, groundwater or even channel into the food chain by crops growing on such a soil (Lin et al., 1998). These heavy metals may adversely affect the soil ecosystem safety, not only agricultural product and water quality, but also the human health (Zhou et al., 2004).

Another popular clean-up method involves augmented bioremediation with the addition of specific microbial strains known to degrade the pollutant. Bacteria and fungi collectively can utilize a vast range of organic molecules. But for bioremediation using microbes at a particular site to be successful, many conditions must be met. These include the ability of the microbes with the desired metabolic activity to survive in that environment, the accessibility or bioavailability of the chemical, and the presence of inducers to activate expression of the necessary enzymes. Many organic pollutants are recalcitrant to degradation and cannot be used as sole carbon source (Doty, 2008). The pollutants are sometimes metabolized by enzymes with other natural substrates; therefore, these substrates sometimes need to be present in order for the genes to be expressed. This requirement is problematic if the inducing chemical is itself a harmful pollutant, such as phenol. Bioremediation also depends on the presence of sufficient carbon and energy sources. Often, thousands of gallons of a food source such as molasses must be pumped down into the site to allow bacterial growth (Doty, 2008).

The use of microorganisms in engineered bioremediation systems has had mixed success. A review of this broad and active field is beyond the scope of this review; a recent book provides an excellent overview of bioremediation of xenobiotics, petroleum, BTEX (benzene, toluene, ethylbenzene, and xylene), explosives, and heavy metals (Fingerman & Nagabhushanum, 2005; Doty, 2008).

Plants are autotrophic organisms capable of using sunlight and carbon dioxide as sources of energy and carbon. However, plants rely on the root system to take up water and other nutrients, such as nitrogen and minerals, from soil and groundwater. As a side effect, plants also absorb a diversity of natural and man-made toxic compounds for which they have developed diverse detoxification mechanisms (Eapen et al., 2007; Van Aken, 2008).

Pollutant-degrading enzymes in plants probably originate from natural defense systems against the variety of allelochemicals released by competing organisms, including microbes, insects and other plants (Singer, 2006; Van Aken, 2008). From this viewpoint, plants can be seen as natural, solar-powered pump-and-treat systems for cleaning up contaminated environments, leading to the concept of phytoremediation (Pilon-Smits, 2005; Van Aken, 2008).

First developed for the removal of heavy metals from soil, the technology has since proven to be efficient for the treatment of organic compounds, including chlorinated solvents, polyaromatic hydrocarbons and explosives (Pilon-Smits, 2005; Salt et al., 1998; Van Aken, 2008). Beyond the removal of contaminants from soil, phytoremediation involves different processes, such as enzymatic degradation, that potentially lead to contaminant detoxification (Pilon-Smits, 2005; Dietz & Schnoor, 2001; Van Aken, 2008). However, despite great promise, rather slow removal rates and potential accumulation of toxic compounds within plants might have limited the application of phytoremediation (Eapen et al., 2007; Van Aken, 2008).

Unlike organic contaminants, metals cannot be degraded. Instead, phytoremediation strategies for metals are based on stabilization, accumulation, and in some cases on

volatilization. The phytostabilization of metals may simply involve the prevention of leaching through the upward water flow created by plant transpiration, reduced runoff due to above-ground vegetation, and reduced soil erosion via the stabilization of soil by plant roots (Vassilev et al., 2004; Martínez et al., 2006).

Phytoremediation, as a cost-effective and environmentally friendly method, is an emerging technology based on the use of plants to remove, transform, clean up or stabilize contaminants including organic pollutants located in water, sediments, or soils (Cunningham et al., 1997; Cherian & Oliveira, 2005; Mello-Farias & Chaves, 2008). This method has attracted growing attention because of its distinctive potential and advantages compared with conventional technologies, such as soil replacement, solidification, and washing strategies (Yang et al., 2005; Mello-Farias & Chaves, 2008). The advantages of phytoremediation over usual bioremediation by microorganisms are that plants, as autotrophic systems with large biomass, require only modest nutrient input and they prevent the spreading of contaminants through water and wind erosion (Pulford & Watson, 2003; Cherian & Oliveira, 2005; Mello-Farias & Chaves, 2008). Plants also supply nutrients for rhizosphere bacteria, allowing the growth and maintenance of a microbial community for further contaminant detoxification (Cherian & Oliveira, 2005; Mello-Farias & Chaves, 2008). Phytoremediation takes advantage of the unique, selective and naturally occurring uptake capabilities of plant root systems, together with the translocation, bioaccumulation and pollutant storage/degradation abilities of the entire plant body. Besides being aesthetically pleasing, phytoremediation is on average tenfold cheaper than other physical, chemical or thermal remediation methods since it is performed *in situ*, is solar driven and can function with minimal maintenance once established (Hooda, 2007).

According to Sarma (2011), there are different strategies of phytoremediation, each having a different mechanism of action for remediating metal-polluted soil, sediment or water, like: 1) Phytoextraction: plants absorb metals from soil through the root system and translocate them to harvestable shoots where they accumulate. Hyperaccumulators mostly used this process to extract metals from contaminated site. The recoveries of extracted metals are also possible through harvesting the plants appropriately; 2) Phytovolatilization: plants used to extract certain metals from soil and then release them into the atmosphere by volatilization; 3) Phytostabilization: plant root microbial interaction can immobilize organic and some inorganic contaminants by binding them to soil particles and as a result reduce migration contaminants to grown water; 4) Phytofiltration: plant roots (rhizofiltration) or seedlings (blastofiltration) absorb or adsorb pollutants, mainly metals from water and aqueous waste streams (Prasad & Freitas, 2003)

Many genes are involved in metal uptake, translocation and sequestration; the transfer of any of these genes into candidate plants is a possible strategy for genetic engineering of plants to improve phytoremediation traits. Depending on the strategy, transgenic plants can be engineered to accumulate high concentrations of metals in harvestable parts. Transfer or overexpression of genes will lead to enhanced metal uptake, translocation, sequestration or intracellular targeting. Genetic engineering of plants for synthesis of metal chelators will improve the capability of plant for metal uptake (Karenlampi et al., 2000; Pilon-Smits & Pilon, 2002; Clemens et al., 2002; Eapen & D´Souza, 2005).

The application of powerful genetic and molecular techniques may surely identify a range of gene families that are likely to be involved in transition metal transport. Considerable progress has been made recently in identifying plant genes encoding metal ion transporters

and their homologous in hyperaccumulator plants. Therefore, it is hoped that genetic engineering may offer a powerful new means by which to improve the capacity of plants to remediate environmental pollutants (Yang et al., 2005, Mello-Farias & Chaves, 2008).

2. Types of pollutants

There is a variety of different pollutants, originated, in most cases, by human action. To facilitate the development of studies on decontamination techniques and also according the different physical and chemical characteristics they present, the different types of contaminants were divided into two major classes: organic and inorganic. These two groups are further subdivided. Organic pollutants include various compounds such as polychlorinatedbiphenyls (PCB's), polycyclicaromatichydrocarbons (PAH's), nitroaromatic (explosives), halogenated hydrocarbons, chlorinated solvents. When compared to inorganic, the organic pollutants are relatively less toxic to plants because they are less reactive and do not accumulate readly. Many of these compounds are not only toxic or teratogenic, but also carcinogenic.

The inorganic contaminants include heavy metals, such as mercury, lead, cadmium, among others; and non-metallic compounds like arsenic and radionuclides like uranium, cesium, chromium, strontium, technetium, tritium, etc. Many metals are essential to growth and development of living forms. However, when in high concentrations, they become extremely toxic, leading the organism to oxidative stress with great production of harmful free radicals, highly dangerous to cells and tissues. Some particularly reactive metals interfere in the structure and function of proteins, and also cause the substitution of other essential nutrients (Garbisuet al. 2002; Pulfort; Watson, 2003; Taiz & Zeiger, 2002).

Many elemental pollutants penetrate the plant through regular systems of nutrient absorption. The plants protect themselves from these xenobiotics through degradation of endogenous toxic organic or sequestering them in the vacuoles (Meagher, 2000).

Different technologies of phytoremediation are compatible with a great number of pollutants. Constructed wetlands have been applied for many inorganics, including metals, nitrates, phosphates, cyanides, as well as organics such as explosives and herbicides (Horne 2000; Schnoor et al., 1995; Jacobson et al, 2003).

There is a special category of plants called hyperaccumulators (described later), for they accumulate a considerable amount of toxic metals and radionucleides in their tissues (phytoextraction), keeping these compounds above the ground surface. This is the main goal of phytoremediation.

2.1 Inorganics

The absorption of any metal by plants depends on the metal relative bioavailability in the contaminated array. Changes in the soil chemistry, such as decreased pH, may increase the availability of many metals for the absorption by the roots. Many plants can absorb significant levels of metals in some soil conditions. Changes in rhizosphere microbial status (e.g.: presence of mycorrhizae) can also have profound effects (positive or negative) on the uptake of metals by the roots (Smith, 1996). The general consensus of researchers in this area, however, is that phytoremediation, especially for heavy metals, will only be economically viable through the use of hyperaccumulators. The research in the past two decades has shown that certain specialized plants have the ability to accumulate more than 3% (dry weight) of heavy metals and over 25% (dry weight) in sap / latex with no apparent

damage to the plant (Baker & Brooks, 1989, Baker et al. 1994; Huang & Cunningham, 1996). The mechanisms that govern this tolerance and absorption of excessive concentrations of metals in leaves were the subject of active research and vary according to the element (Cunningham & Lee, 1995; Huang & Cunningham, 1996). The mechanisms of tolerance include the accumulation of Zn in cell walls; Ni associated with the pectin in large cells; Ni, Co and Zn being chelated by malic acid; phytochelatin associated to Zn, Ni chelation by citrate; and Co associated with oxalate crystals calcium in plant tissues. Knowledge of the mechanisms of tolerance will aid in identifying the genetic characteristics necessary for the transfer of metal tolerance of plants capable of producing greater biomass with deeper rooting. It was suggested that in some cases, the resulting biomass rich in metals (biominery) could be incinerated and have metals economically recycled. This "biomineration" of metals can also be applied as a mining technique for metals with significant economic value (Robinson et al., 1998).

Another type of inorganic compounds that may be susceptible to phytoremediation are radionucleides. The presence of radionucleides in soil and water poses serious risk to human health. These contaminants come from the explosion of atomic bombs or nuclear power plant accidents such as Chernobyl, Ukraine and, more recently in Fukushima, Japan. The selection of an appropriate cleaning technology of these contaminated areas is based on the environmental chemistry of each element, character of deposition and rate of radioactive decay. A variety of physicochemical methods are available, like soil washing, ion exchange, leaching with chelating agents, flocculation and osmosis-ultrafiltration. Recently there has been increasing interest in the use of biological methods to remove radionucleides (Duschenkov, 2003). Negri and Hinchman (2000) reported data in the use of plants for the treatment of ^{3}H, U, Pu, ^{137}Cs and ^{90}Sr.

2.2 Organics

More recently, with the development of the pesticide industry, the metabolic capacity of the plant system began to be assessed. The most modern herbicides are based on the selectivity of crops due to metabolic differences between species of plants. This capability, often created by man, is the cornerstone of the highly profitable market of herbicides. "Desirable" plants rapidly metabolize the herbicide compound in a nontoxic one, while "undesirable" herbs do not, and are therefore dead. This mechanism developed by the natural selection of plants, proves to be potentially exploitable in the remediation of contaminated soils.

This ability of plants to detoxify xenobiotics is widely recognized and with current utility. Besides, plants generally have a metabolic system with differences in the efficiency of degradation of toxic compounds when compared to microorganisms, what makes the union of these two distinct systems in the rhizosphere, an ideal situation for a more efficient phytoremediation. Recent research includes plant selection, alternative patterns of rooting, the composition of exudates produced by the plant and its effect on microbial communities, exudation of specific compounds inducing specific metabolic pathways and, inoculation with rhizosphere microorganisms capable of degrading xenobiotics efficiently (Langenbach, 1994). The plants and their roots can create an environment in the soil which is rich in microbial activity, able to change the availability of organic contaminants or increase the degradation of certain organic compounds, such as hydrocarbons derived from Petroleum.

Siciliano et al. (2003) evaluated the impact of microbial remediation on soil mass and the capacity of microbial community to degrade hydrocarbons in order to determine whether phytoremediation treatments increase the metabolic potential of microbial community by

altering its taxonomic structure. It was found that the best remediation system to reduce hydrocarbons in the soil was obtained by increasing the population of bacteria containing genes for the catabolism of hydrocarbons in the rhizosphere community, thus demonstrating the importance of using microorganisms in phytoremediation. However, it is necessary to identify the species of suitable plants that can beneficially alter microbial diversity for soil remediation. According to Pires et al. (2003), the absorption of herbicides by plants is affected by the compound's chemical properties, environmental conditions and the characteristics of plant species. Actually, the probability of a plant being phytoremediator depends on the type of pollutant; plants should be tested to detect that one with the greater resistance to a specific pollutant. Esteve-Nunez et al. (2001) evaluated trinitrotoluene (TNT), and found that its chemical structure influences its biodegradability. According to these authors, the oxygenated metabolism for aromatic compounds by bacteria does not occur in TNT because of its chemical properties generating compounds not metabolized by microorganisms. However, anaerobic processes have advantages because of the absence of oxygen. Therefore the use of fungi for the bioremediation of TNT has generated considerable interest. Esteve-Nunez et al. (2001) concluded that the remediation of TNT by these organisms is a very valid process; and the rhizoremediation by microbes able to colonize the rhizosphere of plants, will provide a fast and efficient mechanism for the removal of this pollutant. Figure 1 shows some types of organic pollutants.

There are other types of contaminants called Persistent Organic Pollutants (POPs) that resist long in the soil. Some examples are Dichlorodiphenyltrichloroethane (DDT), Polychlorinated biphenyls (PCB), Dioxins, etc. Research has shown that a variety of plants can remove persistent compounds, transporting them to aerial plant tissues (Coutinho & Barbosa, 2007). It is important to highlight that, due to variety of contaminants, the study of pollutant is very important to generate an effective phytoremediation.

Most current methods of cleaning metals and volatile organic compounds not on the soil surface are coarse, expensive and physically destructive (Baker et al., 1994). The remediation by conventional methods of engineering often costs 50 to 500 dollars per ton of soil, and certain specialized techniques can cost up to US$ 1000 (Cunningham & Ow, 1996). Phytoremediation associated with biotechnology is an emerging technology that promises a viable remediation when pollutants: a) are close to the surface, b) are relatively non-leachable, and c) have little immediate risk to the environment (Cunningham & Lee, 1995). The results are more effective in slightly or moderately polluted areas. For heavy contamination, the decontamination time is too long (Robinson et al., 1998). The combination of metal hyperaccumulation and degradation or, increased sequestration of organic compounds with greater biomass and deeper rooting systems can result in a powerful technology of phytoremediation that will provide cheaper, permanent and intrusive remediation. Table 1 shows a summary of the techniques applied to the different types of phytoremediated compounds.

3. Hyperaccumulators

Over recent years a special interest has emerged in the phenomenon of heavy-metal hyperaccumulation since this property may be exploited in the remediation of heavy-metal-polluted soils through phytoextraction and phytomining (Robinson et al., 1997; Martínez et al., 2006).

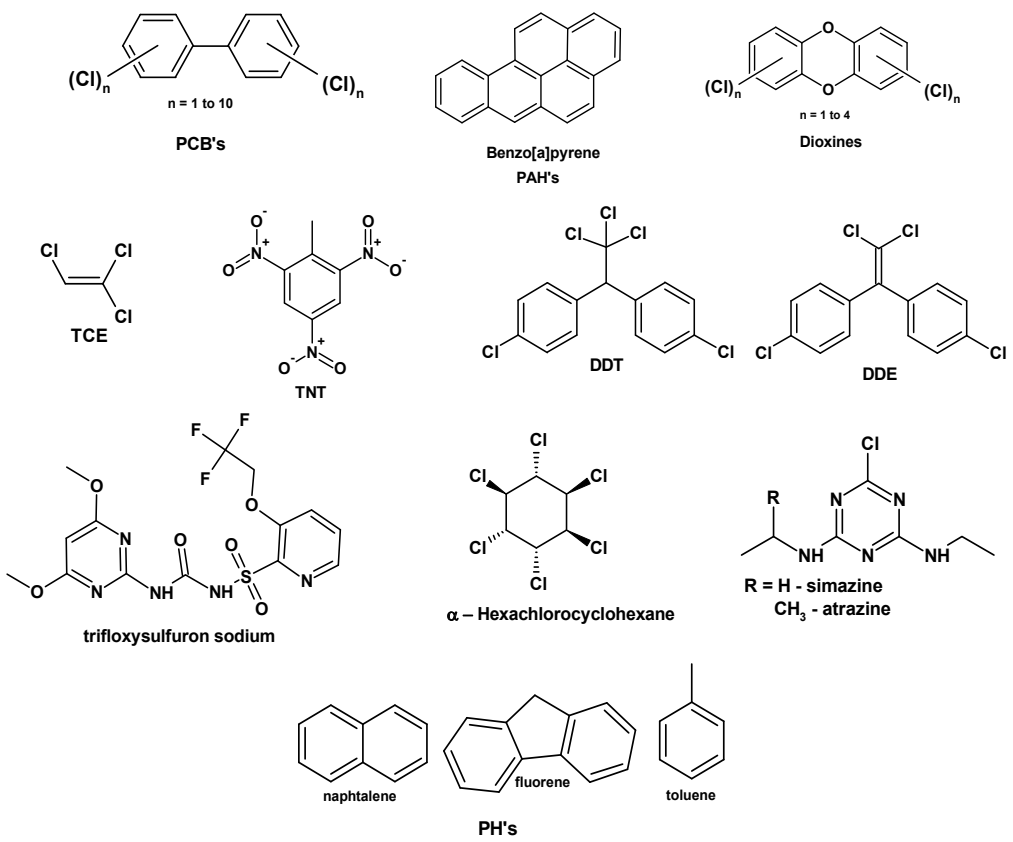

Fig. 1. Different types of organic pollutants

Type of phytoremediation	Chemicals Treated
Phytoaccumulation/extraction	Cd, Cr, Pb, Ni, Zn, radionuclides, BTEX*, penachlorophenol, short chained aliphatic compounds
Phytodegradation/transformation	Nitrobenzene, nitroethane, nitrotoluene, atrazine, chlorinated solvents (chloroform, carbon tetrachloride, etc)
Phytostabilization	Heavy metals in ponds, phenols and chlorinated solvents
Phytostimulation	Polycyclicaromatic hydrocarbon, BTEX, PCB#, tetrachloroethane
Phytovolatilization	Chlorinated solvents, Hg, Se.
Phytofiltration	Heavy metals, organics and radionucleides.

*BTEX = benzene, toluene, ethyl benzene, xylenes;
#PCB = Polychlorinated biphenyl

Table 1. Outline of phytoremediated chemicals (Nwoko, 2010).

The concept of hyperaccumulation was originally introduced to plants containing more than 0.1% (1000 $mg.kg^{-1}$) of Ni in dried plant tissues (Jaffré et al., 1976). At present, the criteria used for hyperaccumulation vary per metal, ranging from 100 $mg.kg^{-1}$ dry mass for Cd, to 1000 $mg.kg^{-1}$ for Cu, Co, Cr, and Pb, to 10000 $mg.kg^{-1}$ for Zn and Mn. These values exhibit a shoot-to-soil metal concentration ratio, the so-called bioaccumulation factor that is higher than 1 (Baker et al., 1994).
An ideal plant for environmental cleanup should have a high biomass production, combined with superior capacity for pollutant tolerance, accumulation, and degradation, depending on the type of pollutant and the phytoremediation technology of choice. Hyperaccumulators are good candidates in phytoremediation, particularly for the removal of heavy metals. Phytoremediation efficiency of plants can be substantially improved using genetic engineering technologies (Cherian & Oliveira, 2005; Mello-Farias & Chaves, 2008).
Transferring the genes responsible for the hyperaccumulating phenotype to higher shoot-biomass-producing plants has been suggested as a potential avenue for enhancing phytoremediation as a viable commercial technology (Pilon-Smits & Pilon, 2002; Martínez et al., 2006).
Some of the plants belonging to *Brassicaceae* such as *Alyssum* species, *Thlaspi* species and *Brassica juncea*, *Violaceae* such as *Viola calaminaria*, *Leguminosae* such as *Astragalus racemosus* are known to take up high concentrations of heavy metals and radionucleides (Reeves & Baker, 2000; Negri & Hinchman, 2000, Eapen & D´Souza, 2005).
To date, there are approximately 400 known hyperaccumulators metal in the world (Reeves & Baker, 2000; Eapen & D´Souza, 2005) and the number is increasing. However, the remediation potential of many of these plants is limited because of their slow growth and low biomass (Chaney et al., 2000; Lasat, 2002; McGrath et al., 2002; Eapen & D´Souza, 2005).

4. Cellular and molecular mechanisms involved in phytoremediation

Exposure to pollutants may cause a series of symptoms in plants. Pollutant action can result in inhibition of cellular activity or rupture of cell structure, due to possible damages of essential components (Coutinho & Barbosa, 2005). Plants show some potential cellular and molecular mechanisms and strategies, which can be involved in detoxification of organic and inorganic pollutants such as herbicides, explosives and heavy metals. These mechanisms can be related to the cell wall composition and root environment, plasma membrane properties and integrity, enzymatic transformation, complexation with ligands and vacuolar compartmentalization (Hall, 2002; Cherian & Oliveira, 2005). Depending on the nature of pollutant (organic or inorganic) plant cells can use one or some of these systems of remediation (Coutinho & Barbosa, 2005; Cherian & Oliveira, 2005; Mello-Farias & Chaves, 2008).

4.1 Cell wall composition and rhizosphere

Organic contaminants, when in contact with roots, may be sorbed to the root structure. The hydrophobic or hydrophilic nature of the organic compounds also determines their possible uptake. Hemicellulose in the cell wall and the lipid bilayer of plant membranes can bind hydrophobic organic pollutants effectively (Pilon-Smits, 2005). In addition, the root uptake of chemicals depends on many factors as plant's uptake efficiency, the transpiration rate, and the chemical concentration in soil water. Further, organic pollutants can be degraded or mineralized by plants, either independently or in association with microorganisms. For example, organics like polycyclic aromatic hydrocarbons (PAHs), polychlorinated

biphenyls, and petroleum hydrocarbons are sufficiently degraded by rhizospheric microbial activity (Olson et al., 2003). Plants have significant metabolic activity in both roots and shoots, and some of the enzymes involved in these metabolic processes (e. g. nitroreductases, dehalogenases, laccases, peroxidases, etc.) are useful in the remediation process (Schnoor et al. 1995; Wolfe & Hoehamer, 2003).
Even though mycorrhizas and ectomycorrhizas are not considered in general reviews of plant metal tolerance mechanisms, they can ameliorate the effects of metal toxicity on the host plant. However, the mechanism involved in conferring this increase of tolerance is not yet well explained; they may be quite diverse and show considerable species and metal specificity since large differences in response to metals have been observed, both between fungal species and to different metals (specially Zn, Cu and Cd) within species (Hall, 2002).
Finally, cell walls may play an important role in detoxifying metals in plant cells of the Ni and Zn/Cd hyperaccumulating plant species. About 60–70% of Ni and/or Zn accumulated is distributed in the apoplast cell walls (Krämer et al., 2000; Li et al., 2005; Yang et al., 2005b). However, molecular bases of metal detoxification by cell walls are not well understood (Yang et al., 2005b).

4.2 Plasma membrane properties and integrity

Although there is no direct evidence for a role for plasma membrane efflux transporters in heavy metal tolerance in plants, recent research has revealed that plants possess several classes of metal transporters that must be involved in metal uptake and homeostasis in general and, thus, could play a key role in tolerance (Yang et al., 2005a). Transport proteins and intracellular high-affinity binding sites mediate the uptake of metals across the plasma membrane. A comprehensive understanding of the metal transport processes in plants is essential for formulating effective strategies to develop genetically engineered plants that can accumulate specific metals (Yang et al., 2005b).
Several classes of proteins have been implicated in heavy metal transport in plants. These include the heavy metal (or CPx-type) ATPases that are involved in the overall metal-ion homeostasis and tolerance in plants, the natural resistance-associated macrophage protein (Nramp) family of proteins, and the cation diffusion facilitator (CDF) family proteins (Williams et al., 2000), zinc–iron permease (ZIP) family proteins, etc. (Yang et al., 2005a; 2005b).
CPx-type heavy metal ATPases have been identified in a wide range of organisms and have been implicated in the transport of essential as well as potentially toxic metals like Cu, Zn, Cd, Pb across cell membranes (Williams et al., 2000; Yang et al., 2005a; 2005b). These transporters use ATP to pump a variety of charged substrates across cell membranes and are distinguished by the formation of a charged intermediate during the reaction cycle (Yang et al., 2005a; 2005b).
Transport of metals and alkali cations across plant plasma membrane and organellar membranes is essential for plant growth, development, signal transduction, and toxic metal phytoremediation (Cherian and Oliveira, 2005).
Another factor concerning plasma membrane seems to be the maintenance of its physical integrity in presence of heavy metals, in order to prevent or reduce their entry in the cell, besides the efflux mechanisms described above (Hall, 2002; Coutinho & Barbosa, 2005).

4.3 Enzimatic transformation

Enzimatic transformation in plants concerns mainly organic pollutants and it can be considered a case of phytotransformation. In this process plants uptake organic pollutants and, subsequently, metabolize or transform them into less toxic metabolites. Once taken up

and translocated the organic chemicals generally undergo three transformation stages: (a) chemical modification (oxidations, reductions, hydrolysis); (b) conjugation (with glutathione, sugars, amino acids); and (c) sequestration or compartmentalization (conjugants are converted to other conjugates and deposited in plant vacuoles or bound to the cell wall and lignin) (Ohkawa et al., 1999; Cherian and Oliveira, 2005).
Plant enzymes that typically catalyze the first phase of the reactions are P450 monoxygenases and carboxylesterases (Coleman et al., 1997; Burken, 2003). The second phase involves conjugation to glutathione (GSH), glucose, or amino acids, resulting in soluble, polar compounds (Marrs, 1996). For instance, detoxification of herbicides in plants is attributed to conjugation with glutathione catalyzed by glutathione S-transferase (GST) (Lamoureux et al., 1991). It was also reported that a group of GSTs mediate conjugation of organics to GSH in the cytosol (Kreuz et al., 1996; Neuefeind et al., 1997). Sometimes organic pollutants, such as atrazine and TNT, are partially degraded and stored in vacuoles as bound residues (Burken & Schnoor, 1997). The third phase of plant metabolism is compartmentalization and storage of soluble metabolites either in vacuoles or in the cell wall matrix. The glutathione S-conjugates are actively transported to the vacuole or apoplast by ATP-dependent membrane pumps (Martinoia et al., 1993). Also, an alternate conjugation-sequestration mechanism for organics exists in plants and involves coupling of a glucose or malonyl group to the organic compound, followed by the transport of the conjugate to the vacuole or the apoplast (Coleman et al., 1997).
Mechanisms as complexation whit ligands and vacuolar compartmentalization are described below.

4.4 Complexation with ligands

Complexation with ligands is a process associated to heavy metal pollutants, and it can be an extracellular or an intracellular molecular event. These ligands can be chelators as organic acids or peptides such phytochelatins (PCs), methallothioneins (MTs) or glutathione (GSH) (Mello-Farias & Chaves, 2008).
Plant tolerance to heavy metals depends largely on plant efficiency in the uptake, translocation, and further sequestration of heavy metals in specialized tissues or in trichomes and organelles such as vacuoles. The uptake of metals depends on their bioavailability, and plants have evolved mechanisms to make micronutrients bioavailable (Cherian and Oliveira, 2005). Chelators such as siderophores, organic acids, and phenolics can help release metal cations from soil particles, increasing their bioavailability. For example, organic acids (malate, citrate) excreted by plants act as metal chelators. By lowering the pH around the root, organic acids increase the bioavailability of metal cations (Ross, 1994). However, organic acids may also inhibit metal uptake by forming a complex with the metal outside the root. Citrate inhibition of Al uptake resulting in aluminum tolerance in several plant species is an example of this mechanism (De la Fuente et al., 1997; Pineros & Kochian, 2001; Papernik et al. 2001). Copper tolerance in Arabidopsis is also the result of a similar mechanism (Murphy et al., 1999).
Intracellular complexation involves peptide ligands, such as metallothioneins (MTs) and phytochelatins (PCs) (Yang et al., 2005b). Chelation of metals in the cytosol by high-affinity ligands is potentially a very important mechanism of heavy-metal detoxification and tolerance (Hall, 2002).
Metallothioneins (MTs) are cysteine-rich proteins that have high affinity to cations such as Cd, Cu, and Zn (Cobbet & Goldsbrough, 2002; Singh et al., 2003; Cherian & Oliveira, 2005).

They confer heavy-metal tolerance and accumulation in yeast. Overexpression of genes involved in the synthesis of metal chelators may lead to enhanced or reduced metal uptake and enhanced metal translocation or sequestration, depending on the type of chelator and on its role and location (Cherian & Oliveira, 2005; Pilon-Smits, 2005). MT proteins were originally isolated as Cu, Cd and Zn binding proteins in mammals. There is now good evidence that four categories of these proteins occur in plants, which are encoded by at least seven genes in *Arabidopsis thaliana* (Cobbett & Goldsbrough, 2002; Hall, 2002; Gratão et al., 2005).

The biosynthesis of MTs is regulated at the transcriptional level and is induced by several factors, such as hormones, cytotoxic agents, and metals, including Cd, Zn, Hg, Cu, Au, Ag, Co, Ni, and Bi (*Yang et al.*, 2005a).

Phytochelatins are a class of post-translationally synthesized (cysteine-rich metal-chelating) peptides that play a pivotal role in heavy-metal tolerance in plants and fungi by chelating these substances and decreasing their free concentrations (Vatamaniuk et al., 1999). PCs have been most widely studied in plants, particularly in relation to Cd tolerance (Cobbett, 2000; Goldsbrough, 2000). PCs consist of only three amino acids, glutamine (Glu), cysteine (Cys), and glycine (Gly). They are structurally related to the tripeptide glutathione (GSH), and are enzymatically synthesized from GSH. PCs form a family of structures with increasing repetitions of the -Glu-Cys dipeptide followed by a terminal Gly, (-Glu-Cys)*n*-Gly, where *n* is generally in the range of 2–5, but can be as high as 11 (Cobbett, 2000; Yang et al., 2005b).

Many plants cope with the higher levels of heavy metals by binding them to PCs and sequestering the complexes inside their cells (Yang et al., 2005a). As mentioned above, PCs are synthesized non-translationally, using glutathione as a substrate by PC synthase, an enzyme that is activated in the presence of metal ions (Cobbett, 2000). So, PCs are structurally related to glutathione (GSH; γ-GluCysGly), and numerous physiological, biochemical, and genetic studies have confirmed that GSH (or, in some cases, related compounds) is the substrate for PC biosynthesis (Cobbett, 2000; Cobbett and Goldsbrough, 2002).

Although PCs clearly can have an important role in metal detoxification, alternative primary roles of PCs in plant physiology have also been proposed. These have included roles in essential metal ion homeostasis and in Fe or sulphur metabolism (Sanita di Toppi & Gabbrielli, 1999; Cobbett and Goldsbrough, 2002). However, there is currently no direct evidence that PCs have functions outside of metal detoxification.

Because of MTs and PCs peptidic nature and because they bind metals in thiolate complexes, these peptide molecules demand a greater input of amino acids (especially cysteine), sulfur and nitrogen from the plant as the level of accumulated metals rise. Their synthesis is energy expensive and requires significant amounts of the growth limiting elements sulfur and nitrogen. Increased synthesis might thus at some point affect plant growth and therefore limit their use as phytoremediators (Tong et al., 2004).

4.5 Vacuolar compartmentalization

The vacuole is generally considered to be the main storage site for metals in yeast and plant cells and there is evidence that phytochelatin–metal complexes are pumped into the vacuole in fission yeast (*Schizosaccharomyces pombe*) and in plants (Tong et al., 2004; Yang et al., 2005b). Compartmentalization of metals in the vacuole is also part of the tolerance mechanism of some metal hyperaccumulators. The Ni hyperaccumulator *Thlaspi goesingense*

enhances its Ni tolerance by compartmentalizing most of the intracellular leaf Ni into the vacuole (Krämer et al., 2000; Tong et al., 2004). High-level expression of a vacuolar metal ion transporter TgMTP1 in *T. goesingense* was proposed to account for the enhanced ability to accumulate metal ions within shoot vacuoles (Persans et al., 2001; Tong et al., 2004; Yang et al., 2005b).

5. Genetically engineered plants for phytoremediation

The genetic and biochemical basis is becoming an interesting target for genetic engineering, because the knowledge of molecular genetics model organisms can enhance the understanding of the essencial metal metabolism components in plants.A fundamental understanding of both uptake and translocation processes in normal plants and metal hyperaccumulators, the regulatory control of these activities, and the use of tissue specific promoters offer great promise that the use of molecular biology tools can give scientists the ability to develop effective and economic phytoremediation plants for soil metals (Chaney et al., 1997; Fulekar et al., 2008). Plants such as *Populus angustifolia, Nicotiana tabacum* or *Silene cucubalis* have been genetically engineered to overexpress glutamylcysteine syntlietase, and thereby provide enhanced heavy metal accumulation as compared with a corresponding wild type plant (Fulekar et al., 2008).

Candidate plants for genetic engineering for phytoremediation should be a high biomass plant with either short or long duration (trees), which should have inherent capability for phytoremediation. The candidate plants should be amicable for genetic transformation. Some of high biomass hyperaccumulators for which regeneration protocols are already developed include Indian mustard (*Brassica juncea*), sunflower (*Helianthus annuus*), tomato (*Lycopersicon esculentum*) and yellow poplar (*Liriodendron tulipifera*) (Eapen & D'Souza, 2005; Mello-Farias & Chaves, 2008).

The application of powerful genetic and molecular techniques may surely identify a range of gene families that are likely to be involved in transition metal transport. Considerable progress has been made recently in identifying plant genes encoding metal ion transporters and their homologous in hyperaccumulator plants. Therefore, it is hoped that genetic engineering may offer a powerful new means by which to improve the capacity of plants to remediate environmental pollutants (Yang et al., 2005a; Mello-Farias & Chaves, 2008).

Brassica juncea was genetically engineered to investigate rate-limiting factors for glutathione and phytochelatin production. To achieve this, *Escherichia coli* gshl gene was introduced. The γ-ECS transgenic seedlings showed increased tolerance to cadmium and had higher concentrations of phytochelatins, γ-GluCys, glutathione, and total nonprotein thiols compared to wild type seedlings (Ow, 1996; Fulekar et al., 2008). Study showed that c-glutamylcysteine synthetase inhibitor, L-buthionine-[S,R]-sulphoximine (BSO), dramatically increases As sensitivity, both in non-adapted and As-hypertolerant plants, showing that phytochelatin-based sequestration is essential for both normal constitutive tolerance and adaptative hypertolerance to this metalloid (Schat et al., 2002; Fulekar et al., 2008).

Some genes have been isolated and introduced into plants with increased heavy metal (Cd) resistance and uptake, like *AtNramps* (Thomine *et al*., 2000), *AtPcrs* (Song et al., 2004), and *CAD1* (Ha et al., 1999) from *Arabidopsis thaliana*, library enriched in Cd-induced cDNAs from *Datura innoxia* (Louie et al., 2003), *gshI*, *gshII* (Zhu et al., 1999a) and PCS cDNA clone (Heiss et al., 2003) from *Brassica juncea*.

There are some examples of transgenic plants for metal tolerance/phytoremediation, as tobacco with accumulation of Cd, Ca and Mn transformed with gene *CAX-2* (vacuolar

transporters) from *A. thaliana* (Hirschi et al., 2000); *A. thaliana* tolerant to Al, Cu, and Na with gene *Glutathione-S-transferase* from tobacco (Ezaki et al., 2000); tobacco with Ni tolerance and Pb accumulation with gene *Nt CBP4* from tobacco (Arazi et al., 1999); tobacco (Goto et al., 1998) and rice (Goto et al., 1998; 1999) with increased iron accumulation with gene *Ferretin* from soybean; *A. thaliana* and tobacco resistant to Hg with gene *merA* from bacteria (Rugh et al., 2000; Bizily et al., 2000; Eapen & D'Souza, 2005); indian mustard tolerant to Se transformed with a bacterial glutathione reductase in the cytoplasm and also in the chloroplast (D´Souza et al., 2000); transgenic *A. thaliana* plants expressing SRSIp/ArsC and ACT 2p/γ-ECS together showed high tolerance to As, these plants accumulated 4- to 17-fold greater fresh shoot weight and accumulated 2- to 3-fold more arsenic per gram of tissue than wild plants or transgenic plants expressing γ-ECS or ArsC alone (Dhankher et al., 2002; Mello-Farias & Chaves, 2008).

Even though there is a variety of different metal tolerance mechanisms, and there are many reports of transgenic plants with increased metal tolerance and accumulation, most, if not all, transgenic plants created to date rely on overexpressing genes involved in the biosynthesis pathways of metal-binding proteins and peptides (Zhu et al., 1999b; Mejäre & Bülow, 2001; Bennett et al., 2003; Gisbert et al., 2003), genes that can convert a toxic ion into a less toxic or easier to handle form, or a combination of both (Dhankher et al., 2002; Yang et al., 2005b; Mello-Farias & Chaves, 2008).

At least three different engineering approaches to enhanced metal uptake can be envisioned (Clemens et al., 2002), which include enhancing the number of uptake sites, alteration of specificity of uptake system to reduce competition by unwanted cations and increasing intracellular binding sites. Each metal has specific molecular mechanism for uptake, transport and sequestration (Eapen & D'Souza, 2005; Mello-Farias & Chaves, 2008).

New metabolic pathways can be introduced into plants for hyperaccumulation or phytovolatilization as in case of *MerA* and *MerB* genes which were introduced into plants which resulted in plants being several fold tolerant to Hg and volatilized elemental mercury (Bizily et al., 2000; Dhankher et al., 2002; Eapen & D'Souza, 2005) developed transgenic *Arabidopsis* plants which could transport oxyanion arsenate to aboveground, reduce to arsenite and sequester it to thiol peptide complexes by transfer of *Escherichia coli ars C* and *γ-ECS* genes (Eapen & D'Souza, 2005).

Alteration of oxidative stress related enzymes may also result in altered metal tolerance as in the case of enhanced Al tolerance by overexpression of glutathione-S-transferase and peroxidase (Ezaki et al., 2000; Eapen & D'Souza, 2004). Overexpression of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase led to an enhanced accumulation of a variety of metals (Grichko et al., 2000; Eapen & D'Souza, 2005).

According to Eapen & D'Souza (2005), it is essential to have plants with highly branched root systems with large surface area for efficient uptake of toxic metals. Experiments had shown that *Agrobacterium rhizogenes* could enhance the root biomass in some hyperaccumulator plants (Eapen, unpublished work). The hairy roots induced in some of the hyperaccumulators were shown to have high efficiency for rhizofiltration of radionuclide (Eapen et al., 2003) and heavy metals (Nedelkoska and Doran, 2000; Eapen et al., unpublished work).

Nowadays there are many different examples of genes that have been used for the development of transgenic plants for metal tolerance and/or phytoremediation, as shown on Table 2.

6. Advantages and disadvantages of phytoremediation

Admittedly, phytoremediation has benefits to restore balance to a stressed environment, but it is important to proceed with caution. Plants enjoy enormous reduction in energy cost and utilization by virtue of deriving energy from solar radiation. The plant tolerates a wide range of environmental conditions.

Gene transferred	Origin	Target plant species	Effect
MT2 gene	Human	Tobacco, oil seed rape	Cd tolerance
MT1 gene	Mouse	Tobacco	Cd tolerance
MTA gene	Pea	A*rabidopsis*	Cu accumulation
CUP-1 gene	Yeast	Cauliflower	Cd accumulation
CUP-1 gene	Yeast	Tobacco	Cu accumulation
γ-Glutamylcysteine synthetase	*E. coli*	Indian mustard	Cd tolerance
Glutathione synthetase	Rice	Indian mustard	Cd tolerance
Cysteine synthetase	Rice	Tobacco	Cd tolerance
CAX-2 (vacuolar transporters)	A*rabidopsis*	Tobacco	Accumulation of Cd, Ca and Mn
At MHX	A*rabidopsis*	Tobacco	Mg and Zn tolerance
Nt CBP4	Tobacco	Tobacco	Ni tolerance and Pb accumulation
FRE-1 and FRE-2	Yeast	Tobacco	More Fe content
Glutathione-s-Transferase	Tobacco	*Arabidopsis*	Al, Cu, Na tolerance
Citrate synthase	Bacteria	*Arabidopsis*	Al tolerance
Nicotinamine amino transferase (NAAT)	Barley	Rice	Grew in iron deficient soils
Ferretin	Soybean	Tobacco	Increased iron accumulation
Ferretin	Soybean	Rice	Increased iron accumulation
Zn transporters ZAT (At MTPI)	*Arabidopsis*	*Arabidopsis*	Zn accumulation
Arsenate reductase γ- glutamylcysteine synthetase	Bacteria	Indian mustard	As tolerance
Znt A-heavy metal transporters	*E. coli*	*Arabidopsis*	Cd and Pb resistance
Selenocysteine methyl transferase	*A. bisculatus*	*A. thaliana*	Resistance to selenite
ATP sulfurylase CAPS		Indian mustard	Se tolerance

Gene transferred	Origin	Target plant species	Effect
Cystathione-gamma synthase (CGS)		Indian mustard	Se volatilization
Glutathione-S-transferase, peroxidase		*Arabidopsis*	Al tolerance
Glutathione reductase		*B. juncea*	Cd accumulator
ACC-deaminase	Bacteria		Many metal tolerance
YCF1	Yeast	*Arabidopsis*	Cd and Pb tolerance
Se-cys lyase	Mouse	*Arabidopsis*	Se tolerance and accumulation
Phytochelatin synthase (Ta PCS)	Wheat	*Nicotiana glauca*	Pb accumulation

Table 2. Selected examples of transgenic plants for metal tolerance/phytoremediation (from Eapen & D'Souza, 2005)

The molecular composition of plants, mainly related to their enzyme and protein profiles, is of great interest to phytoremediation, because this technology can exploit plant molecular and cellular mechanisms of detoxification, through the use of genetic engineering tools.
The nature of plants is still an advantage because they are able to develop, over time, complex mechanisms to absorb nutrients, detoxify pollutants and control the local geochemical conditions. The plants play an important role in regulation water contant in soil avoiding the penetration of liquids by infiltration, which is the main mechanism of entry of contaminants. Plant roots supplement microbial nutrients and provide aeration to the soil, increasing consequently microbial population compared to non-vegetated area. Above all, phytoremediation gives better aesthetic appeal than other physical means of remediation.
On the other hand, phytoremediation has several limitations that require further intensive research on plants and soil conditions. A major disadvantage is that this method of detoxification is too slow or only seasonally effective. Regulatory agencies often require significant progress in remediation to be made in only a few years, making most phytoremediation unsuitable. In many cases, like trichloroethylene and carbon tetrachloride, the concentration of pollutant is not reduced satisfactorily. Besides, in some contaminated sites, the pollutants can reach phytotoxic concentration, making the plant ineffective. For this reason, recent studies have been conducted with the aim of increasing the phytoremediation potential of plants using genetic engineering (Danh et al. 2009). In phytoremediation technology, multiple metal contaminated soil and water require specific metal hyperaccumulator species and therefore, a wide range of research prior to the application. Other factors are also tied to the success of phytoremediation such as the existence of a pollutant in a bio-available form. If the metal is strongly linked to the organic soil it will not be available to the plant. Moreover, the plants are quite specific to certain pollutants. Hyperaccumulators of Cd and Zn (*Thlaspi caerulescens*) can be sensitive to other metals, such as Cu, not allowing the detoxification of polluted areas with different pollutants (Mijovilovich et al., 2009). Despite the current limitations, present day phytoremediation technology is used worldwide and several researchers are working to

overcome these limitations. Table 3 resumes advantages and limitations of some of the sub-process of phytoremediation.

7. Perspectives on biotechnology - based phytoremediation

The environmental contamination by pollutants, organic or inorganic, has great importance due to its impacts on human and animal health. Thus, the most effective and inexpensive technologies to promote detoxification are necessary in the recovery of affected biomes. Great efforts have been made in identifying plant species and their detoxification mechanisms more efficient on those places. The mechanisms of pollutant uptake, accumulation, exclusion, among others, vary according to each plant species and are very important, for they will determine its specific role in phytoremediation.
Plants can have their detoxification capabilities significantly enhanced through the identification of specific genes in certain promising species and the transmission of these to other species, using genetic engineering tools. This can play a significant role in the more effective detoxification of contaminated sites by improving the cost-benefit.

Advantage	Limitation
Phytoextraction	
The plant must be able to produce abundant biomass in short time. e. g.: in a greenhouse experiment, gold was harvested from plants.	Metal hyperaccumulators are generally slow-growing and bioproductivity is rather small and shallow root systems. Phytomass after process must be disposed off properly
Phytostabilization	
It circumvents the removal of soil, low cost and is less disruptive and enhances ecosystem restoration/re-vegetation	Often requires extensive fertilization or soil modification using amendments; long-term maintenance is needed to prevent leaching.
Phytovolatilization	
Contaminant/Pollutant will be transformed into less-toxic forms. e. g.: elemental mercury and dimethyl selenite gas. Atmospheric processes such as photochemical degradation for rapid decontamination/transformation.	The contaminant or a hazardous metabolite might accumulate in plants and be passed on in later products such as fruit or lumber. Low levels of metabolites have been found in plant tissue.
Phytofiltration/rhizofiltration	
It can be either *in situ* (floating rafts on ponds) or *ex situ* (an engineered tanks system); terrestrial or aquatic.	pH of the medium to be monitored continually for optimizing uptake of metals; chemical speciation and interactions of all species in the influent need be understood; functions like a bioreactor and intensive maintenance is needed.

Table 3. Advantages and limitations of some of the phytoremediation sub-processes (Prasad, 2004; Gratão et al. 2005)

Studies on phytoremediation are developed in order to benefit the environment. Several pollutants are bringing some kind of harm to all habitats. Thus, the use of specific techniques already represents hope. The necessary mechanisms are different, however, the organisms, especially plants, have specific ways for the removal, detention or conversion of specific pollutants. The study and subsequent evaluation of the interaction between the soil and its microorganisms, plant and pollutant is very necessary and guiding.
All things considered, more studies must be carried out in this area to better know the phytoremediation capacity of living organisms and their possible use in combating pollution through plant transformation technology.

8. References

Alexander, M. (1999). *Biodegradation and bioremediation*. Ithaca, NY, USA: Academic Press.

Arazi, T.; Sunkar, R.; Kaplan, B. & Fromm, H.A. (1999). Tobacco plasma membrane calmodulin binding transporter confers Ni^{+} tolerance and Pb^{2+} hypersensitivity in transgenic plants. *Plant Journal*, Vol.20, pp. 171-182.

Baker, A.J.M. & Brooks, R.R. (1989). Terrestrial higher plants which hyperaccumulate metallic elements – a review of their distribution, ecology and phytochemistry. *Biorecovery*, Vol.1, pp. 81-126.

Baker, A.J.M.; McGrath, S.P.; Sidoli, C.M.D. & Reeves, R.D. (1994). The possibility of *in situ* heavy metal decontamination of polluted soils using crops of metal accumulating plants. *Resources, Conservation and Recycling*, Vol.11, pp. 42-49.

Baudouin, C.; Charveron, M.; Tarrouse, R. & Gall, Y. (2002). Environmental pollutants and skin cancer. *Cell Biology and Toxicology*, Vol.18, pp. 341-348.

Bennett, L.E.; Burkhead, J.L.; Hale, K.L.; Terry, N.; Pilon, M. & Pilon-Smits, E.A.H. (2003). Analysis of transgenic Indian mustard plants for phytoremediation of metalcontaminated mine tailings. *Journal of Environmental Quality*, Vol.32, pp. 432-440.

Bizily, S.P.; Rugh, C.L. & Meagher, R.B. (2000). Phytodetoxification of hazardous organomercurials by genetically engineered plants. *Nature Biotechnology*, Vol.18, pp. 213-217.

Burken, J.G. & Schnoor, J.L. (1997). Uptake and metabolism of atrazine by poplar trees. *Enviromental Science and Technology*, Vol.31, pp. 1399-1406.

Burken, J.G. (2003). Uptake and metabolism of organic compounds: green-liver models. In: *Phytoremediation*: *Transformation and Control of Contaminants*, McCutcheon, S.C. & Schnoor, J. L. (Ed.), 59-84, Wiley: New York.

Chaney, R.L.; Malik, M.; Li, Y.M.; Brown, S.L.; Angle, J.S. & Baker, A.J.M. (1997). Phytoremediation of soil metals. *Current Opinion in Biotechnology*, Vol.8, pp. 279-284.

Chaney R.L.; Li, Y.M.; Brown, S.L.; Homer, F.A.; Malik, M.; Angle, J.S.; Baker, A.J.M.; Reeves, R.D. & Chin, M. (2000) Improving metal hyperaccumulator wild plants to develop phytoextraction systems: approaches and progress. In: *Phytoremediation of contaminated soil and water*, Terry, N. & Banuelos, G. (Ed.) 129-158, Boca: Lewis Publishers.

Cherian, S. & Oliveira, M.M. (2005). Transgenic Plants in Phytoremediation: Recent Advances and New Possibilities. *Environmental Science & Technology*, Vol.39, No.24, pp. 9377-9390.

Clemens, S.; Palmgren, M.G. & Krämer, U. (2002). A long way ahead: understanding and engineering plant metal accumulation. *Trends in Plant Science*, Vol.7, pp. 309– 314.

Cobbett, C.S. (2000). Phytochelatin biosynthesis and function in heavy-metal detoxification. *Current Opinion in Plant Biology*, Vol.3, pp. 211-216.

Cobbett, C. & Goldsbrough, P. (2002). Phytochelatins and Metallothioneins: Roles in Heavy Metal Detoxification and Homeosteasis. *Annual Review of Plant Biology*, Vol.53, pp. 159-182.

Coleman, J.O.D.; Blake-Kalff, M.M.A. & Davies, T.G.E. (1997). Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. *Trends in Plant Science,* Vol.2, pp. 144-151.

Coutinho, D.H. & Barbosa, A.R. (2007). Fitorremediação: Considerações Gerais e Características de Utilização. *Silva Lusitana*, Vol.15, No.1, pp. 103 - 117, Portugal.

Cunningham, S.D. & Lee, C.R. (1995). Phytoremediation: Plant-based remediation of contaminated soils and sediments. In: *Bioremediation: Science and Applications*, Skipper, H.D. & Turco, R.F. (Ed.), 145-156, SSSA Special Publication 43, Soil Science Society of America, Madison, WI.

Cunningham, S.D. & Ow, D.W. (1996). Promises and prospects of phytoremediation. *Plant Physiology*, Vol.110, No.3, pp. 715-719.

Cunningham, S.D.; Shann, J.R.; Crowley, D.E. & Anderson, T.A. (1997). Phytoremediation of contaminated water and soil: Phytoremediation of soil and water contaminations. *ACS Symposium Series*, Vol.664, pp. 2-17.

Danh, L.T.; Truong, P.; Mammucari, R.; Tran, T. & Foster, N. (2009). Vetiver grass, *Vetiveria zizanioides*: A choice plant for phytoremediation of heavy metals and organic wastes. *International Journal of Phytoremediation*, Vol.11, pp. 664-691.

De la Fuente, J.M.; Ramirez-Rodriguez, V.; Cabrera-Ponce, J.L. & Herrera-Estrella, L. (1997). Aluminium tolerance in transgenic plants by alteration of citrate synthesis. *Science*, Vol.276, pp. 1566-1568.

Dhankher, O.P.; Li, Y.; Rosen, B.P.; Shi, J.; Salt, D.; Senecoff, J.F.; Sashti, N.A. & Meagher, R.B. (2002). Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and g-glutamylcysteine synthetase expression. *Nature Biotechnology*, Vol.20, pp. 1140-1145.

Dietz, A.C. & Schnoor, J.L. (2001). Advances in phytoremediation. *Environmental Health Perspectives*, Vol.109, pp. 163–168.

Doty, S.L. Enhancing phytoremediation through the use of transgenics and endophytes. (2008). *New Phytologist,* Vol.179, pp. 318–333.

D´Souza, M.P.; Pilon-Smits, E.A.H. & Terry, N. (2000). The physiology and biochemistry of selenium volatilization by plants. In: *Phytoremediation of toxic metals: using plants to clean up the environment*, Raskin, I. & Ensley, B.D. (Ed.) 171- 188.

Dushenkov, S. (2003). Trend in phytoremediation of radionuclides. *Plant and Soil*, Vol.249, pp. 167-175.

Eapen, S.; Suseelan, K.; Tivarekar, S.; Kotwal, S. & Mitra, R. (2003). Potential for rhizofiltration of uranium using hairy root cultures of *Brassica juncea* and *Chenopodium amaranticolor*. *Environmental Research*, Vol.91, pp. 127–33.

Eapen, S. & D'Souza, S.F. (2005). Prospects of genetic engineering of plants for phytoremediation of toxic metals. *Biotechnology Advances*, Vol.23, pp. 97– 114.

Eapen, S.; Singh, S. & D´Souza, S.F. (2007). Advances in development of transgenic plants for remediation of xenobiotic pollutants. *Biotechnology Advances*, Vol.25, pp. 442–451.

Estevez-Núnez, A.; Caballero, A. & Ramos, J.L. (2001). Biological degradation of 2,4,6-trinitrotoluene. *Microbiology and Molecular Biology Reviews*, Vol.65, No.3, pp. 335-352.

Ezaki, B.; Gardner, R.C.; Ezaki, Y. & Matsumoto, H. (2000). Expression of aluminium induced genes in transgenic *Arabidopsis* plants can ameliorate aluminium stress and/or oxidative stress. *Plant Physiology*, Vol.122, pp. 657-665.

Fingerman, M. & Nagabhushanum, R. (2005). Bioremediation of aquatic and terrestrial ecosystems. Enfield, NH, USA: Science Publishers.

Fulekar, M.H.; Singh, A. & Bhaduri, A.M. (2008). Genetic engineering strategies for enhancing phytoremediation of heavy metals. *African Journal of Biotechnology*, Vol.8, No.4, pp. 529-535.

Garbisu, C.; Hernandez, A.J.; Barrutia, O. Alkorta, J. & Bacerril, J.M. (2002). Phytoremediation: a technology using green plants to remove contaminants from pollutes areas. *Reviews of Environmental Health*, Vol.17, No.3, pp. 173-188.

Gisbert, C.; Ros, R.; De Haro, A.; Walker, D.J.; Bernal, M.P.; Serrano, R. & Navarro-Avino, J. (2003). A plant genetically modified that accumulates Pb is especially promising for phytoremediation. *Biochemical and Biophysiscal Research Communities*, Vol.303, pp. 440-445.

Glass, D. (1999). U.S. and International Markets for Phytoremediation, 1999–2000. Needham, MA, USA: D. Glass Associates, Inc.

Goldsbrough, P. (2000). Metal tolerance in plants: the role of phytochelatins and metallothioneins. In: *Phytoremediation of contaminated soil and water*, Terry N. & Bañuelos G. (Ed.), 221-233, CRC Press LLC.

Goto, F.; Yoshihara, T. & Saiki, H. (1998). Iron accumulation in tobacco plants expressing soybean ferritin gene. *Transgenic Research*, Vol.7, pp. 173-80.

Goto, F.; Yoshihara, T.; Shigemoto, N.; Toki, S. & Takaiwa, F. (1999). Iron accumulation in rice seed by soybean ferritin gene. *Nature Biotechnology*, Vol.17, pp. 282-286.

Gratão, P.L.; Prassad, M.N.V.; Cardoso, P.F.; Lea, P.J. & Azevedo, R.A. (2005). Phytoremediation: green technology for the clean up of toxic metals in the environment. *Brazilian Journal of Plant Physiology*, Vol.17, No.1, pp. 53-64.

Grichko, V.P.; Filby, B. & Glick, B,R. (2000). Increased ability of transgenic plants expressing the bacterial enzyme ACC deaminase to accumulate Cd, Co, Cu, Ni, Pb and zinc. *Journal of Biotechnology*, Vol.81, pp. 45– 53.

Ha, S.B.; Smith, A.P.; Howden, R.; Dietrich, W.M.; Bugg, S.; O'Connell, M.J.; Goldsbrough, P.B. & Cobbett, C.S. (1999). Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. *Plant Cell*, Vol.11, pp. 1153-1163.

Hall, J.L. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany*, Vol.53, pp. 1-11.

Heiss, S.; Wachter, A.; Bogs, J.; Cobbett, C. & Rausch, T. (2003). Phytochelatin synthase (PCS) protein is induced in *Brassica juncea* leaves after prolonged Cd exposure. *Journal of Experimental Botany*, Vol.54, pp. 1833-1839.

Hirschi, K.D.; Korenkov, V.D.; Wilganowski, N.L. & Wagner, G.J. (2000). Expression of Arabidopsis CAX2 in tobacco altered metal accumulation and increased manganese tolerance. *Plant Physiology*, Vol.124, pp. 125-33.

Hooda, V. (2007). Phytoremediation of toxic metals from soil and waste water. *Journal of Environmental Biology*, Vol.28, No.2, pp. 367-376.

Horne, A.J. (2000). Phytoremediation by constructed wetlands: In: *Phytoremediation of contaminated soil and water*, Terry, N. & Bañuelos, G. (Ed.), 13-40, Boca Raton: Lewis.

Huang, J.W. & Cunningham, S.D. (1996). Lead phytoextraction: species variation in lead uptake and translocation. *New Phytologist*, Vol.134, No.1, pp. 75-84.

Jaffré, T.; Brooks, R.R.; Lee, J. & Reeves, R.D. (1976). *Sebertia acuminata*: a hyperaccumulator of nickel from New Caledonia. *Science*, Vol.193, pp. 579–580.

Jacobson, M.E.; Chiang, S.Y.; Gueriguian, L.; Weshtholm, L.R. & Pierson, J. (2003). Transformation kinetics of trinitrotoluene conversion in aquatic plants. In: *Phytoremediation: Transformation and control of contaminants*, McCutcheon, S.C. & Schnoor, J.L. (Ed.), 409-427,Wiley, NY.

Karenlampi, S.; Schat, H.; Vangronsveld, J.; Verkleij, J.A.C.; Van der Lelie, D.; Mergeay, M. & Tervahauta, A.L. (2000). Genetic engineering in the improvement of plants for phytoremediation of metal polluted soils. *Environmental Pollution*, Vol.107, pp. 225–31.

Knasmuller, S.; Gottmann, E.; Steinkellner, H.; Fomin, A.; Pickl, C.; Paschke, A.; God, R. & Kundi, M. (1998). Detection of genotoxic effects of heavy metal contaminated soils with plant bioassays. *Mutation Research*, Vol.420, pp. 37-48.

Krämer, U.; Pickering, I.J.; Prince, R.C.; Raskin, I. & Salt, D.E. (2000). Subcellular localization and speculation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. *Plant Physiology*, Vol.122, pp. 1343-53.

Krämer, U. & Chardonnens, A.N. (2001). The use of transgenic plants in the bioremediation of soils contaminated with trace elements. *Applied Microbiology and Biotechnology*, Vol.55, pp. 661-672.

Kreuz, K.; Tommasini, R. & Martinoia, E. (1996). Old enzymes for a new job: herbicide detoxification in plants. *Plant Physiology*, Vol.111, pp. 349-353.

Lamoureux, G.L.; Rusness, D.G.; Schroder, P. & Rennenberg, H. (1991). Diphenyl ether herbicide metabolism in a spruce cell suspension culture: the identification of two novel metabolites derived from glutathione conjugation. *Pesticide Biochemistry and Physiol*ogy, Vol.39, pp. 291-301.

Langenbach, T. (1994). Degradação de xenobiontes: potencialidades e limites. In: Microrganismos de importância agrícola, Araújo, R.S. & Hungia, M. (Ed.), 218-236, Brasília: EMBRAPA-SPI.

Lasat, M.M. (2002). Phytoextraction of toxic metals: a review of biological mechanisms. *Journal of Environmental Quality*, Vol.31, pp. 109– 20.

Li, T.Q.; Yang, X.E.; He, Z.L.; Yang, J.Y. & Long, X.X. (2005). Zn accumulation and subcellular distribution in leaves of Zn hyperaccumulator species of *Sedum alfredii* Hance. *Pedosphere*, Vol.16, No.5, pp. 616-623.

Lin, C.F.; Lo, S.S.; Lin, H.Y. & Lee, Y.C. (1998). Stabilization of cadmium contaminated soils using synthesized zeolite. *Journal of Hazardous Material*, Vol.60, pp. 217-226.

Louie, M.; Kondor, N. & Witt, J.G. (2003). Gene expression in cadmium-tolerant *Datura innoxia*: Detection and characterization of cDNAs induced in response to Cd^{2+}. *Plant Molecular Biology*, Vol.52, pp. 81-89.

Marrs, K.A. (1996). The functions and regulation of glutathione S-transferase in plants. *Annual Review of Plant Physioogy and Plant Molecular Biology*, Vol.47, pp. 127-158.

Martínez, M.; Bernal, P.; Almela, C.; Vélez, D.; García-Agustín, P.; Serrano, R. & Navarro-Aviñó, J. (2006). An engineered plant that accumulates higher levels of heavy metals than *Thlaspi caerulescens*, with yields of 100 times more biomass in mine soils. *Chemosphere*, Vol.64, pp. 478–485.

Martinoia, E.; Grill, E.; Tomasini, R.; Kreuz, K. & Amrehin, N. (1993). ATP-dependent glutathione S-conjugate 'export' pump in the vacuolar membrane of plants. *Nature,* Vol.364, pp. 247-249.

McGrath, S.P.; Zhao, F.J. & Lombi, E. (2002). Phytoremediation of metals, metalloids and radionuclides. *Advances in Agronomy*, Vol.75, pp.1–56.

Meagher, R.B. (2000). Phytoremediation of toxic elemental and organic pollutants. *Current Opinion of Plant Biology*, Vol.3, pp. 153–162.

Mejäre, M. & Bülow, L. (2001). Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. *Trends in Biotechnology*, Vol.19, pp. 67-73.

Mello-Farias, P.C. & Chaves, A.L.S. (2008). Biochemical and molecular aspects of toxic metals phytoremediation using transgenic plants. In: *Transgenic Approach in Plant Biochemistry and Physiology*, Tiznado-Hernandez, M.E.; Troncoso-Rojas, R. & Rivera-Domínguez, M. A. (Ed.) 253-266, Research Signpost, Kerala, India.

Mijoviloch, A.B.; Leitenmaier, W.; Meyer-Klauche, P.M.; Kroneck, B.; Götz, B. & Kupper, H. (2009). Complexation and toxicity of copper in higher plants. II. Differents mechanisms for copper versus cadmium detoxification in the cooper-sensitive cadmium/zinc hyperaccumulator Thlaspi caerulescens (Ganges Ecotype). Plant Physiolology, Vol.151, pp. 715-731.

Murphy, A.S.; Eisenger, W.R.; Shaff, J.E.; Kochian, L.V. & Taiz, L. (1999). Early copper-induced leakage of K+ from Arabidopsis seedlings is mediated by ion channels and coupled to citrate efflux. Plant Physiology, Vol.121, pp. 1375-1382.

Nedelkoska, T.J. & Doran, P.M. (2000). Hyperaccumulation of cadmium by hairy roots of Thlaspi caerulescens. Biotechnology Bioengineering, Vol.67, No.5, pp. 607– 15.

Negri, M.C. & Hinchman, R.R. (2000). The use of plants for the treatment of radionuclides. In: Phytoremediation Of Toxic Metals: Using Plants to Clean Up the Environment, Raskin, I. & Ensley, E.D. (Ed.), 107-132, New York: Wiley.

Neuefeind, T.; Reinemer, P. & Bieseler, B. (1997). Plant glutathione S-transferases and herbicide detoxification. Biological Chemistry, Vol.378, pp. 199-205.

Nwoko, C.O. (2010). Trends in phytoremediation of toxic elemental and organic pollutants. *African Journal of Biotechnology*, Vol.9, No.37, pp. 6010-6016.

Ohkawa, H.; Imaishi, H. & Shiota, N. (1999). In: *Pesticide Chemistry and Biosciences: The Food-Environment Challenge*, Brooks, J.T. & Roberts, T.R. (Ed.), 259-264, Royal Society of Chemistry, Cambridge, UK.

Olson, P.E.; Reardon, K.F. & Pilon-Smits, E.A.H. (2003). Ecology of rhizosphere bioremediation. In: *Phytoremediation: Transformation and Control of Contaminants*, McCutcheon, S.C. & Schnoor, J.L. (Ed.), 317-354, Wiley: New York.

Ow, D.W. (1996). Heavy metal tolerance genes-prospective tools for bioremediation. *Resources, Conservation and Recycling*, Vol.18, pp. 135-149.

Papernik, L.A.; Bethea, A.S.; Singleton, T.E.; Magalhaes, J.V.; Garvin, D.F. & Kochian, L.V. (2001). Physiological basis of reduced Al tolerance in ditelosomic lines of Chinese Spring wheat. *Planta*, Vol.212, pp. 829-834.

Persans, M.W., Nieman, K. & Salt, D.E. (2001). Functional activity and role of cationefflux family members in Ni hyperaccumulation in *Thlaspi goesingense*. *The Proceedings of the National Academy of Sciences USA*, Vol.98, pp. 9995-1000.

Pilon-Smits, E. Phytoremediation. (2005). *Annual Review of Plant Biology,* Vol. 56, pp. 15-39.

Pilon-Smits, E. & Pilon, M. (2002). Phytoremediation of metals using transgenic plants. *Critical Reviews in Plant Science*, Vol.21, pp. 439–456.

Pilon-Smits, E. (2005). Phytoremediation. *Annual Reviews of Plant Biology*, Vol.56, pp. 15–39.

Pineros, M.A. & Kochian, L.V. (2001). A patch-clamp study on the physiology of aluminium toxicity and aluminium tolerance in maize. Identification and characterization of Al^{3+} induced anion channels. *Plant Physiology,* Vol.125, pp. 292-305.

Pires, F.R.; Souza, C.M.; Silva, A.A.; Procópio, S.O. & Ferreira, L.R. (2003). Fitorremediação de solos contaminados com herbicidas. *Planta Daninha*, Vol.21, No.2, pp. 335-341.

Prasad, M.N.V. (2004) Phytoremediation of metals in the environment for sustainable development. *Proceedings of the Indian National Science Academy*, Vol.70, pp. 71-98.

Prasad, M.N.V. & Freitas, H. (2003). Metal hyperaccumulation in plants: Biodiversity prospecting for phytoremediation technology. *Electronic Journal of Biotechnology*, Vol.6, pp. 275-321.

Pulford, I.D. & Watson, C. (2003). Phytoremediation of heavy metal-contaminated land by trees - a review. *Environment International*, Vol.29, pp. 529-540.

Robinson, B.H.; Brooks, R.R.; Howes, A.W.; Kirkman, J.H. & Gregg, P.E.H. (1997). The potential of the high biomass nickel hyperaccumulator *Berkheya coddii* for phytoremediation and phytomining. *Journal of Geochemical Exploration*, Vol.60, pp. 115–126.

Robinson, B.H.; Leblanc, M.; Petit, D.; Brooks R.R.; Kirkman J.H. & Gregg P.E.H. (1998). The potential of *Thlaspi caerulescens* for phytoremediation of contaminated soils. *Plant and Soil*, Vol.203, pp. 47–56.

Reeves, R.D. & Baker, A.J.M. (2000). Metal-accumulating plants. In: *Phytoremediation of toxic metals using plants to clean up the environment*, Raskin, I. & Ensley, B.D. (Ed.) 193-230, John Wiley and Sons, New York.

Rugh, C.L.; Bizily, S.P. & Meagher, R.B. (2000). Phytoremediation of environmental mercury pollution. In: *Phytoremediation of toxic metals using plants to clean up the environment*, Raskin, I. & Ensley, B.D. (Ed.) 151-171, New York Wiley and Sons, New York.

Sanita di Toppi, L. & Gabbrielli, R. (1999). Response to cadmium in higher plants. *Environmental and Experimental Botany*, Vol.41, pp. 105-30.

Schnoor, J.L.; Licht, L.A.; McCutcheon, S.C.; Wolf, N.L. & Carreira, L.H. (1995). Phytoremediation of organic and nutrient contaminants. *Environmental Science and Technology*, Vol.29, pp. 318-323.

Siciliano, S.D.; Germida, J.J.; Banks, K. & Green, C.W. (2003). Changes in Microbial Communit Composition and during a polyaromatic hydrocarbon phytoremediation field trial. *Applied and Environmental Microbiology*, Vol.69, No.1, pp. 483-489.

Siqueira, J.O. (1996). *Avanços em fundamentos e aplicações de micorrizas*. (edition) Lavras: UFLA, 290p.

Ross, S.M. (1994). *Toxic Metals in Soil-Plant Systems*, Wiley & Sons: Chichester, UK.

Salt, D.E.; Smith, R.D. & Raskin, I. (1998) Phytoremediation. *Annual Review of Plant Physiology and Plant Molecular Biology*, Vol.49, pp. 643–668.

Sarma, H. (2011). Metal hyperaccumulation in Plants: A Review Focusing on Phytoremediation Technology. *Journal of Environmental Science and Technology*, Vol.4, No.2, pp. 118-138.

Schat, H.; Llugany, M.; Voojis, R.; Harley-Whitaker, J. & Bleeker, P.M. (2002). The role of Phytochelatin in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. *Journal of Experimental Botany*, Vol.53, pp. 2381-2392.

Schnoor, J.L.; Licht, L.A.; McCutcheon, S.C.; Wolfe, N.L. & Carreira, L.H. (1995). Phytoremediation of contaminated soils and sediments. *Environmental Science and Technology*, Vol.29, 318-323.

Singer, A. (2006). The chemical ecology of pollutants biodegradation. In: *Phytoremediation and Rhizoremediation: Theoretical Background*, Mackova, M.; Dowling, D. & Macek, K. (Ed.), 5–21, Springer.

Singh, O.V.; Labana, S.; Pandey, G.; Budhiraja, R. & Jain, R.K. (2003). Phytoremediation: an overview of metallic ion decontamination from soil. *Applied Microbiology and Biotechnology*, Vol.61, pp. 405-412.

Song, W.Y.; Martinoia, E.; Lee, J.; Kim, D.; Kim, D.Y.; Vogt, E.; Shim, D.; Choi, K.S.; Hwang, I. & Lee, Y. (2004). A novel family of cys-rich membrane proteins mediates cadmium resistance in *Arabidopsis*. *Plant Physiology*, Vol.135, pp. 1027-1039.

Taiz, L. & Zeiger, E. (2002). *Plant Physiology*; Sinauer: Sanderland, M.A., pp. 1-690.

Thomine, S.; Wang, R.; Ward, J.M.; Crawford, N.M. & Schroeder, J.I. (2000). Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to *Nramp* genes. *Proceedings of the National Academy of Sciences USA*, Vol.97, pp. 4991-4996.

Tsao, D.T. (2003). Overview of phytotechnologies. *Advances in Biochemical Engineering and Biotechnology*, Vol.78, pp. 1–50.

Tong, Y.P.; Kneer, R. & Zhu, Y.G. (2004). Vacuolar compartimentalization: a second generation approach to engineering plants for phytoremediation. *Trends in Plant Science*, Vol.9, No.1, pp. 7-9.

Van Aken, B. (2008). Transgenic plants for phytoremediation: helping nature to clean up environmental pollution. *Trends in Biotechnology*, Vol.26, No.5, pp. 225-227.

Vassilev, A.; Schwitzguebel, J.P.; Thewys, T.; Van der Lelie, D. & Vangronsveld, J. (2004). The use of plants for remediation of metal-contaminated soils. *The Scientific World Journal*, Vol.4, pp. 9–34.

Vatamaniuk, O.K.; Mari, S.; Lu, Y.P. & Rea, P.A. (1999). At PCS1, a phytochelatin synthase from *Arabidopsis*: isolation and *in vitro* reconstitution. *The Proceedings of the National Academy of Sciences USA*, Vol. 96, No.12, pp. 7110- 7115.

Williams, L.E.; Pittman, J.K. & Hall, J.L. (2000). Emerging mechanisms for heavy metal transport in plants. *Biochimica et Biophysica Acta*, Vol.1465, pp. 104-26.

Wolfe, N.L. & Hoehamer, C.F. (2003). Enzymes used by plants and microorganisms to detoxify organic compounds. In: *Phytoremediation: Transformation and control of contaminants*, Mc-Cutcheon, S.C. & Schnoor, J.L. (Ed.), 158-187, Wiley: New York.

Yang, X.; Jin, X.F.; Feng, Y. & Islam, E. (2005a). Molecular Mechanisms and Genetic bases of Heavy Metal Tolerance/Hyperaccumulation in Plants. *Journal of Integrative Plant Biology*, Vol.47, No.9, pp. 1025-1035.

Yang, X.; Feng, Y.; He, Z. & Stoffella, P. (2005b). Molecular mechanisms of heavy metal hiperaccumulation and phytoremediation. *Journal of Trace Elements in Medicine and Biology*, Vol.18, pp. 339-353.

Zhou, Q.X.; Kong, F.X. & Zhu, L. (2004). *Ecotoxicology*. Science Press, p. 454, Beijing, China.

Zhu, Y.L.; Pilon-Smits, E.A.H.; Jouanin, L. & Terry, N. (1999a). Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. *Plant Physiology*, Vol.119, pp. 73-79.

Zhu, Y.L.; Pilon-Smits, E.A.H.; Tarun, A.S.; Weber, S.U.; Jouanin, L. & Terry, N. (1999b). Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing g-glutamylcysteine synthetase. *Plant Physiology*, Vol.121, pp. 1169-77.

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