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Plants for the Future

Edited by Hany El-Shemy

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Notice

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Meet the editor

Professor Hany A. El-Shemy received his two Ph.D. degrees in Biochemistry and Genetic Engineering from the University of Cairo, Egypt, and the University of Hiroshima, Japan. He became an Assistant Professor in the Biochemistry Department of Cairo University, Egypt, in September 1996 and advanced to Associate Professor in September 2002 as well as Full Professor in March 2007. His research interests are in the fields of plant biotechnology and medicinal plants (molecular biology). He received 2 patents, wrote 8 international books, published more than 70 SCI journal papers, delivered 35 conference presentations, and served as the technical committee member as well as chair in many international conferences. He received several awards, including State Prize awarded by the Academy of Science, Egypt (2004), Young Arab Researcher Prize awarded by the Shuman Foundation, Jordan (2005), State Excellence Prize awarded by the Academy of Science, Egypt (2011), and Cairo University Prizes 2007, 2010, 2014. He served as an expert for the African Regional Center of Technology, Dakar, Senegal, and as a Visiting Professor at the Pan African University, African Union, Nairobi, Kenya. He was appointed Acting Vice President of the Academy of Scientific Research and Technology, Cairo, Egypt, from November 2013 to November 2014. Currently, he is working as the Dean of the Faculty of Agriculture, Cairo University, and Coordinator of Egypt-Africa Scientific Relations, Ministry of Scientific Research, Egypt.

Contents

Preface

The world has come to understand only recently the importance of plants in our life. Therefore, we have brought together such book chapters that will help strengthen the scientific background of the readers on plants and deliver the message regarding plants for the future, in food security, health, industry, and other areas.

The book has been divided into seven chapters on plant sciences. Some chapters explain the genetic background of legume-rhizobium symbiosis and RNase genes. In addition, molecular farming (MF) of plants has been discussed in one chapter including its applications.

The biochemical parameters especially in tomato have also been discussed to understand the mode of action using both techniques biochemically and genetically. Metabolic engineering has also been taken into consideration in order to accomplish the plant sciences phenomena.

This book will add to the scientific knowledge of the readers on the molecular aspects of plants.

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Section 1

Applications on Plant Molecular Biology

Chapter 1

Understanding the Genetics of Clubroot Resistance for Effectively Controlling this Disease in Brassica Species

Arvind H. Hirani and Genyi Li

Additional information is available at the end of the chapter

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Abstract

Clubroot disease is one of the most serious diseases of Brassica species, which is caused by soil-borne pathogen *Plasmodiophora brassicae* Woronin. Clubroot disease has a long history on vegetable crops belonging to the Brassica species; most recently, this disease is also invading rapeseed/canola crop around the globe. The clubroot disease causes significant yield and quality losses in highly infected fields. Clubroot pathogens invade into the host plant roots and infect root tissues with the formation of abnormal clubs, named as galls, which results in incompetent plant roots to intake water and nutrients and eventually dead plants. As it is a soil-borne disease and accomplishes its disease cycle in two different phases and both phases are highly efficient to damage root system as well as to release more inoculum, there are many challenges to control this disease through chemical and other cultural practices. In general, clubroot disease can be effectively managed by developing resistant cultivars. In this chapter, various resistance sources of clubroot disease in different Brassica species have been discussed with potential applications in canola/rapeseed breeding programs worldwide. Importance of gene mapping and molecular marker development efforts by different research studies for clubroot in *B. rapa*, *B. oleracea*, and *B. napus* has been stressed. Transcriptomic and metabolomic changes occurring during host–pathogen interactions are also covered in this chapter, which would enhance our understanding and utilization of clubroot resistance in Brassica species.

Keywords: brassica species, clubroot resistance, molecular marker development, marker-assisted selection

1. Introduction

The crops in the *Brassicaceae* family are the most economically and nutritionally important for human consumption after cereals [1]. Based on utilities, Brassica species are broadly categorized into oilseed, vegetable, and sources of condiments. In the Brassica crops, canola/rapeseed is the second largest vegetable oil crop after soybean in the world [2]. Globally, rapeseed/canola has been cultivated in about 36.4 M ha with total production approximately 72.5 M tonnes [2]. Among the Brassica species, *B. napus*, *B. rapa*, *B. juncea*, and *B. carinata* provide about 15% of edible vegetable oil supplies around the world [3]. In addition, Brassica species such as *B. oleracea*, *B. rapa*, and *B. napus* supply nutritionally rich green leafy, stem and root vegetables for human daily diets. Brassica species also possess rich genetic diversity with respect to both speciation and the ample morphotypes [4], which designate important species to be investigated for genetic evaluation of plant kingdom. The Brassica crops, however, have significant impact by biotic stresses including diseases and pests, which challenge production and productivity of these crops.

Clubroot is one of the most threatening disease affecting global production and productivity of cruciferous crops including canola/rapeseed and Brassica vegetables. Clubroot disease is caused by the soil-borne obligate biotroph pathogen *P. brassicae* in Brassica crops. Cultivation of different Brassica oilseed and vegetable crops fulfills the host range requirement of the clubroot pathogen, which leads to wide spreading of the pathogen throughout the world. Since the emergence of the clubroot pathogen in vegetable crops of Brassica species, management of the disease has been a big challenge due to the obligate biotroph nature of the pathogen.

2. Impact of clubroot disease in Brassica species

The clubroot disease is not a new one in Brassica crops, it has been historically considered as the most important disease [5]. The origin of the clubroot disease is unknown, but it appears as ancient as its host. Earlier literatures reported the existence of clubroot disease in the 13th century in Spain, and later in 17th century, clubroot disease was also observed in England and subsequently it spread in Scotland, France, Germany, Poland, and other European countries. In Russia, clubroot was first reported in Brassica vegetable crops in 1872 [6]. In Japan, the disease was first recorded in 1890s and now it is one of the major constrains in Chinese cabbage and other Brassica vegetable production [7]. Similarly, this disease was first reported in Australia in the early 1890s [8]. Most of the earlier clubroot disease infections were reported on Brassica vegetable crops.

Mustard/rapeseed crops have similar cultivation history like other Brassica vegetables in different parts of the world; however, there was no evidence of clubroot disease in rapeseed/mustard crop in earlier time. Before three decades, about 2.5% canola/rapeseed crops were reported with clubroot disease in 18 countries [9, 10]. Since then, canola/rapeseed cultivation expanded significantly due to health benefit properties of its oil. This suggests that clubroot

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disease is relatively new in canola/rapeseed compared to other Brassica vegetable crops in which this disease is known from as early as 13th century.

In Canada, canola/rapeseed is the second most important crop next to the wheat and it is mainly cultivated in the Prairie Provinces. Canola is economically the most important crop for the Canadian agriculture, food, and animal industries. The crop supplies nutritionally healthy edible oil to the food industries [11] along with nutritionally balance seed meal to animal industries. Annually, over 8 M ha canola/rapeseed crop has been grown with greater than 15.6 MT productions in Canada [12]. Canola crop contributes more than $15 billion each year to the Canadian economy [13]. In Canada, clubroot disease has been a problem on Brassica vegetables in producing areas including Ontario, Quebec, British Columbia, and the Atlantic Provinces. Clubroot has been periodically reported in few cases on Brassica vegetables in Alberta and Manitoba over the past 80 years [14]. This situation, however, entirely changed with the discovery of about 12 infected canola fields in Alberta in 2003. Annual survey carried out in Alberta, Saskatchewan, and Manitoba have revealed that clubroot is a much more widespread and serious disease in Canadian canola because canola is one of the major crops in the Prairie Provinces. In 2011, clubroot disease has been confirmed in over 800 fields distributed in most part of the Alberta [15], and from two fields in Saskatchewan [16]. Clubroot disease also reported in the North Dakota state in a few canola fields having patches of >80% plant mortality [17].

Clubroot disease has caused different degree of yield losses in canola/rapeseed fields depending on pressure of the disease and nature of genetic inheritance (susceptible/moderately resistance/ resistance) of canola cultivars planted. Clubroot can cause up to 100% yield loss in heavily infected fields when susceptible canola cultivars are planted [18]. Similarly, about 90% yield loss and 5–6% reduction in oil content was reported in clubroot-infected canola field in Quebec [19]. In a previous publication, Dixon [20] has extensively reviewed clubroot infection in three major Brassica species, *B. oleracea*, *B. rapa*, and *B. napus* based on the survey data [10], and suggested that greater than 10% fields were infected in Australia, Canada, Czechoslovakia, Finland, Germany, Ireland, Netherlands, New Zealand, Norway, Poland, Scotland, United States, and Wales in the early 1980s.

In Asian countries, clubroot disease is widespread in the Brassica species cultivating regions in India, China, Nepal, Bangladesh, Pakistan, Indonesia, and Bhutan. In India, North Eastern part has become widespread due to frequent cultivation of cauliflower and yellow sarson, which are susceptible to clubroot. Similarly, China, Bangladesh, and Nepal are high-risk regions for the clubroot disease, especially for Brassica vegetables, mustard, and rapeseed production.

3. Disease cycle and symptoms

The pathogen *P. brassicae* Wornonin is an obligate biotrophic protist belonging to the class phytomyxea. The pathogen can infect primary and secondary roots at the early stage of plant growth and development that causes significant yield and quality losses. The life cycle of *P.*

brassicae consists of two phases; in a primary phase, under favorable conditions, resting spores germinate and produce primary zoospores that penetrate in root hairs and mass production of secondary zoospores occurs in the root hairs. The resting spores are about 3 μm in size and subspherical to spherical in shape and the surface of each resting spore is covered with spines [21]. Mass of primary zoospores is released from each resting spore, spindle-shaped or pyriform, 2.8–5.9 μm long, and biflagellate. When the zoospores come in contact with the surface of a root hair, it penetrates in the cell wall and it is also called root hair infection. Secondary phase of life cycle occurs in the root cortex as a result secondary plasmodia and gall formation occurs as a result restriction in water and nutrient uptake by plants (Figure 1) [21, 22]. The life cycle study of *P. brassicae* in *A. thaliana* reported uninucleate and binucleate myxamoeboid structure production within host cytoplasm that caused cell wall burst and production of secondary plasmodia [23]. During pathogen infection, secondary plasmodia proliferate in roots and plant hormone, especially auxin and cytokinin, biosynthesis altered in the root tissues that causes gall formation (Figure 2) [21]. Infected plants become stunted, yellowish in color, and eventually wilt, which causes severe reduction in yield and quality of crops [24]. Mature secondary plasmodia subsequently develop into resting spores that can survive for 20 years or more [25]. Clubroot disease pressure can significantly increase in those fields where crop rotation frequently includes canola/rapeseed or other Brassica crops. Acidic soil with high soil moisture is the most favorable condition for resting spore germination and subsequent secondary infection.

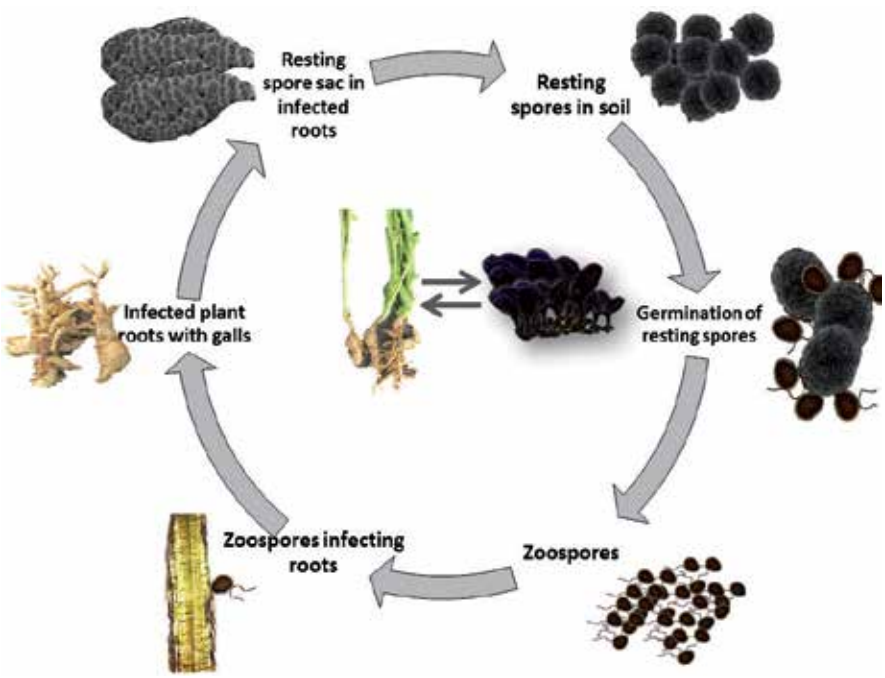

Figure 1. Life cycle of *P. brassicae* and club like gall formation on the roots of Brassica host plant.

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Figure 2. Clubroot disease symptoms in highly infected fields. (a) Clubroot-resistant breeding line of *B. rapa* showed no clubroot symptoms on roots, (b) clubroot disease symptoms with large galls on primary and secondary roots in turnip rape line of *B. rapa* under field conditions, (c) clubroot galls on roots of broccoli in *B. oleracea*, and (d) highly infected field of Chinese cabbage in Henan province of China.

4. Biology of *P. brassicae*

The genus *Plasmodiophora* is a monophyletic group with uncertain systematic affinities. The species belonging to this genus possess unique features such as cruciform nuclear division, parasitism, obligate nature, biflagella, heterocont zoospores, and environmentally resistance and long-living resting spores [26]. In this genus, the economically significant member is *P. brassicae*, which hosts Brassica species to cause clubroot disease. The pathogen shows a wide biological range and its populations usually consist of a mixture of different pathotypes [27–30]. Soil environmental factors such as physical, chemical, and biological properties of soil may differentially influence the survival of some physiological races of the pathogen [21, 31]. In European, field isolates of *P. brassicae* display great variation and show a tendency to overcome different resistance sources from either *B. rapa* or *B. oleracea*.

To enhance our understanding of the pathogenicity factors of *P. brassicae* causing clubroot disease on different Brassica hosts, several molecular techniques and tools are employed to determine *P. brassicae* genome size, structure, and number of possible functional genes in the whole genome. Several studies reported use of pulse-field gel electrophoresis (PFGE) to determine the karyotypes for *P. brassicae*. Ito et al. [32] used sheroplasts and differentiated 13 chromosomal bands in the range of 1.9 Mb to 750 kb. Bryan et al. [33] used isolated plasmodia and differentiated six chromosomal bands in the range of 1.7 Mb to 680 kb. Similarly, Graf et al. [34] distinguished 16 chromosomal bands in the range of 2.2 Mb to 680 kb. Based on these studies, it is estimated that the *P. brassicae* total genome size can be 18–20.3 Mb [35].

On the other hand, several molecular marker techniques were employed to investigate virulent pattern of the *P. brassicae* population derived from single-spore isolate or field isolates [36–38]; however, the number of distinguishing patterns were very low and that could not correlate with virulence patterns. In continuous efforts, two RAPD markers [39] and one SCAR marker [40] were identified, which correlate to isolates of pathotype 1. Yet there are no sets of molecular markers that can distinguish other pathotypes from field isolates which make clubroot-resistance breeding intriguing.

5. Host–pathogen interactions

a. During resting spore germination

In soil environment, host–pathogen interactions begin at the early seedling stage when host plant root exudates are present, which induces germination of resting spores [41] and releasing of primary zoospores. The role of root exudates as stimulants for resting spore germination was examined and confirmed in different research studies [42–44]. In contrast, substantial studies by Kowalski and Bochow [45] reported that the stimulant effect for germination is not confined to the specific host of *P. brassicae*. This finding was supported by the evidence of root exudates from Brassica host (broccoli) and non-Brassica host (ryegrass), both stimulated spore germination [46]. Studies also reported that some specific stimulants such as caffeic acid, coumalic acid, corilagin, and others could stimulate resting spore germination in Chinese cabbage [47, 48]. All these studies suggest that the Brassica species have unique root characteristics which permit pathogen invasion and subsequent infection for the disease development. Resting spore germination is observed stimulated by root exudates in other species, but zoospores could not establish primary infection.

b. During disease development and gall formation

Earlier studies reported that regulation of phytohormones plays an importance role in the formation of massive galls on roots. Rapid increase in both cytokinin and auxin biosynthesis was observed during secondary infection and gall formation in the infected roots of *B. rapa* [49–51]. Brassica species contain high aliphatic, indole, and aromatic glucosinolates may play a vital role in disease development and gall formation because conversion of indole-3-methyl glucosinolate to indole-3-acetonitrile is thought to be the main pathway of auxin synthesis in

infected root tissues [52, 53]. Studies suggested that induction in nitrilase activity which cleavages indole-3-acetonitrole to indole-3-acetic acid occurred in infected roots [54]. Elevated cytokinin biosynthesis was also observed in secondary plasmodia during gall formation [51, 55]. Pedras et al. [56] reported production of 45 different metabolites in *B. napus* infected by *P. brassicae*, which suggested that canola roots under biotic stress produce a complex blend of phytoalexins and other antimicrobial metabolites as defensive mechanisms. However, limited information about metabolomic interaction between host and pathogen is available during gall formation in both susceptible and complete clubroot resistance disease reactions.

6. Identification of clubroot resistance in Brassica species and their relatives

Brassica species are the major sources that are used to identify clubroot resistance. In the *Brassica* genus, three diploid species are the natural progenitors of three amphidiploid species, which is the famous triangle of U, explaining the evolutionary relationship of Brassica species. The close evolutionary relationship of Brassica species suggests that it is relatively easy to transfer clubroot resistance from species to species through interspecific hybridization and gene introgression. Extensive searching for the clubroot resistance has been performed in Brassica species, especially *B. rapa*, *B. oleracea*, and *B. napus*, and the European turnips in *B. rapa* are found to contain dominant resistance and those clubroot resistance sources have been widely used *in B. rapa* and *B. napus* breeding.

In *B. rapa*, there are various types of vegetables such as Chinese cabbage, Shanghai Pak-choy, and turnip. Clubroot disease causes heavy yield losses in Chinese cabbage production in Eastern Asian countries, especially in Japan, South Korea, and China. Fortunately, European turnip contains dominant clubroot resistance which is commonly used in Chinese cabbage hybrid cultivar development through crosses of Chinese cabbage and resistant European turnips. The clubroot resistance in European turnips has been extensively tested and genetically analyzed under field conditions or using artificial inoculation under controlled environmental conditions [57]. Before 1960, breeders in the Netherlands developed various clubroot-resistant turnip cultivars which were used to control the most serious disease in fodder turnip production, and also those clubroot-resistant turnip cultivars were used to differentiate pathogen and study clubroot infection under different field conditions [57–59]. For example, the European Clubroot Differential (ECD) set has been selected [59] and are currently used by other researchers.

B. oleracea vegetables such as cabbage, broccoli, and cauliflower are tested to identify clubroot resistance. As the clubroot resistance in *B. oleracea* was analyzed, the results in genetic analyses showed that susceptibility was dominant over resistance, and recessive genes were inferred to explain the inheritance of clubroot resistance in diallel analysis [60]. In another diallel analysis of F_1 kale populations, it was also found that additive effects are inferred based on the assessment of broad sense inheritability [61]. In addition, there are several other investigations on the clubroot resistance in *B. oleracea*; and in most cases, recessive inheritance of clubroot

resistance was identified. For example, 71 accessions of cabbage, broccoli, and curly kale were tested and most of them showed some levels of resistance to clubroot, while all the F_1 populations of these resistant and susceptible *B. oleracea* accessions were susceptible [62, 63]. Further analysis indicated that multiple loci are involved in the clubroot resistance in *B. oleracea*, but it was not easy to determine how many loci control clubroot resistance in the analysis of F_1, F_2, and backcross populations of *B. oleracea* [64]. Moreover, 44 landraces of Portuguese coles (*B. oleracea*) were tested to identify clubroot resistance and three accessions showed high levels of clubroot resistance [65].

In *B. napus*, rutabaga cultivars are identified to contain dominant clubroot resistance. In one report, the clubroot resistance in rutabaga was suggested to be controlled by one dominant resistance gene [66]. To investigate the diversity of clubroot pathogen (*P. brassicae*), the Williams differential set was suggested; [67] and in this set, there are two rutabaga accessions that show clubroot resistance in several reports [66, 68, 69]. Vigier et al. [70] tested 31 cultivars and breeding lines of spring canola under controlled environmental conditions and found that several Swedish accessions showed clubroot resistance, but the resistance was not recovered in the subsequent progenies. In another report, the clubroot resistance from rutabaga was transferred into cabbage through interspecific hybridization and results indicated that all the F_1 hybrids were resistant to clubroot disease [71].

Radish (*Raphanus sativus*) is a Brassica relative and there are several reports that focus on the identification and transfer of clubroot resistance to Brassica species. Rowe [72] tested 68 radish cultivars and breeding lines collected from several countries and found that all Japanese and most Dutch radish cultivars were completely resistant to clubroot. Akaba et al. [73] used *B. napus*–radish chromosome additional lines to analyze clubroot resistance and found that one chromosome additional line, the c-type, showed a high level of clubroot resistance. More recently, quantitative resistance loci (QTL) mapping for clubroot resistance in radish has been performed and one major gene on one linkage group was found to control the high level of clubroot resistance in radish [74].

As discussed earlier, European turnips contain dominant clubroot resistance genes which makes gene mapping easier than in *B. oleracea* varieties. To control clubroot disease, Chinese cabbage hybrid cultivars were developed by introducing clubroot resistance from European turnips into Chinese cabbage in Japan [75]; and currently, clubroot-resistant Chinese cabbage cultivars containing turnip clubroot resistance genes are being used in Japan, South Korea, and China.

7. Genetic mapping of clubroot resistance

All the first generation of molecular markers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and simple sequence repeats (SSR) are used to map clubroot resistance in various Brassica species [76–83]. Landry et al. [80] used RFLP markers detected two QTLs in *B. oleracea*. Figdore et al. [79] used RFLP markers and associated several linkage groups to clubroot resistance in *B. oleracea*. Grandclément and Thomas [82] used RAPD markers and

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analyzed QTL for clubroot resistance in broccoli and cauliflower, and identified some RAPD markers significantly linked to clubroot resistance. Moreover, Voorrips et al. [83] used RFLP and AFLP markers, detected two genes for clubroot resistance in *B. oleracea* doubled haploid lines. Rocherieux et al. [84] performed QTL analysis in *B. oleracea* and detected isolate-specific and broad spectrum QTLs, suggesting that the clubroot resistance in *B. oleracea* is genetically complicated and molecular marker-assisted selection might be not so effective. More recently, Nagaoka et al. [24] performed QTL mapping using a DH line population between resistant cabbage and susceptible broccoli and detected two major QTL for clubroot resistance on chromosomes O2 and O5 and minor QTLs on chromosome O2, O3, and O7. Similar to the conclusion drowned from conventional genetic analysis, clubroot resistance in *B. oleracea* is most likely due to the effects of multiple minor genes and molecular marker-assisted selection might be not as effective as that in Chinese cabbage which contains dominant resistance genes introduced from European turnips.

In Chinese cabbage, major clubroot resistance loci that are introduced from European turnips have been mapped (Table 1). These clubroot resistance loci are named as *Crr1*, *Crr2*, *Crr3*, and *Crr4* and also *CRa*, *CRb*, *CRc*, and *CRk* in a dozen of investigations, suggesting that there might be eight independent loci [81, 85–87]. Two clubroot resistance loci, *Crr1* and *Crr2* were mapped using SSR markers [75, 88] and a third locus, *Crr3* was identified using RAPD markers, which suggested that there are three independent clubroot resistance loci in Chinese cabbage [85, 87]. Using RFLP markers, a genetic map was constructed and a clubroot resistance locus, *CRa*, was mapped on linkage group 3 [77], and SCAR and CAPS markers were used to map another locus, *CRb*, on chromosome R3 [81]. Moreover, three clubroot resistance loci *CRa*, *CRc*, and *CRk* have been added in the list of clubroot resistance through molecular marker-assisted selection [89].

Due to the complex genome structure of *B. napus*, QTL mapping for clubroot resistance is necessary in *B. napus* (Table 1). It is quite common to find the clubroot resistance in *B. napus* that does not segregate as a typical Mendelian trait as in *B. rapa*. Although one major locus was mapped on chromosome N3, two minor QTL on chromosomes N12 and N19 were identified for clubroot resistance in *B. napus* [90]. In another report, [91] a DH line population derived from a cross of clubroot-resistant synthetic *B. napus* and susceptible canola was used to perform QTL mapping for clubroot resistance. The synthetic *B. napus* contains dominant clubroot resistance from European turnip ECD4 and may be medium resistance from *B. oleracea*. They identified a total of nineteen QTLs on chromosomes N02, N03, N08, N13, N15, N16, and N19 for clubroot resistance, and surprisingly, there were four QTLs with LOD values of over 11, of which three were located on chromosome N3 and one on N19 and the proportion of the phenotypic variance explained by each QTL was over 40%. Their data suggested that the major QTLs might come from the C genome of *B. oleracea*, which is contradictory to previous reports where major clubroot resistance genes in *B. napus* come from the A genome of *B. rapa*.

8. Fine mapping and cloning of clubroot resistance genes

As the whole genome sequencing and molecular marker development in Brassica species advances [92], those previously identified clubroot resistance loci in Chinese cabbage have

been fine mapped and some clubroot resistance genes have been eventually cloned (Table 1). The *Crr3* locating on chromosome R3 was first mapped to a small genetic region between 0.35 cM genetic distance using 888 F_2 individual plants [85]. In another report, the clubroot resistance locus *CRa* has been further analyzed to identify the candidate gene [93]. Over 1,600 F_2 individual plants were used to select 80 recombinants using two closely linked molecular markers. Further analysis of those recombinants allowed identifying one open reading frame located on chromosome R3, which belongs to a typical resistance gene family and encodes a TIR-NBS-LRR protein [93]. More recently, there are two other independent reports that focused on fine mapping of clubroot resistance loci on chromosome R3. The *CRb* clubroot resistance locus which was described to be effective to *P. brassicae* isolates No. 14, a very aggressive isolate in Japan, has been fine mapped [94]. Using over 2,000 F_2 individual plants and F_3 progeny testing, 92 F_2 recombinants between two closely linked molecular markers were identified. The analysis of these 92 F_2 recombinants suggested that the *CRb* clubroot resistance locus might be the same as the *CRa* locus and the *CRa* and *CRb* clubroot resistance loci are different from the clubroot resistance locus *Crr3* [94]. Similarly, gene mapping of five Chinese cabbage cultivars was performed and all these hybrid cultivars were found to contain the same clubroot resistance locus on chromosome R3 [95]. They further fine mapped the clubroot resistance locus in Chinese cabbage to a 187 kilo-base pair (kb) chromosomal region using a large segregating population with over 8,000 individual plants. Molecular markers which are closely linked to the mapped clubroot resistance locus have been developed and those molecular markers can be used in marker-assisted selection to breed Chinese cabbage with clubroot resistance.

Characterization of clubroot resistance genes offers opportunities for further understanding clubroot resistance and interactions of resistance genes and pathogens. Hatakeyama et al. [86] cloned one clubroot resistance gene *Crr1a* on chromosome R8 and confirmed the resistance through plant transformation. Some transgenic *B. rapa* plants are resistant while others are susceptible, suggesting that the *Crr1a* gene might not explain the whole clubroot resistance in the original locus. They also found that *Crr1a* and *Crr1b* were tandem repeats in the same locus and both genes encode typical resistance gene proteins with TIR-NBS-LRR structures.

Based on the previous reports and whole genome sequencing data, clubroot resistance loci on chromosome 3 in *B. rapa* also contain multiple genes that encode TIR-NBS-LRR proteins. The complexity of those clubroot resistance loci needs to be investigated further. When a clubroot resistance locus contains multiple genes encoding the similar proteins, it becomes challenging to know how each individual gene plays a role in the clubroot resistance and how they contribute to the differences of alleles from various resistant sources. It is necessary to further dissect those complex clubroot resistance loci and investigate each individual gene to understand the functional properties of those loci. Therefore, gene functional analysis for clubroot resistance is still an important research focus in Brassica species.

9. Understanding the mechanism of clubroot disease resistance

The formation of galls on primary and secondary roots is typically characteristic of clubroot disease. The modification of root structure and decaying of root galls eventually damages plant

root systems so the plants may completely die or dramatically reduce productivity. Arabidopsis is a model plant and relative to the Brassica species, thus it has been successfully used in clubroot research. Malinowski et al. [96] investigated the relationship of cell division, gall formation, and clubroot disease development in Arabidopsis. Using those genes involved in cell division as molecular markers, their data suggested that reducing gall formation by inhibiting cell division would not prevent pathogen from finishing the life cycle while large galls may help pathogens produce more resting spores.

The expression of genes involved in the progression of clubroot disease may change so transcriptome analysis can be used to pinpoint the dynamic changing of gene expression in metabolic pathways for clubroot disease development. Schuller et al. [97] used laser microdissection and microarray analysis to check the changes of gene expression and found that the genes involved in the metabolism of plant hormones, especially auxin, cytokinin, and brassinosteriod, and plant defense-related hormones such as jasmonate and ethylene were differentially regulated. In another microarray analysis in Arabidopsis, Jubault et al. [98] observed that the major differences of gene expression in partial resistance interaction and susceptible interaction of the same Arabidopsis accession inoculated with two different clubroot isolates. The results showed that reduced or delayed metabolomic changes by pathogen and early induced classical defense responses were the major scenarios leading to partial clubroot phenotype instead of full susceptibility. More recently, Chu et al. [99] used RNA sequencing technology to identify over 2,000 genes that were expressed differentially in clubroot-resistant and susceptible plants. They found that those genes involved in defense responses such as jasmonic acid, ethylene, callose deposition, and indole glucosinolates were upregulated, and the expression of some genes in the pathway of salicylic acid did not show changes while the genes in the auxin biosynthesis and cell growth and development showed reduced expression in clubroot-resistant plants. By inducing clubroot resistance with an endophytic fungus, *Heteroconium chaetospira*, Lahlali et al. [100] detected the upregulation of genes involved in plant defense interaction such as PR-2 and genes in phenylpropanoid biosynthesis, and in the metabolism of plant hormones such as jasmonic acid, auxin, and ethylene using qPCR. Moreover, Verma et al. [101] performed miRNA analysis using miRNA-based microarray to detect differentially expressed miRNA during clubroot development. They further predicted the targets of those differentially expressed miRNA which belong to transcription factors, plant hormone-related and stress-related genes. In general, the data collected in those reports are quite preliminary and more research are required to know how each individual dominant clubroot resistance gene interacts with some avirulence genes in pathogen and eventually the interaction changes the expression of downstream genes which leads to clubroot resistance.

10. Transferring clubroot resistance through molecular marker-assisted selection in canola

Canola is one of the most important oilseed crops, and clubroot disease becomes a major limiting factor in canola production worldwide [102]. To develop resistant canola cultivars, several resistant sources such as European turnips, Chinese cabbage, and rutabaga cultivars

are available and the resistance in these sources are dominant, which makes it easier to transfer clubroot resistance through interspecific and intraspecific hybridization. Rutabaga cultivars have been identified as clubroot-resistant sources [66, 67, 69]. However, the genetics of clubroot resistance in rutabaga is complicated so it is difficult to develop molecular markers that are closely linked to the dominant clubroot resistance genes.

In Chinese cabbage, the dominant clubroot resistance from European turnips has been successfully used to develop clubroot-resistant Chinese cabbage. Since the gene mapping has been performed extensively in Chinese cabbage, molecular markers closely linked to clubroot resistance loci that are used in gene mapping can be easily selected to transfer clubroot resistance genes in the development of Chinese cabbage cultivars through molecular marker-assisted selection. Since canola, the amphidiploid *B. napus*, has a very complex genome, most of the molecular markers developed in *B. rapa* may not be polymorphic and cannot be directly used in canola. Additional efforts are required to develop molecular markers in canola when the mapped clubroot resistance loci in Chinese cabbage are transferred into canola. Currently, most clubroot resistance genes in European turnips have not been intensively investigated and mapping and cloning of these clubroot resistance genes in European turnips will allow using these genes effectively and efficiently in canola breeding.

Brassica species (R sources)	**Populations**	**LG**	**QTL/genes**	**Reference**
B. rapa (Chinese cabbage)	BC_1	A03	*Rcr1* fine mapped	[103]
B. rapa (Chinese cabbage)	F_2	A03	*CRb* fine mapped	[104]
B. rapa (G004 line)	F_2	A08	*Crr1a* fine mapped	[86]
B. rapa (Chinese cabbage)		A03	*CRa* fine mapped	[93]
B. oleracea (Anju)	DH	O2, O5,	pb-Bo(Anju)1, pb-Bo(GC)1	[24]
B. napus (synthetic line)	DH	N02, N03, N08, N13, N15, N16 and N19	Nineteen QTL identified on different LGs	[91]
B. oleracea (kale)	$F_{2:3}$	LG1, 2, 5	Nine QTL (Pb-Bo1 to Pb-Bo9) with phenotypic variance 20-88%	[84]
B. rapa (Shinki)	F_2	A03	*CRb*	[81]
Brassica oleracea (Bindsachsener)	DH	-	Two QTL (pb-3 and pb-4)	[83]
B. rapa (Chinese cabbage)	F_2, BC_1	A03	CR gene fined mapped	[95]
B. rapa (turnip line)	F_2	A03, A08	Two major QTL (Pb-Br3, Pb-Br8 and	[105]
B. rapa (European turnip)	$F_{2:3}$	A03	*Crr3*	[85, 87]
B. rapa (G004)	F_2	A06	*Crr4*	[88, 106]
B. rapa (Chinese cabbage)	F_2	A03 and A02	*CRk and CRc*	[107]

Table 1. Clubroot resistance QTL/gene mapped/fine mapped in different Brassica species by different research studies

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11. Management of clubroot resistance for effective utilization

The Brassica genomes (A, B, and C genome) are crucially important to provide novel genetic inheritance for economically important traits that can be used for the overall improvement of crop production and quality. For example, single genome of diploid *B. rapa* (A-genome) holds more than 230 R-gene sequences in 16 gene families [108], among which over 8 genetic loci have been identified in different research studies, which have functional properties for clubroot disease resistance. There are possibilities of the existence of more R-genes specific to the clubroot disease resistance and their allelic variations in different genetic pools or wild relative species. Effective utilization of resistance loci and their allelic variations may enhance the durability of resistance against clubroot disease in different Brassica species.

As a long history of clubroot disease revealed relatively high evolutionary patterns of the pathogen, *P. brassicae*. In various cultivating geographical regions of Brassica crops, persistence of *P. brassicae* pathotypes with high levels of pathogenicity poses challenges to breed durable clubroot-resistant cultivars. The breakdown of clubroot-resistant cultivars has become a serious problem in Chinese cabbage and leafy cabbage in China, Korea, and Japan [109, 110]. Effective management of resistance genetic resources in breeding novel cultivars could enhance the performance of resistance loci in different Brassica species for sustainable, more durable, and cost-effective control of the clubroot disease.

12. Summary and prospects of clubroot disease control

Crop plants are always challenged by various biotic and abiotic stresses. In agriculture cropping system, plant protection is being delivered using different approaches such as chemical control, various agronomic practices, biological control, integrated pest management (IPM), and cultivation of resistance cultivars. Among these approaches, resistance cultivars are the most economical, environmentally sustainable solution to control different diseases including clubroot in Brassica species. Previous studies suggested that the inheritance of clubroot resistance is either qualitative or quantitative in Brassica species. Recently mapped clubroot resistance genetic loci and closely linked molecular marker to these loci can be used for marker-assisted selection in clubroot resistance breeding of Brassica species. Extensive use of recently available resistance sources can be combined with molecular tools and new technologies such as gene/QTL mapping, fine mapping, gene cloning, comparative genomics and analysis of transcriptomic profiles through next-generation sequencing, which could enhance our understanding of clubroot resistance mechanism. Novel information can help controlling clubroot disease in an effective way, so yield losses would be reduced and the quality of Brassica crop product would be improved.

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Chapter 2

Molecular Farming in Plants

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Additional information is available at the end of the chapter

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Abstract

Plant molecular farming describes the production of recombinant proteins and other secondary metabolites in plants. This technology depends on a genetic transformation of plants that can be accomplished by the methods of stable gene transfer, such as gene transfer to nuclei and chloroplasts, and unstable transfer methods like viral vectors. An increasing quest for biomedicines has coincided with the high costs and inefficient production systems (bacterial, microbial eukaryotes, mammalian cells, insect cells, and transgenic animals). Therefore, transgenic plants as the bioreactors of a new generation have been the subject of considerable attention with respect to their advantages, such as the safety of recombinant proteins (antibodies, enzymes, vaccines, growth factors, etc.), and their potential for the large-scale and low-cost production. However, the application of transgenic plants can entail some worrying concerns, namely the amplification and diffusion of transgene, accumulation of recombinant protein toxicity in the environment, contamination of food chain, and costs of subsequent processing. The given threats need to be the subject of further caution and investigation to generate valuable products, such as enzymes, pharmaceutical proteins, and biomedicines by the safest, cheapest, and most efficient methods.

Keywords: Molecular farming, transgenic plants, biomedicines, protein stability

1. Introduction

Molecular farming is a biotechnological program that includes the genetic modification of agricultural products to produce proteins and chemicals for commercial and pharmaceutical

purposes. A vast majority of developing countries cannot afford the high costs of medical treatments resulted from the existing methods. Hence, we need to produce not only the new drugs but also the cheaper versions of the present samples in the market. Molecular farming can offer efficient solutions for the current growing need for the biomedicines [1]. Plants provide an inexpensive and simple system for the production of valuable recombinant proteins on large scale, and compared to the other production systems, they have numerous advantages in terms of economy, safety, and applicability. Though using transgenic plants has entailed some sorts of limitations and concerns, the optimization has been operated for solving the existing problems. Normally, the production of pharmaceutical proteins has been largely concentrated by the technology of molecular farming in plants, also plants can be used for the production of food supplements, biopolymers, industrial enzymes, and proteins in the investigations (avidin, β-glucuronidase, etc.). Prior production systems, including bacteria, microbial eukaryotes (yeasts, double-stranded fungi), animal cells, and transgenic animals, as a result of their limitations, were replaced by transgenic plants. The primary recombinant pharmaceutical proteins, extracted from the plants (hormones of human growth), and the first recombinant antibodies were generated from transgenic plants, respectively, in 1986 and 1989 [2, 3]. In 1997, the first recombinant protein, avidin (egg protein) was produced in a transgenic maize for industrial uses [4]. These applications proved that plants can be converted into bioreactors to produce a wide range of recombinant proteins. Many years had already passed when it was proved that plants were even able to produce several complexes of functional mammal proteins with the pharmacological functions, such as human serum proteins, growth regulators, antibodies, vaccines, hormones, cytokines, and enzymes [5]. An increasing request for the biomedicines was aligned with the high costs and inefficiency of existing production systems [6] including yeasts, bacteria, animal cells [7], and transgenic animals [8].

The aim of this study is to review the technologies of molecular farming, limitations and advantages of plant systems, challenges, bio-security, public acceptance of molecular farming.

2. The strategies of plant transformation

Plant molecular farming depending on the production of transgenic plants has been operated by two general methods as the following:

2.1. Stable or permanent expression systems

a. Stable nuclear transformation: Stable nuclear transformation refers to the integration of genes or nominated foreign genes into the nuclear genome of plants, which results in the change of genetic structures and consequent expression of transgenes after integration with the host genomes. The largest amount of recombinant proteins has been produced by one of the most common methods of stable nuclear transformation. A method exploited for aggregating proteins in dried beans of maize culminates in a long-term storage in the beans at the room temperature without decomposition of proteins [9]. In addition, it has a considerable

potential for producing crops like cereals that actually grow everywhere. However, a long production cycle for some crops and their potential collisions with natural species or food products have restricted the wide acceptance of this method [10].

b. Stable plastid transformation: Plastid transformation offers a remarkable solution in comparison to that of nuclear transformation since it has numerous advantages including preventing transgene escape through amphimixis (because plastids are inherited through the maternal tissue in the majority of species.) and absence of chloroplasts in pollen and consequent improbability of their transfer, which reduces environmental concerns [11, 12]. The transformed transgenic plants with homoplasmic chloroplasts (all chloroplasts carry transgenes) were selected after several generations of plant regeneration from bombarded leaf explants. Selection was conducted on a medium containing spectinomycin or combined with streptomycin. The researchers [13, 14] have already extracted a human pharmaceutical protein, more than 3% to 6%, from the total soluble proteins in the chloroplasts of tobacco. Recently, Oey et al. [75] reported a very high level (70% of an entire soluble protein) for a protein antibiotic with the chloroplast system, which, till today, has been the highest concentration of recombinant proteins. Despite this, the great potential of plastid transformation has some functional limitations. Although this technology has been developed in other species such as tomatoes, lettuce, soy, and eggs [15, 16], in the current situation, chloroplast transformation only in tobacco is practically possible, but unfortunately this plant is inedible and full of poisonous alkaloids; in addition, long lasting storage in refrigerators will bring about changes in protein stability [9].

c. Plant cell suspension culture: This method involves the removal of cell walls and gene transfer to the obtained protoplasts and suspension culture. The purification system and its downstream processing are cheaper and easier [17]. In addition, the use of suspension culture can decrease heterogeneity in proteins and sugar (N-glycans) regarding the uniformity of the type and size of cells [5]. Furthermore, as a fast system there is no need for the production of transgenic plants; however, the cell lines can be produced after a few months [18, 19]. Some samples of plant suspension cultures can be used for producing biomedicines, including vaccines of Newcastle disease virus of chicks approved by the Center for Veterinary Biology and recombinant human glucocerebrosidase for treating diabetes (www.protalix.com) [19]. Though this method is cheaper, safer, and easier in comparison to the other methods of genetic manipulation, cell suspension has not yet been suggested as an optimal production choice of production in plant systems. This is due to a belief that the ultimate products and their usability are constrained by reducing the level of recombinant proteins during the stationary phase because of the enhanced proteolytic activity [20].

2.2. Temporary or transient expression systems

A transient production may be the fastest system for plant molecular farming [21]. Nowadays, these are the systems routinely applied for verifying expression constructs during a few weeks for a significant amount of proteins [22]. The given systems include the following methods:

a. Agrobacterium transformation method: Infiltration of recombinant agrobacterium suspension into tobacco leaf tissue is achieved without stable gene transfer, which facilitates the transfer of T-DNA to a very high percentage of cells, where the transgenes are expressed at a high level without a stable transfer of genes. Presently, this method has been very efficient for the production of clinical biomedicines with a fast expansion [22-24].

b. Viral infection methods: The viral infection method depends on the capability of plant viruses, such as tobacco mosaic virus and X potato virus, which functions as a vector to convey foreign genes into plant genomes without fusing with the genome of that plant [25].

c. Magnifection system: Expression systems based on viral vectors and agrobacterium methods suffer from some constraints for the co-expression of two or more polypeptides required for the production of *hetero*-oligomeric proteins [26]. Thus, a new transient expression system known as MagnICON technology has been developed by Icon Genetics Company. This method includes removing coat proteins (responsible for systemic movement) of non-competitive virus stains and systemic delivery of the derived viral vectors to all of the plants using agrobacterium as the medium of primary infection. This method not only optimizes the infection but also significantly increases proliferation, and finally results in the co-expression of several polypeptides and the rise of functional protein production more than 100 times.

3. The advantages of utilizing transgenic plants as bioreactors

Comparison of different expression systems (see Table 1) reveals the advantages of plants in comparison with other expression systems as follows:

- The healthiness of derived products (plants cannot be the host of human pathogens and bacterial toxins).
- Capability of post-translational processing (respecting the features of eukaryotic cells).
- The possibility of using breeding methods and sexual crosses to obtain active recombinant multi-chain proteins (therefore, there is the possibility of producing antibodies without application of a double transformation).
- Reducing the costs of production (plants can produce biological materials by the use of carbon dioxide, solar energy, and inorganic materials. Moreover, the scale of production can be manipulated regarding scalability).
- Reducing the costs of storage and transportation of recombinant proteins (when they are produced in dry textures like grains).
- Removing the purification step (when the plant tissues containing recombinant protein are edible) [1].

Characteristics	Bacteria	Mammalian cell culture	Transgenic plants	Plant cell culture
Production cost	Average	High	Low	Low
Post-translational modifications	No	Yes	Yes	Yes
Function	High	Average	High	High
Protein stability	Yes	Yes	Yes in seeds	Yes

Table 1. Comparison of Various Expression Systems for Producing Recombinant Proteins

4. The limitations and optimization of plant production systems

4.1. The problem of product shortage or the same recombinant proteins

4.1.1. 3.1.1. Optimization of expression of transcripts

To optimize the expression of transcripts, a widely used strategy is the use of building promoters, such as cauliflower mosaic virus 35S RNA promoter and maize 1-ubiquitin promoter, respectively, suitable for spilt-cotyledons and single-cotyledons [27]. Tissue-specific and organ-specific promoters are used for stimulating the expression of transgenes (antigen vaccine HBsAgM, single-chain variable fragment Maureen G4, and Human interferon-α) in some tissues or organs, such as tubers, seeds, and fruits [28, 29]. The given specific expression of tissues prevents the accumulation of recombinant proteins in vegetative organs, which can have a negative impact on plant growth; for example, *palatine* is a gland-specific promoter; i.e., the protein is expressed in the gland but not in leaves; and also ubiquitin promoter is specified for the embryonic tissues of plants. Transcription factors (e.g., AlcR) can act as the invigorator of promoters to increase the level of transgene expression [30]. The stability of transcripts of genes can be achieved by co-expression of the specified gene and an RNA silencing inhibitor [31].

4.1.2. Optimization of translation

Expression constructs can be designed for guaranteeing the efficiency of translation and the sustainability of transcripts. As an instance, the removal of 5' untranslated region and natural ′3 for foreign genes and introducing the leader sequence of tobacco mosaic virus RNA, RUB13 rice polyubiquitin gene, alfalfa mosaic virus, or tobacco viruses in the expressions, all, individually, have shown a significant increase in the level of transgene expressions [32, 33].

In addition to the leader sequences, expression cassettes can be designed with the AU-rich sequences in 3' untranslated regions, which may change or be removed as the editing sites for ensuring the stability of transcript. It is also proved that every organism shows codon usage deviations that may be the subject of importance for adapting the coding sequence of heterologous genes for the host gene to optimize the efficiency of translation. In this regard, the site of initial translation from heterologous protein to pair with Kozak consensus sequence, with

the application of GCTTCCTCC sequence, started after codon or ACC, or ACA had been changed before that. It is better to unscientifically estimate codon changes rather than their real amount considering the changes in the expression level of transgenes in similar systems and the use of similar structures. To this end, an increase of codon combinations of (A/G)(a/c)(a/g)AUG and (A/g)(u/C)(g/C)AUG for the optimal operation of translation was, respectively, reported in Arabidopsis and rice. The given change in transgene expression could be due to the position effect, number of transgene copies, or gene silencing.

Regarding the effect of position, expression cassettes can be designed to have nuclear matrix attachment regions for ensuring the transgene insertion in proper sites for stimulating transcription factors for promoters. Furthermore, the problem of position effect can be prohibited by targeting the transgene to plastids. To optimize the production of single-cotyledon transgene, the strategies that include the use of specific genetic elements containing cAMP response elements for a simultaneous transfer with transgene in T-DNA are used. In addition, one new technology, including the structure of an artificial autonomous mini-chromosome, can genetically materialize excellent possibilities with several advantages, namely genetic stability due to the absence of gene silencing and position effect.

4.1.3. Optimization of protein stability

To optimize the stability of recombinant proteins, known as the most important limiting factor for the function of molecular farming [34], the targeting of proteins into certain intracellular parts is demanded. The intracellular targeting not only increases protein stability but also determines the processing type of dependent protein. This can be applied for the optimization of isolation and procedures of purification by the fusion proteins and targets with high affinity [27]. Targeting of proteins can be done by the following pathways and organelles,

- The intracellular parts, like *protein storage vacuoles,* have been discovered for the accumulation of recombinant proteins [30].
- Cathepsin D *inhibitor* can act as the agent of stability *of protein structures* to protect the targeted recombinant proteins in the cytosol of plants [35]. Recombinant protein production through this signal has been proved to be very effective and economical [36-38].
- To protect proteins from cytosolic degradation, these proteins can be targeted by fusion to a C-terminal tail without a forced passing through the lumen of the endoplasmic reticulum to the membrane surface [39]. To enhance the ease of purification, proteins can be fused to *oleosin* proteins as oil bodies in order to target protein expression with the oil bodies of seeds.
- The proteins, like in glycosylation, that do not need post-translation modifications for their activity, can be targeted to chloroplast since post-translation modifications are not conducted in these organelles [40].
- Targeting for accumulation in endoplasmic reticulum is accomplished by two methods: one is adding SEKDEL endoplasmic reticulum signals to the end of C-protein, and the other is using fused N or C signals with y-zein. Endoplasmic reticulum is an oxidizing environment with high amounts of *chaperone proteins and low levels of proteases.* This pathway is suitable

for the proteins that need post-translational modifications (e.g., glycosylation) [76]. The breakdown of proteins by proteases (proteolytic degradation) outside the cell is another noticeable factor for investigating the plant-based production of biomedicines.

4.2. Challenge of glycosylation (protein quality)

Glycosylation refers to the covalent binding of sugars to proteins in order to increase close-packing, biological activity, solubility, and biological functionality [5]. Glycosylation takes place in plants in the secretory pathway of endoplasmic reticulum and golgi apparatus. The glycosylation patterns of plants and animals differ in the composition of N-glycans; plants add residues of α (1, 3) fucose and β-(1,2) xylose to N-glycans of their protein, but animals add residues of (1 and 6) fucose, glucose, and sialic acid to N-glycans. These differences can be problematic for humans when medical animal proteins extracted from plants are used (Krupp et al., 2003); consequently, a correct human N-glycosylation demands a plant engineering. A number of strategies for changing the pattern of N-glycosylation in plants have been elaborated as following [71]:

- The use of purified enzymes of β-(1,4) galactosyltransferase and Sialyltransferase for making glass transitions in the recombinant proteins derived from plants.
- Co-expression of β-(1,4) galactosyltransferase human enzyme with the target transgene in transgenic plants.
- Prohibiting the activity of fucosyltransferase and xylyltransferase enzymes.
- Targeting pharmaceutical proteins to the endoplasmic reticulum in order to avoid the addition of protective N-glycans.

4.3. Selecting appropriate host plants

Major economical factors in appointing an appropriate host include the total biomass yield, storage characteristics, ease of transport, value of recombinant proteins, maintenance costs, its availability for workers, required area, duration of production cycle, cost of subsequent products, and edibility [27, 34]. In addition to the economical analysis, a sufficient host should be appropriate in terms of transformation and regeneration [34, 72]. In addition to the high potential of tobacco for transformation and regeneration, it has the majority of the aforementioned economic benefits [27, 41, 42]. However, tobacco (except the *cultivar 81 V9*) [43] contains high amounts of toxic combinations, nicotine and other alkaloids, that cannot be removed during the purification process. In spite of this, alternative forage crops like alfalfa and lettuce are being investigated and discovered as a suitable host for molecular farming [44]. However, forage plants generally suffer from the problem of instability of expressed proteins, by which drying and freezing of the leaves and immediate processing following the harvest have been inevitable [27]. The seed-based expression of proteins is considered to be more ideal regarding the fact that it neither affects the growth of plants nor needs the freezing of leaves or immediate processing after harvest, and it allows the long-term storage of proteins at a limited temperature without decreasing the level of activity [45, 46]. In this regard, grains, especially rice and

corn, have been cited as the superior ones. Maize has abundant advantages, such as having the highest rate of biomass yield among food crops and ease of transformation and production increase [47]. The high amount of protein (20%-40%) in the grains of legumes with remarkable levels of self-pollination in soy and peas is the main reason for transgenes of these plants for protein accumulation [48-50].

5. Predicting the intracellular localization of the recombinant protein

The importance of intracellular localization of proteins is due to the functional consequences of proteins. Therefore, the problem of intracellular localization of amino acid sequences has been the subject of great attention in the community of bioinformatics. Thus, various methods, like searching for targeted signals, have been presented with respect to a prediction that various proteins are produced in different intercellular segments [51].

6. Proteins and biomedicines produced in plants

Plants are able to produce those bacterial and viral recombinant antigens that preserve the capability of making the structures Type IV similar to those witnessed in mammalian systems, and the post-translational modifications are operated to maintain the biological activity of proteins. The most important issue is vaccine production in the edible tissues of transgenic plants, which is a very safe and effective method in vaccination.

The biomedicines produced in plants are as follows:

- Antigens for the production of edible vaccines: Antigens, used for generating an immune response resulting in immunity against diseases in human proteins, are expressed from different pathogens in plants. Those vaccines derived from plants have been so far induced immunity against rabies virus, hepatitis B, rotavirus, HIV, and other pathogens.
- Monoclonal antibodies: Widespread application of antibodies has lead to the study of new methods in order to strengthen efficiency and reduce the cost of producing antibodies. Among the studied methods, using transgenic plants as bioreactors are known as the most efficient one. While designing therapeutic antibodies in the production of recombinant expression systems, the apprehension of the functioning mechanisms of antibodies is essential. Although the primary function of antibodies is actualized by binding to antigens, it does not act as a protective performance. Some antibodies have a direct neutralizing impact, for instance blocking the bacteria or the active sites of the pathogenic factors such as enzymes. The antibodies produced in plants incorporate Immunoglobulin G (IgG) and Immunoglobulin A (IgA), IgA and IgG shimmer molecules, IgG and IgA secreted molecules, Single-Chain variable fragment, fragment antigen-binding, and second variable of heavy and light chains [52-54].

- Pharmaceutical proteins: Some samples of biomedicines recently expressed in plants include *erythropoietin, interferon*, hirudin, aprotinin, Leu-enkephalin, somatotropin of human growth hormone [55, 56].
- Non-pharmaceutical proteins derived from plants or industrial proteins belong mainly to the enzymes that include avidin, trypsin, aprotinin, β-*glucocerebrosidase, peroxidase* and *cellulose,* etc., listed by Basaran and Rodriguez-Cerezo [73] and now available in the market. Molecular farming of destructive enzymes of the cell walls such as cellulose, *hemicellulase,* xylanase, and particularly *ligninase* provide a great status for the biofuel industry respecting cellulosic ethanol [57, 58].

7. Molecular farming and metabolic engineering, an opportunity for producing plants with a high technology

Molecular farming and metabolic engineering make the production of new high-tech products possible. There is a driving force backing molecular farming that makes its costs much less than traditional farming. *Chlamydomonas reinhardtii*, as a unicellular alga, is one of the most recent production projects examined by Franklin and Mayfield. *C. Reinhardtii* is the only plant whose transformation was operated in its all segments containing DNA (nucleus, plastid, and mitochondria). Unique features of the moss system bring about the possibility of removing target genes and purification of the proteins secreted from the culture medium. The target gene was omitted to get rid of the nuclear genes for glycosylation. The first step towards the long-term goals of reengineering mechanism in modifications of plant proteins is setting a new standard in all systems of plant expressions in order to humanize the biomedicines produced in plants [59].

8. Purification and downstream processing of the recombinant proteins

Recovery usually includes the process and breakdown of plant tissues, protein extraction, solid-liquid separation, and protein concentration while purification encompasses safety protection, liquid-liquid extraction, membrane filtration, chromatography, etc. The processing of leaves requires a particular attention; leaves should be processed immediately after the harvest or frozen to prevent protein degradation by proteases, whereas seeds can be stored for a long period of time due to the less probability of destruction of recombinant proteins expressed in seeds. Using the secretory systems of cells can also be beneficial since disintegrating plant cells throughout recovery is not required; thus, the release of phenolic compounds can be avoided while the recombinant proteins can be unstable in culture mediums. Another way of facilitating the recovery of proteins is utilizing continuous labels. Protein labels must be removed after purification so that the structure of purified protein can change into its natural position. The technology of oleosin fusion, through which the gene sequence of recombinant proteins is fused to the sequence of a special internal oil protein called oleosin in

safflower and canola, is separated after the digestion of internal protease following protein purification [1].

9. Costs of subsequent processing

The costs of subsequent processing of the recombinant proteins derived from plants have been estimated about 80% of the total production costs [60, 61]. This is why so much attention has been paid at sufficient strategies for reducing the costs to the least amount. The application of watery textures like tomatoes as a production system has been expanded because of their potential for reducing the costs via the ease of extracting from their textures in comparison with those of dry tissues like grains [34, 62]. In addition, tomatoes are highly regarded as a reputed host crop in terms of its bio-safety because these plants grow in greenhouses without worrying about the preservation of transgenic plants.

Nowadays, oil bodies of oilseed agricultural products, like the seeds of safflower and mustard, are being exploited by the application of oleosin fusion technology developed by SemBioSys Genetics in order to facilitate the purification of recombinant proteins and reduction of subsequent costs (http://www.sembiosys.com/). The strategies including targeting of recombinant proteins for the seeds of oilseed agricultural products as an oleosin fusion facilitate the extraction of fused proteins from oil bodies and the release of the recombinant proteins from their fusion partner; one example can be the accumulation and purification of biologically active human insulin, apolipoprotein *A-I* (Milano) and human growth hormone in safflower [63-65].

There are several recombinant proteins derived from plants that were the basic idea of edible vaccines, directly eaten as fruits (tomatoes and bananas) and vegetables (lettuce and carrots); accordingly, no processing costs will be demanded by the elimination of processing, [66]. Bananas, as a fruit host in agricultural products, have particularly attracted lots of customers for the production of edible vaccines, especially for developing countries. This has been widely developed in such countries because of long distance transports and cooling requirements [42]. Apart from the mentioned advantages, high digestibility and palatability of bananas have won a wide public acceptance for the vaccination of children [67, 68]. The sufficiency of potatoes, eaten in raw or low processed forms, for edible vaccines has resulted in their wide production. Potatoes, like seeds, have the advantage of production stability due to a special molecular environment allocated in glands [69].

10. Bio-safety and the challenges in the domain of protein production and biomedicines in molecular farming

The risks of transgenic plants are divided into two categories: one category directly affects humans and the other endangers environment and other organisms. The attack of immune

system can disable these medicines and lead to the stimuli for the allergic reactions, some of which have been elaborated as follows:

- There are some concerns in terms of environmental pollution about the entrance of transgenes into the food chain, which requires a sound management and supervision.
- The other concern refers to the grain transformations using agrobacterium since grains are important crops in the production of pharmaceutical protein.
- The reactions of immune system can disable the medicines produced in plants and be the stimuli for allergic reactions [70].

11. Perspectives upon the commercial production of medicines and pharmaceutical proteins in molecular farming

The development stages and subsequent commercialization of the products is the subject of consideration in the second phase of clinical trials. A number of small biotechnology companies have aimed to commercialize the antibodies produced in plants. It has been estimated that the increasing annual need for secretory IgA will be 13%, and a rate of $25 billion was predicted as the annual income of producing IgA in crops. While there have been great advances in the field of biomedicine production in plants on large scales, fundamental studies are demanded to pave the way for the commercialization of these products. The present problems include the difficulty of low yield of protein, the possibility of harmful effects on the function/performance of proteins due to the differences in glycosylation patterns, and the severe potential impact of expressing plants of biomedicine plants on the environment (e.g., concerns upon genetic limitations) [74].

12. Conclusion

The aim of molecular farming is to produce large quantities of active and secure pharmaceutical proteins with lower prices. With the scientific advances in the field of bio-technology, nowadays, gene transfer methods in plants have considerably developed. These transgenic plants in comparison with other microbial and animal expression systems have various advantages in terms of easy production, cost, safety, etc. for producing pharmaceutical biomolecules. So far, lots of valuable pharmaceutical proteins and antibodies have been produced by the help of this method, which remarkably has helped the treatment of patients especially in developing countries where the production and preservation costs of such medicines cannot be afforded. However, there are some disputes, such as public acceptance, transgene escape and biosecurity, clinical and commercialization investigations of products, etc., which has made it a challenging area, but it is hoped that in near future molecular farming will witness great achievements with the researchers and scholars' efforts.

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Section 2

Abiotic Stress

Chapter 3

Biochemical Parameters in Tomato Fruits from Different Cultivars as Functional Foods for Agricultural, Industrial, and Pharmaceutical Uses

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Additional information is available at the end of the chapter

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Abstract

Tomato and tomato based products are an important agricultural production worldwide. More than 80 % of grown tomatoes in the worldwide are processing in the products such as tomato juice, paste, puree, catsup, sauce, and salsa. Tomato fruit is rich in phytochemicals and vitamins. Tomato nutritional value, color, fruit and flavor of their products depends mainly on lycopene, β-carotene, ascorbic acid and sugars and their ratio in fruits. Epidemiological studies and the results associated with the consumption of tomato products against the prevention of chronic diseases such as cancer and cardiovascular disease, confirming the tomato products as a functional food, and show that lycopene and β-carotene acts as an antioxidant. In order to increase the amount of these elements in tomato fruit, it is important to evaluate and investigate tomato genotypes influence to the carotenoids accumulation. Studies have confirmed that the carotenoid content in tomato fruits is determined by genotypic characteristics. In this work the main attention will be focused on from the biochemical and physical properties in tomato of different varieties, chemical and physical properties, to functional properties of supercritical fluid extraction of lycopene from tomato processing by products supercritical fluid tomato extracts.

Keywords: biochemical composition, physical properties, phytochemicals, nondestructive analysis, supercritical fluid extraction

1. Introduction

Tomato is one of the most valuable and popular vegetables worldwide. It is desirable that tomatoes are fertile and disease resistant, and each cultivar differs in fruit size, shape, taste, colour, and skin and flesh firmness. Tomatoes must also be resistant to transportation conditions to meet market requirements and consumer needs, as there is an increased demand for large-fruit salad-type tomato varieties. Tomatoes and tomato-based products are an important agricultural commodity worldwide. More than 80% of tomatoes grown worldwide are processed into products such as tomato juice, paste, puree, catsup, sauce, and salsa. Tomato fruit is rich in organic acids, sugars, dietary fibre, pectic substances, proteins, fats, minerals (potassium, phosphorus, sulphur, magnesium, calcium, iron, copper, and sodium), vitamins (B1, B2, B3, PP, C, provitamin A, I, and H), and carotenoids possessing antioxidant qualities (lycopene, β-carotene, etc.). Due to the importance of vegetables in the human diet, it is recommended to consume 400 -500 grams daily, 140 to 150 kg per year of various vegetables, including 25 to 32 kg of tomatoes for an adult human [1, 2]. The nutritional value, colour, and flavour of tomatoes and their products depend mainly on lycopene, β-carotene, ascorbic acid and sugars, and their ratios in the fruits. The two most important carotenoids in tomato fruits are lycopene, which determines the fruit's red colour, and β-carotene, which accounts for approximately 7% of tomato carotenoids [3]. Therefore, tomato products and their quality can be characterised by the contents of these elements. Humans gets 85% of their lycopene from tomatoes and tomato products [4], which is the reason why tomatoes are used in functional food products [5], and sometimes as functional foods [2, 6]. Epidemiological studies and other studies associated with the consumption of tomato products for the prevention of chronic diseases, such as cancer and cardiovascular disease, confirm that tomato products are functional foods and show that lycopene and β-carotene act as an antioxidant [7, 8]. In order to increase the amounts of these elements in tomato fruits, it is important to evaluate and investigate the influence of tomato genotypes on carotenoid accumulation. Previous studies have confirmed that the carotenoid content in tomato fruits could be determined by genotypic characteristics [9, 10]. This paper focuses on the biochemical and physical properties of tomatoes of different varieties, their chemical and physical properties, and the functional properties of supercritical fluid extraction of lycopene from tomato processing.

2. Tomato biochemical composition

Currently, the food industry advocated increasingly in synthetic antioxidants changes by the 'safer natural mixtures'. This option has been made available through the worldwide consumer preference for natural antioxidants, some of which are added intentionally during processing and some exist inherently in foods. Between them, carotenoids comprise the group of the most abundant micronutrients in fruits and vegetable; also, their dietary consumption is related with a lower frequency of some cancer types of as well as reinforces prevention against the cardiovascular diseases [11-13].

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Flower and fruit colour is caused by different types of pigments belonging to the terpenoid and phenylpropanoid classes. Carotenoids, chlorophylls, and anthocyanins are the main three groups of pigments. Colour characteristics, in some plants, can be determined by domestication of agronomic traits, while in others, the increase of these pigments in tissues can occur naturally. This could be applied to tomato (*Solanum lycopersicum* L.), which has several carotenoids, such as lycopene and β-carotene, among others. The amount of these carotenoids is principally determined by the tomato cultivar and genotype [14, 15]. It has been established that carotene, nitrates, and sugar amounts in fruits and root crop vegetables depend on plant genotype, meteorological conditions, fertilisation, and soil composition [16-19]. The levels of the essential antioxidant vitamins, in contrast with other antioxidative defences, are determined mainly by the plant's dietary supply. One major vitamin for enriching human diets is the antioxidant vitamin C (ascorbic acid). This vitamin can counteract the oxidising effects of lipids by scavenging free radicals that have been found to be major promoters of certain diseases. Recently, it has been demonstrated that carotenoids react cooperatively and synergistically with vitamin C, serving to regenerate a pro-oxidant radical carotenoid following the antioxidant reduction of a radical species [11]. Vitamin C usually found in vegetables and fruits, and it is a natural antioxidant. Ascorbic acid plays an important role in biochemical processes such as the formation of collagen, absorption of iron, and its involvement in the immune response and the synapses. However, a high amount of this antioxidant for human could be painful and cause adverse effect. Thus, the precise determination of ascorbic acid in various plant species or cultivars is very important [21-25]. There seems to be little doubt that acids and sugar not only contribute to the sweetness and sourness of tomatoes but are also major factors in overall flavour intensity. Since the lack of flavour is a common complaint about fresh market tomatoes, increases in sugar and acid contents could make a contribution to improve tomato flavour [16].

Nitrate is very important for plant functions and nutrition. It is a part of the nitrogen cycle and occurs naturally. Human exposure to nitrate is mainly exogenous through the consumption of vegetables, and to a lesser extent through water and other foods. Vegetables are the major vehicles for the entry of nitrate into the human body. Ever-increasing concerns over nitrate toxicity have directed a number of countries to institute maximum allowable threshold concentrations of nitrate-N in vegetables [26].

Tomatoes and tomato products are major sources of lycopene compounds and are also considered an important source of carotenoids and vitamins in the human diet [27, 28]. Therefore, considerable work has been conducted to increase their levels in tomatoes through breeding programmes [29]. The amount of carotenes and their antioxidant activity as well as their biochemical composition are significantly influenced by the tomato variety and maturity [20, 30]. The importance of genotype selection for high nutritional value is outlined first, followed by the optimisation of environmental conditions and agricultural practices [31]. Normalised values for lycopene contents of different tomato cultivars in California ranged from 8.4 to 17.2 mg 100 g^{-1}, representing a 100% difference from the lowest to the highest values [32]. According to Viskelis, the highest amount of lycopene (over 10 mg 100 g^{-1}) was found in the Lithuanian cultivar 'Rutuliai', which was 1.6 times higher than that of the hybrid 'Admiro' and twice as high as the hybrid 'Kassa' [33].

Based on the multiannual data [34] of 10 cultivars ('Viltis', 'Milžinai', 'Skariai', 'Laukiai', 'Vėža', 'Pažar', 'Vilina', 'Ruža', 'Ranij 310', and 'Elbrus') investigated in Lithuania (Fig. 1), the highest level of lycopene was established in cultivars the 'Ranij 310' (13.56 mg 100 g^{-1}) and 'Elbrus' (12.57 mg 100 g^{-1}).

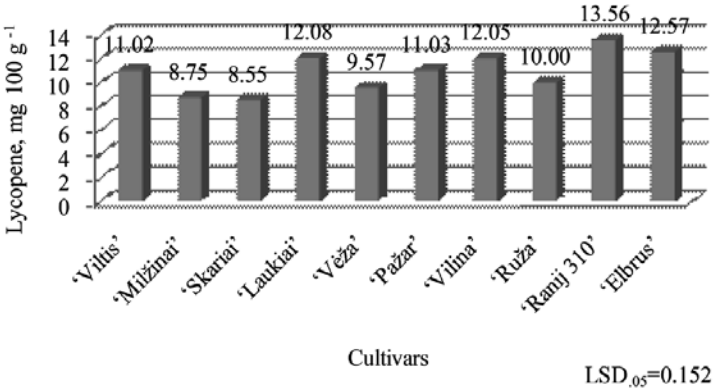

Figure 1. The amount of lycopene in tomato fruits.

The least amount of lycopene was found in the fruits of the cultivars 'Skariai' (8.55 mg 100 g^{-1}) and 'Milžinai' (8.75 mg 100 g^{-1}). In other cultivars, the lycopene amount had varied from 9.57 ('Vėža') to 12.08 mg 100 g^{-1} ('Laukiai'). Lycopene is the most abundant carotene in red tomato fruits and accounts for up to 90% of the total carotenoids. Typical red-pigmented tomato fruits also contain a lesser amount of β-carotene and other carotenoids. β-Carotene occurs in tomato fruits in various amounts from 0.23 to 2.83 mg 100 g^{-1} [35]. Our studies showed (Fig. 2) that significantly higher amounts of β-carotene were accumulated by two cultivars, 'Ranij 310' and 'Elbrus', with 2.34 and 2.16 mg 100 g^{-1}, respectively. The least amount of β-carotene was found in the cultivar 'Vėža' (1.33 mg 100 g^{-1}). Most of the investigated cultivars had similar amounts of β-carotene, which varied from 1.43 to 1.70 mg 100 g^{-1}.

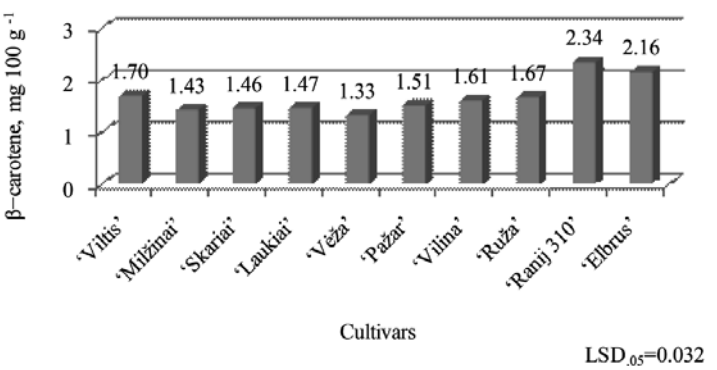

Figure 2. The amount of β-carotene in tomato fruits.

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Tomatoes are a good dietary source of ascorbic acid (vitamin C); however, the ascorbic acid content in tomatoes varies greatly. Many factors contribute to this variation, and environmental growing conditions and cultivar genotype have been reported as having major effects on ascorbic acid composition [21, 22]. There is a wide variation of ascorbic acid content in different cultivars. According to Mathews, the vitamin C values for 41 cultivars ranged from 10.7 to 20.9 mg 100 g^{-1} (23). Ten years of data presented by a Lithuanian scientist showed that the average amount of ascorbic acid was 16.20 mg 100 g^{-1} in different tomato cultivars [36]. According to our data (Fig. 3), the cultivar 'Vilina' had a significantly higher amount (15.9 mg 100 g^{-1}) of ascorbic acid compared with the other eight cultivars, except for the value of the cultivar 'Laukiai', which was not significantly different (12.2 mg 100 g^{-1}). The least amount of ascorbic acid was found in the cultivar 'Viltis' (7.8 mg 100 g^{-1}).

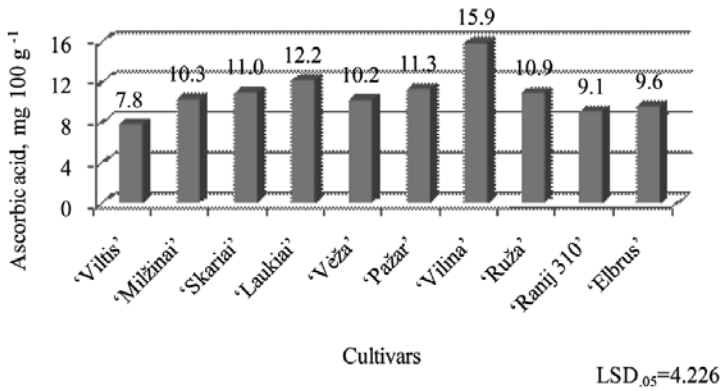

Figure 3. The amount of ascorbic acid in tomato fruits.

Sugars and acids are particularly important taste constituents of tomatoes. The sugar content of ripe tomatoes averages 3% [37], but in Lithuanian-grown tomatoes, the average amount of total sugar is 4.37% [2]. Other researchers have reported that the amounts of total sugar varied little for different cultivars and ranged from 4.01% to 4.17% [33]. In our research, the total sugar content had a small amount of variation, from 4.32% in cultivar 'Viltis' to 5.03% in cultivar 'Elbrus' (Fig. 4).

The nitrate content in vegetables may range from 1 to 10000 mg kg^{-1}. The various reasons for this wide range are the excessive use of fertilisers, crop variety, types of N-fertilisers, light and temperature conditions, and lack of water [26]. A combination of these factors accounts for the different nitrate values reported for vegetables in different countries. A complicating factor for the nutritional exploitation of vegetables is the presence of nitrate (nitrite), which is antinutritional and toxic in nature. Nitrate content is an important quality characteristic of vegetables [38]. Amr and Hadidi reported that cultivar had a significant effect ($P \leq 0.05$) on the nitrate content of greenhouse grown tomatoes [39]. Tomatoes accumulate low contents (100 -150 mg kg^{-1}) of nitrates. This was demonstrated in our investigation (Fig. 5); all cultivars had a low content of nitrates compared with other vegetables, and the amount of nitrates ranged from 55 ('Vilina') to 91 mg kg^{-1} ('Elbrus').

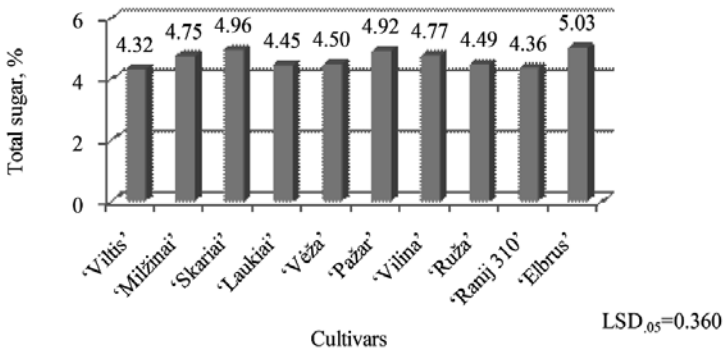

Figure 4. The amount of total sugar in tomato fruits.

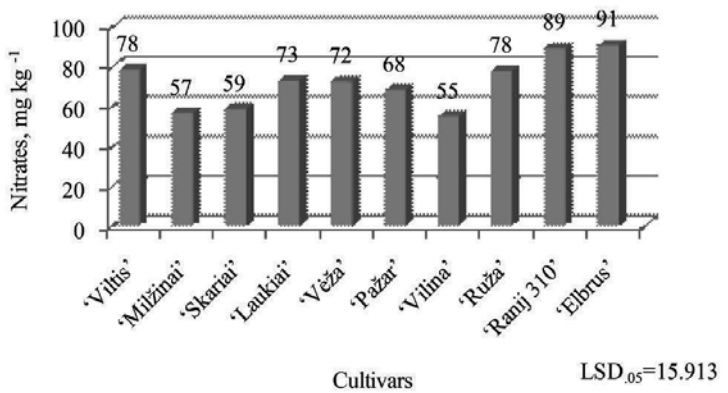

Figure 5. The amount of nitrates in tomato fruits.

2.1. Fruit biochemical composition of organic tomato

Consumers are becoming more interested in the environmental problems caused by agricultural activities, and there is an increased focus on the health risks resulting from the use of various chemicals. The growing phytosanitary problems and the unrelenting use of pesticides has led to new challenges in food safety. The sustainability of conventional agriculture is being questioned, and changes are needed in agricultural sciences. Currently, the development of organic growing systems is rapidly emerging as a national priority, and many countries have certified organic agriculture programs. The organic food market is developing dynamically in many countries, and therefore, studies concerning the nutritive value of organically grown products are becoming increasingly important [40, 41].

Tomatoes are an excellent plant for the comparison of fruit quality attributes between conventional and organic systems due to their global value and popularity. Unfortunately, organic cultivation has a markedly negative effect on yield, and organic fruits have more visible defects compared with conventionally grown fruits [42]. However, consumers expect organic food to have a higher nutritional value, to be healthier, or simply to be safer and less risky. Scientists

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have reported that conventional crops have higher levels of protein and vitamin E, carotenoids, and alkaloids, while organically grown crops tend to have more phytic acid, phenolic compounds, glucosinolates, and vitamin C. However, studies have shown that the relative impact of adopting organic production methods on food quality and safety may change over time, according to changes of soil characteristics and plant cultivars [43]. Organic foods are perceived by many consumers as safer and healthier compared with conventional ones. Organic farming enhances the long-term natural fertility of the soil, minimises soil pollution, and avoids the use of mineral fertilisers and pesticides, which lead to positive health effects for livestock and humans consuming organic foods. Fruits and vegetables have positive health benefits and contain significant amounts of biologically active compounds that may be responsible for these effects [44].

Tomato fruit quality composition varies due to a wide variety in species, stage of ripeness, year of growth, climatic conditions, light, temperature, soil, fertilisation, irrigation, and other conditions of cultivation. The amount of total and soluble solids in tomato fruits are a major economic parameter for their nutrition value. The average dry matter content of a ripe fresh fruit ranges between 5.0% and 7.5% [45]. Earlier studies noticed statistically significant differences in the content of dry matter in organic tomato fruits. Studies have reported that organic tomatoes contain, on average, 7.86% dry matter in fresh tomato fruits, compared with 5.07% dry matter in conventionally grown tomatoes. An investigation of different cultivars showed that cherry tomato contained the highest levels of dry matter compared with other tomatoes [42].

An investigation of different farming systems was carried out by the Institute of Horticulture Lithuanian Research Centre for Agriculture and Forestry [46]. Tomatoes were grown using two different farming systems (organic and conventional) in unheated greenhouses in natural soil, using the tomato cultivar 'Vilina' and two tomato hybrids, 'Benito' and 'Tolstoi'. Organic tomato plants were sprayed twice (on the 7th and 21th of July) with an organic fertiliser based on seaweed extract (*Ascophyllum*, 15% w/w). Conventional tomatoes were grown under conventional tomato growing technologies adopted by the Institute of Horticulture [46].

The results of this research agreed with previous data that higher amounts of dry matter and soluble solids were present in the organically grown tomatoes of all investigated cultivars compared with the conventionally grown fruits (Fig. 6) but determined that the differences were not statistically significant. The amount of dry matter varied from 6.64 (cv. 'Vilina') to 9.06% (cv. 'Benito H') in organically grown tomato fruits and from 6.37 (cv. 'Vilina') to 8.44% (cv. 'Benito H') in conventionally grown tomatoes. The highest amount of soluble solids (4.47%) was detected in fruits of the tomato hybrid 'Tolstoi' grown using the organic system. In organic tomatoes, the average amount of dry matter was 7.77%, and the amount of soluble solids was 4.40%, while conventional fruits had 7.30% dry matter and 4.30% soluble solids.

Previous studies noted that the lycopene content in organic tomatoes was lower than in conventional ones, but found a significantly higher level of β-carotene in organic fruits [42]. Schulzova and Hajslova [47] investigated the impact of fertilisation systems on biologically active compounds in tomatoes and determined that the amounts of carotenoids varied depending on the farming system and cultivar. In their experiment, levels of β-carotene ranged

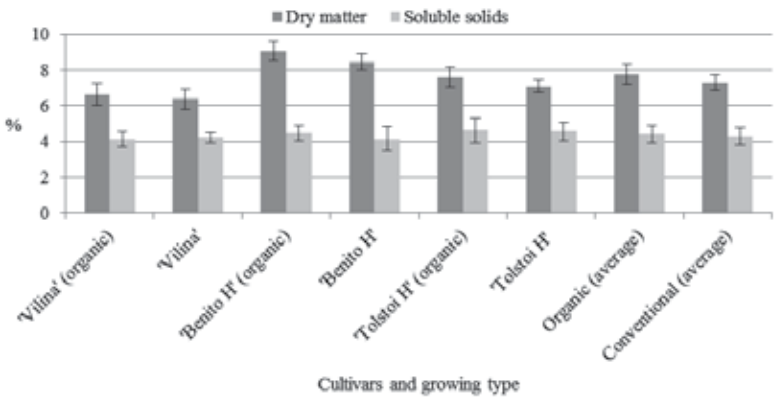

Figure 6. The influence of farming systems on the amount of dry matter and soluble solids in tomato fruits.

from 5.4 to 9.8 mg kg^{-1} and levels of lycopene ranged from 137 to 286 mg kg^{-1}. However, Riahi and colleagues [48] investigated the impact of conventional and organic production systems on the quality of field tomatoes and did not find any significant differences in the amount of lycopene for all cultivars.

The data (Fig. 7) from this study show that tomato hybrids, grown organically, accumulated significantly higher amounts of lycopene in their fruits compared with those under conventional farming, but there were no significant difference in the amount of lycopene in cv. 'Vilina' fruits. The organic fruit of the tomato hybrid 'Tolstoi' had 5.85 mg 100 g^{-1} of lycopene, while conventional tomato fruits had 4.58 mg 100 g^{-1} of lycopene. The average amount of lycopene in organic fruits was significant higher compared with conventional tomatoes. A comparison of β-carotene in organic and conventional tomatoes showed that a significant higher amount (0.21 mg 100 g^{-1}) was found only in organic fruits of the hybrid 'Tolstoi'.

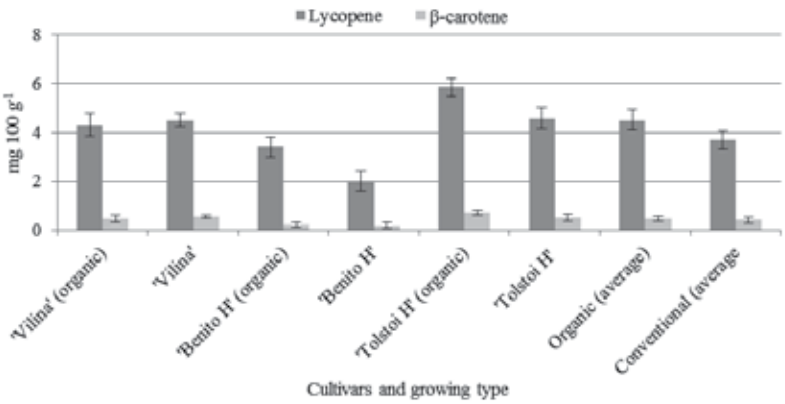

Figure 7. The influence of farming systems on the amount of carotenoids in tomato fruits.

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2.2. Tomato ripening impact on fruit biochemical composition

The fruit quality and biochemical composition of tomatoes can be determined by fruit maturity at harvest. That is particularly problematic when tomatoes are picked green because it is difficult to distinguish between mature and immature green tomatoes. Thus, the chosen harvest time determines tomato fruit quality and biochemical composition. Normally, advanced mature green tomatoes will usually achieve much better flavour at the table-ripe stage compared with fruits picked at the immature or partially mature stages, which are more susceptible to physical injuries and water loss because of their thin skin. During ripening on the vine, tomato fruits accumulate sugars, carotenoids, and ascorbic acid [1, 49]. Fruit texture is another very relevant attribute of tomato quality in common with biochemical composition. Tomato firmness is closely related to the susceptibility of fruit to physical damages at harvest time and during storage. In addition, this characteristic can be tested very easy, by human fingers, and that can be the most important factor for consumer [49, 50].

Plant genotype, growing conditions, and fruit ripeness can have a major influence on carotenoids content in tomato fruits [2, 34, 51]. On the basis of scientific data, the lycopene amount can vary widely in fully ripened tomatoes. For example, Heinonen and colleagues [52] detected 3.1 mg 100 g^{-1} lycopene, while others reported that the average amount of lycopene was 9.27 mg 100 g^{-1} [7], or varied from 3.1 to7.7 mg 100 g^{-1} in fresh tomato fruits [3]. The investigation of the impact of tomato ripening on fruit biochemical composition was carried out at the Lithuanian Research Centre for Agriculture and Forestry [53]. To evaluate the impact of fruit ripening on tomato quality, tomatoes were picked at different ripening stages: I—100% green tomato fruits, II—early stage of ripeness (10% -30% coloured tomato fruits), III—tomato fruits gained colour specific to the cultivar (60% -90% coloured tomato fruits), and IV—fully ripened (over 90% coloured tomato fruits). The research was conducted on 5 tomato (*Lycopersicon esculentum* Mill.) varieties: 'Aušriai', 'Skariai', 'Milžinai', 'Vilina', and 'Vėža'. It was found that the highest amount of accumulated lycopene was detected in fully ripened fruits and varied from 9.21 ('Milžinai') to 12.69 mg per 100 g^{-1} ('Vilina') (Fig. 8). In the green tomato fruits detected, lycopene levels were the lowest ones and varied from 0.25 ('Milžinai') to 0.72 mg 100 g^{-1} ('Vėža').

The similar tendencies were observed with β-carotene content (Fig. 9). In the green tomato fruits detected, β-carotene amount was lowest and ranged from 0.20 ('Milžinai') to 0.47 mg 100 g^{-1} ('Vėža'), while in fully ripened tomatoes, detected β-carotene levels were highest and varied from 1.40 ('Vėža') to 1.69 mg 100 g^{-1} ('Vilina'). According to these experiment, it is possible to make conclusion that levels of lycopene and β-carotene increase sequentially in tomato fruits during their ripening, except in varieties 'Vilina' (between II and III stages) and 'Milžinai' (between III and IV stages) fruits where a small increase in lycopene and β-carotene was detected, but these values were not statistically insignificant.

Fruit flavour of tomato is mainly determined by acids and sugars quantity and the ratio of the two. The flavour is more enjoyable with more sugars and less acids [54, 55]. Tomato quality changes during the fruit ripening time. There are less ascorbic acid and more organic acids in the tomatoes at the beginning of the fruit ripening, and there are the high levels of total sugar and dry matter in fruits at the end of tomato ripening. However, data regarding total sugar

and ascorbic acid amount in tomatoes within their ripening vary greatly; some authors say that tomato ascorbic acid levels increase rapidly throughout ripening [33], while others report that there were no significant differences [56].

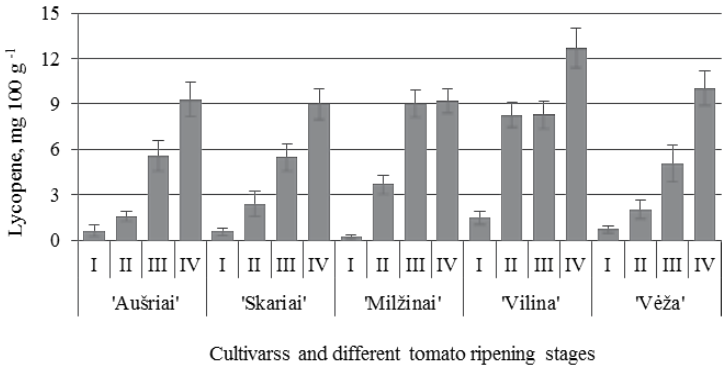

Figure 8. Amount of lycopene in different cultivars at different tomato ripening stages.

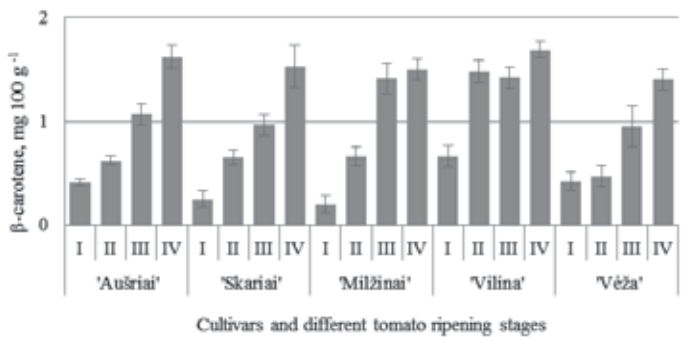

Figure 9. Amount of β-carotene in different cultivars at different tomato ripening stages.

Studies have shown that amounts of ascorbic acid (Fig. 10) and total sugars (Fig. 11) throughout tomato ripening increased in some investigated cultivars, while in others decreased. In fully ripened tomato fruits, the average amount of ascorbic acid varies from 10 to 20 mg 100 g^{-1}. However, some scientists note that the average amount of ascorbic acid is 25 mg 100 g^{-1} in fresh tomatoes [57]. According to this study, it was found that ascorbic acid increased rapidly within tomato ripening only in cv. 'Vilina' fruits and the highest amount of ascorbic acid was accumulated in fully ripe tomatoes and reached 20.4 mg 100 g^{-1}. Throughout the ripening period of tomatoes, there were no trends found of ascorbic acid accumulation in other cultivars. The lowest levels of ascorbic (in all ripening stages) were found in cv. 'Milžinai and varied from 3.8 to 4.2 mg 100 g^{-1}. It is possible to make a conclusion that the amount of ascorbic acid mainly depends on tomato genotype and lees influenced by fruit ripening stage. Thus, the

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amount of total sugar varied independently of the tomato ripening stage. The highest levels of total sugar were detected in fully ripe tomatoes in three of the five investigated cultivars, and the established amount varied from 4.71% to 5.14%.

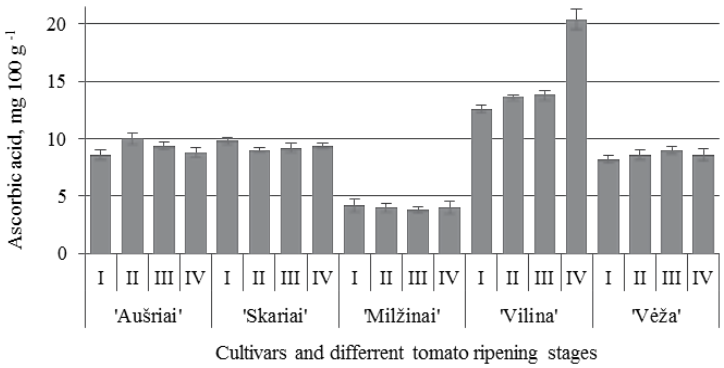

Figure 10. Ascorbic acid content in different tomato cultivars at different ripening stages.

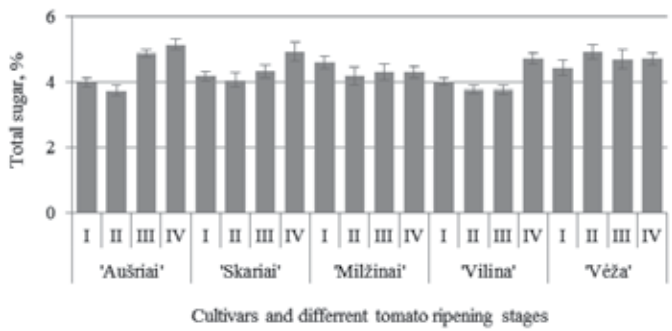

Figure 11. Total sugar content in different tomato cultivars at different ripening stages.

3. Tomato physical properties

In additional to chemical composition, texture and fruit colour are also very important quality attributes of fruit vegetables. Firmness is related to the susceptibility of fruit to physical damage within harvest and storage. For fresh tomatoes, the two quality attributes—texture and skin colour—are very important to buyers and consumers. Texture is influenced by flesh firmness and skin strength. The degree of fruit firmness has been used as an indication of fruit quality, and firmness may be the final index by which the consumers decide to purchase a given batch of tomatoes [14, 58, 59]. Therefore, fruit firmness and colour are the main elements for external tomato quality evaluation. According to provisions, All marketable tomatoes

should have firmness over 1.45 N mm^{-1}, but the means of fruits for home use should be higher 1.28 N mm^{-1} [60]. Thus, there are two possible minimum limits for tomato fruit firmness considering to market regulations and for home use. Previous investigations have shown that the growing system affected fruit firmness, but only in some tomato cultivars [48].

3.1. Fruit physical properties of organic tomato

Experimental evidence (Fig. 12) has indicated that higher tomato fruit skin and flesh firmness were found in hybrids grown conventionally [44]. Conventional tomato fruits of hybrid 'Benito' had significant stronger skin (294.3 N cm^{-2}) and flesh (53.8 N cm^{-2}) firmness, meanwhile organic fruits skin firmness reached 273.1 N cm^{-2} and flesh firmness—41.5 N cm^{-2}. There were no significant differences found in tomato cv. 'Vilina' skin and flesh firmness between organic and conventional fruits, but skin and flesh firmness of organic tomato fruits was slightly stronger. Average data of fruit firmness showed that conventional tomato fruits had stronger skin and flesh compared with organic ones, but difference was not significant.

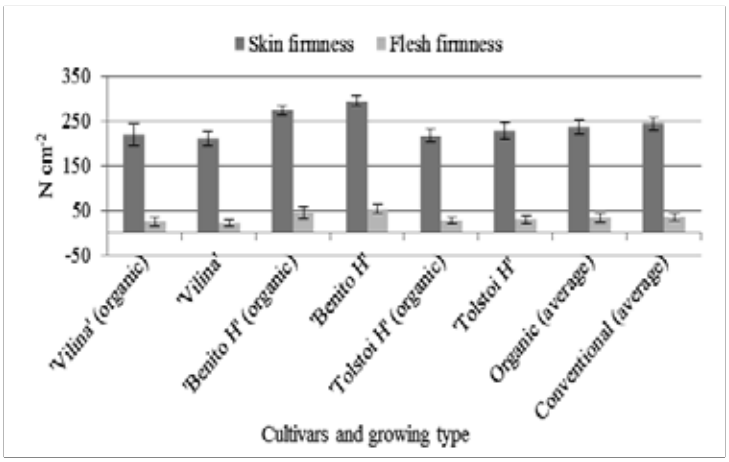

Figure 12. The influence of farming systems on tomato fruit skin and flesh firmness.

People identification of colours is sufficiently complex where sensations like brightness, intensity, lightness, and others modify the perception of the primary colours (red, blue, and yellow) and their combinations, meaning that in many ways colour definition is a matter of subjective interpretation. Colour scale gives measurements of colour in units of approximately visual uniformity across the colour solid. According to the Hunter scale, lightness measured by value L^* and varies from 100 for perfect white to 0 for black, approximately as the human eye would appreciate it. Value a^* measures greyness when zero, greenness when negative, and redness when positive; value b^* measures greyness when zero, blueness when negative, and yellowness when positive. C indicates colour pureness, and $h°$ indicates colour tone [61].

Colour development in tomato fruits is temperature sensitive with better plastid conversion occurring above 12°C and below 30°C [62]. Scientists found that index b^* suffered big changes

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if tomatoes were ripened to high temperatures (over 30°C) and yellowing took place due to the inhibition of lycopene synthesis and the accumulation of yellow/orange carotenoids. Otherwise, at low temperatures (below 12°C), chlorophyll is not degraded and lycopene accumulation does not start. This could be related to abnormal ripening conditions; changes in the *b** values may compensate *a** magnitudes, depending on their mathematical relationship, leading to misleading results [14, 63]. According to average data of colour indexes (Table 1), it was established significant increase in tomato fruit colour tone (by 4.78 U) and pureness (by 2.46 U) in conventional tomato, meanwhile organic tomato fruits distinguished with significant higher value of colour index *a** (redness) by 4.18 U. There were no significant differences found in colour index *b** and chroma (*C*) values between organic and conventional tomato fruits.

Cultivar	*L**		*a**		*b**		*C*		h^o	
'Vilina' (organic)	39.65	± 1.05	29.05	± 2.02	28.12	± 2.90	40.55	± 1.62	43.99	± 2.38
'Vilina'	38.50	± 1.15	28.41	± 2.10	26.95	± 1.91	39.20	± 2.28	43.50	± 2.43
'Benito H' (organic)	42.36	± 1.25	34.29	± 1.67	32.38	± 2.58	47.23	± 1.94	43.31	± 1.86
'Benito H'	49.64	± 1.40	24, 70	± 1.72	36.49	± 1.69	44.11	± 1.19	55.88	± 2.81
'Tolstoi H' (organic)	43.12	± 1.25	28.36	± 1.34	30.50	± 2.22	41.67	± 2.22	47.03	± 1.84
'Tolstoi H'	44.36	± 1.24	26.06	± 2.57	30.19	± 1.18	39.93	± 2.11	49.30	± 2.72
Organic (average)	41.71	± 1.18	30.57	± 1.68	30.33	± 2.57	43.15	± 1.93	44.78	± 2.03
Conventional (average)	44.17	± 1.26	26.39	± 2.13	31.21	± 1.59	41.08	± 1.82	49.56	± 2.65

Table 1. Farming systems influence on colour indexes in tomato fruits

3.2. Tomato fruit colour and firmness changes during ripening

Tomato fruit ripening provides positive and negative features to the final product. Even if ripening provides desired taste, texture and colour, considerable costs, and harvest losses result from negative ripening features. The increase of fruit pathogen susceptibility related with ripening is a main factor to production losses before and after harvest. This change is genetically regulated fruit physiology, and it necessitates use of various fumigants and pesticides in attempts to minimise losses. In addition to being potentially harmful and wasteful of energy and to the environment, such practices represent main costs in agricultural output. Eventually, ripening imparts abundant nutritional and quality parameters upon a very important component of the human diet, fruit [1, 64, 65].

One of the most important parameter of all complex attributes of fruit quality is fruit colours. The complexity of tomato fruit colour is subject of both environmental and genetic regulation due to the presence of a various carotenoid pigment system with appearance conditioned by pigment types and quantity [30].

Tomato fruits are generally consumed at their last ripening stage, which appears when fruit reach the full red colour but before it softening. Thus, it is possible to say that tomato colour

is one of the most important external parameter to appreciate ripeness level and postharvest life and is a main contributor in the final consumer's purchase [49, 50, 66].

Thompson and coworkers [67] compared the colour measurements of tomato fruits (measurements were taken at fruit equatorial) with the homogeneous ones from the same region and reported that the hue (h^o) of homogeneous tomatoes was a better indicator for lycopene content than fruit surface hue. The earlier colorimetric investigation showed that the ratio between the chromatic coordinates (a^*/b^*) separated better than the tomato colour index in the fruits of the different varieties as a function of their external colour [49, 60, 68].

The study of tomato fruit colour changes during ripening [69, 70] revealed that colour index L has a tendency to decline (Table 2) from 49.5 till 44.7 (after 10 days) in tomato fruits during ripening on vine. The polynomial trendline of fruit ripening time and colour index L revealed that the determination coefficient (R^2) was 0.9504.

Period (days)	L		a^*		b^*		C		h^o	
0	49.65	±2.11	-3.23	±4.16	27.54	±1.06	27.99	±0.98	96.67	±4.57
2	49.50	±2.57	-3.42	±4.29	27.39	±1.95	27.87	±2.02	96.87	±4.94
4	49.58	±2.38	-0.87	±8.88	26.92	±1.35	28.05	±2.57	92.75	±6.99
6	48.12	±3.66	5.41	±11.62	31.85	±2.84	34	±2.82	81.25	±5.21
8	45.52	±3.02	7.43	±10.26	24.54	±1.95	27.29	±2.08	74.53	±6.42
10	44.68	±3.52	11.2	±11.55	24.13	±1.99	28.61	±2.20	66.92	±5.38

Table 2. Tomato fruit colour changes during ripening on vine

At the beginning of tomato ripening, colour index a^* was negative. Positive value of index a^* was detected only on the 6th day of experiment. Hence, colour index a^* has a tendency to increase during tomato ripening and that was expressed by a polynomial trendline where the coefficient of determination (R^2) reached 0.9592. Colour index b^* has distinguished on the 6th day when reached 31.9 value, but there were no significant differences between the rest measurements. A significant increase in chroma value (C) on the 6th day was established, and it reached 34.0. The comparison of the rest measurements showed that chroma had varied in small range, and there were no big differences. The experiment showed that hue angle has a tendency to decline during tomato fruit ripening on vine from 96.80 to 66.92, and it was expressed by polynomial trendline where the coefficient of determination (R^2) reached 0.9739. Previous studies showed that tomato fruit lightness (L^*), at different ripening stage, varied from 42.3 to 50.7, chroma (C*)—from 32.5 ('Brooklyn H') to 44.1 ('Benito H'), colour index b^* (yellowness)—from 28.8 ('Rutuliai') to 36.5 ('Benito H'), colour index a^* (redness)—from 12.9 ('Brooklyn H') to 26.1 ('Tolstoi H'), and hue angle (h^o)—from 49.3 ('Tolstoi H') to 66.6 ('Brooklyn H') [70].

Maturation of tomato fruits continues after their harvest, so they may quickly overripe, which affects fruit quality and reduce their realisation time. The quality of tomato texture is determined by tomato skin and flesh firmness and their relationship. Tomato fruit firmness is

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strongly correlated with fruit quality parameters (colour, shape, appearance, etc.). Fruit firmness is used as a parameter in determining the quality of tomatoes. The hardness of the fruit can be a crucial factor to the consumer choice. The transportability of fruit is very important factor because the stronger fruits are less vulnerable to harvesting, sorting, packaging, and transporting production [71]. The assessment of data showed that the skin (Fig. 13) and fruit flesh (Fig. 14) firmness value of tomato fruits went down throughout ripening period.

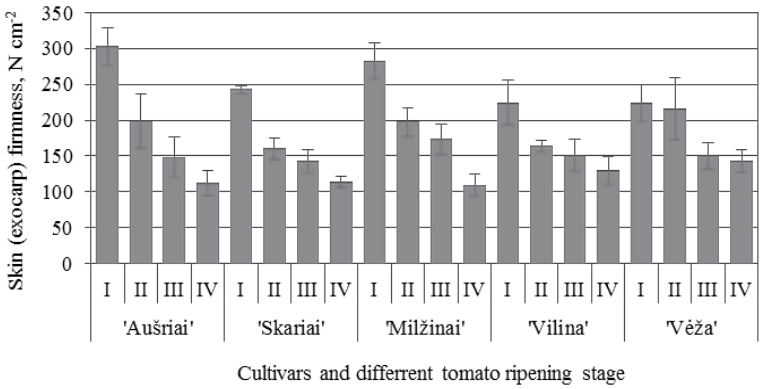

Figure 13. Fruit skin firmness of different cultivars at different tomato ripening stages.

Fruit skin firmness varied from 109 N cm^{-2} in fully ripened tomatoes (cv. 'Milžinai') to 303 N cm^{-2} in green fruits of the cv. 'Aušriai'. Tomato flesh firmness varied from 6.0 N cm^{-2} (fully ripened cv. 'Vilina') to 68.0 N cm^{-2} (green tomatoes cv. 'Milžinai'). The comparison of fully ripened tomatoes revealed that fruits of cv. 'Vėža' had the strongest skin and fruits of cv. 'Aušriai' had the strongest flesh. A significant increase of tomato flesh firmness between the III and the IV ripening stages in 'Skariai' fruits was also found.

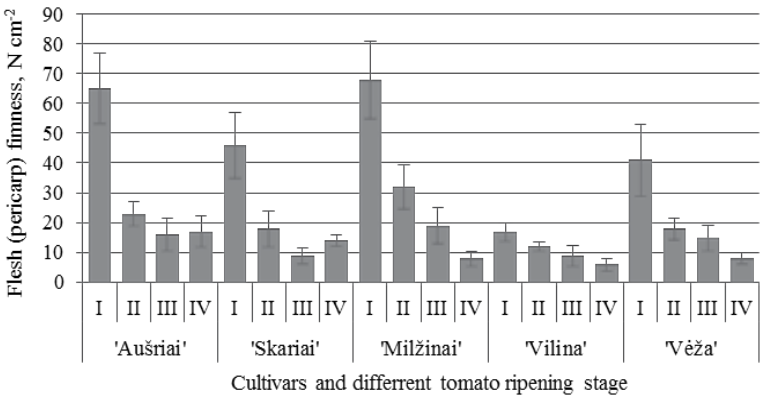

Figure 14. Tomato flesh firmness in different tomato cultivars at different ripening stages.

4. Nondestructive determination of tomato fruit in different ripening stages — Techniques to analyse properties and quality of plants

From an agricultural point of view, the optimal picking time for tomatoes is when 85% -90% of the fruits are red or almost red. Typically, tomato fruits are harvested when they are light red in colour. Such fruits are less injured mechanically during the harvesting process. It is known that tomato colour depends on the pigment (lycopene, carotene, xanthophylls, and chlorophyll) concentration and distribution, so the proper time of tomato picking affects the overall fruit and yield quality. The optimal colour of tomato fruits is when they are rich in carotenoids and low in carotene. Fruits that are harvested too early have a poorer quality because organic and mineral accumulation in their tissues is not finished. Such fruits are entirely unsuitable for storage. Therefore, fruit picking time is determined by the size, colour, texture and flesh firmness [2, 72].

Producers focus their attention on fruit and vegetable quality and aim to avoid poor quality production. The genotype of selected cultivars has a great influence on fruit quality, but the degree of maturity is also very important. Packaging factories use ethylene gas (natural fruit hormone) to speed up the tomato ripening processes. It has been established that during the fruit ripening process, ethylene gas is produced naturally. It increases the permeability of the cell protoplasm. Ethylene then enters the cell, and the air activates the biochemical processes of ripening. Therefore, in order to speed up the ripening of fruit, ethylene gas is employed. The action of ethylene helps to ripen tomatoes within 4-6 days, under the same conditions without ethylene gas fruit maturation time is nearly three times longer [73, 74].

Growers select commercial tomato varieties and hybrids that are resistant to diseases and pests in order to obtain more high-quality fruits. However, picking tomato fruits before they are technically mature may have a decisive influence on their final quality and taste. Commercial growers are concerned with producing adequate amounts of high-quality products, but they are not as concerned about fruit taste; however, tomato flavour is one of the most important indicators for consumers [2, 69].

In tomatoes, individual elements of biochemical composition are typically determined by chemical analysis methods (spectroscopy, high performance liquid chromatography, thin layer chromatography, and so on). Carotenoid (lycopene and β-carotene) extraction from tomato as well as its biochemical analysis requires a large quantity of various organic solvents. Lycopene extraction with organic solvents is a good method for qualitative and quantitative analysis, but this extraction method is not cost-efficient and is time consuming [75, 76]. To facilitate and simplify the determination of biochemical substances without tomato damage, it may be possible to use nondestructive methods, such as colour coordinate spectrophotometry and near-infrared (NIR) spectroscopy method based on the transmittance principle, using near-infrared wavelength spectrophotometer.

Biochemical analyses using modern detection methods require not only specialised and expensive equipment but also professional and technical personnel, causing many inconveniences for growers, producers, and researchers. Agriculture, plant breeding, and food industry

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should use a simple, inexpensive, reliable, and rapid method for the detection of biochemical substances in tomatoes [77, 78]. Therefore, attention has been given to three-dimensional colorimetry, where the assessment of reflection values is rescaled and compared with the values of biochemical elements. The nondestructive prediction of individual biochemical elements is very important in tomato breeding and in the development of new varieties to improve fruit quality, because it is possible to predict the amount of biochemical elements on the plant without fruit damage. This could significantly speed up the process of selection and hybridisation [79, 80].

Prediction accuracy depends on the amount of accurate accumulated data, which is obtained by chemical analysis. Therefore, it is important to collect a large database of research results so that predicted data would more closely resemble observed data [75, 79].

For that reason, a study of tomato fruit ripening processes was conducted, and calibration curves for dry matter, soluble solids, organic acids, skin and flesh firmness, lycopene and β-carotene, ascorbic acid, and sugar content were created according to data from NIR and biochemical analysis methods. The investigation looked at different tomato cultivars of different fruit ripening stages. The study examined 10 different tomato cultivars and hybrids including 'Tamina', 'Money Maker', 'Saint Pierre', 'Tocayo H', 'Polfast H', 'Brooklyn H', 'Tolstoi H.', 'Benito H', 'Tourist H', and 'Rutuliai'. In order to get more and varied types of data, the dynamics of biochemical elements during fruit ripening were also observed. Therefore, the tomato fruit investigations were made with fruits of six different ripening stages [80].

During the investigation, tomato fruit biochemical composition and texture analysis were conducted using near-infrared (NIR) spectroscopy performed in parallel with normal biochemical and texture analyses. It assessed the values of reflection (nondestructive method) compared with the biochemical and fruit texture values (destructive methods).

During the first year of the experiment, calibration graphs were created, and the statistical reliability of these graphs was evaluated during the second year.

Biochemical analyses were conducted using the following methods. Ascorbic acid was determined by titration with 2.6-dichloroindophenol sodium salt solution, soluble solids were determined with a digital refractometer (ATAGO, PAL-1, Japan), dry matter by gravimetrically after drying at 105°C to a constant weight, and sugars by the AOAC method. Organic acid content, expressed as citric acid, was determined by titration with a 0.1-N sodium hydroxide solution, and carotenoids were measured using HPLC.

Tomato texture was measured using a texture analyser (TA.XTPlus, Stable Micro Systems, Godalming, United Kingdom). To pierce the tomato peel and the pulp of the fruit (unpeeled skin), a P/2 probe (2 mm diameter flat probe tip) was used, and the data were processed using 'Texture Exponent' software.

The near-infrared (NIR) spectroscopy method, based on transmittance, was used for nondestructive measurements, using a near-infrared wavelength spectrophotometer (NIR Case NCS001A, SACM SCImola Imola, Italy).

Calibration graphs were created using 'SACM NCS (NIR Calibration Software) Vers. 3.0 RC 1' software.

Calibration graphs of dry matter, soluble solids, organic acids, skin and flesh firmness, lycopene and β-carotene, ascorbic acid, and sugar content were created according to NIR and chemical analysis data. The created graphs make it possible to determine the amount of these elements in a nondestructive manner. The tomato skin firmness calibration graph is shown in Fig. 15.

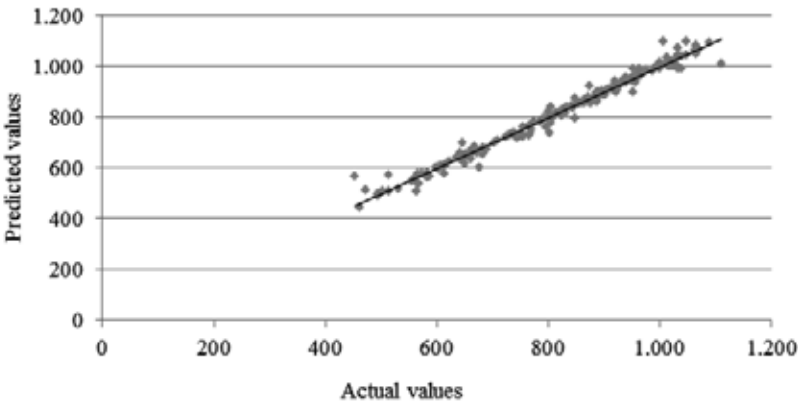

Figure 15. Calibration graph of tomato skin firmness.

Such calibration graphs allow the determination of the strength of the tomato fruit and amount of biochemical elements very quickly and inexpensively, and also offers great opportunities to producers, manufacturers, and food industry.

On the second year of the study, the reliability of the newly created calibration graphs was assessed. Again, normal biochemical analysis and nondestructive measurements using near-infrared (NIR) spectroscopy were performed. The reliability of the obtained results was evaluated statistically (Table 3).

Based on the obtained results, a strong correlation between normal and nondestructive analytical methods in measuring of soluble solids ($r = 0.9251$), lycopene ($r = 0.8701$), β-carotene ($r = 0.9486$), ascorbic acid ($r = 0.8052$), skin strength ($r = 0.9906$) and the pulp strength ($r = 0.9369$) was found. The average correlation was observed in dry matter ($r = 0.6480$), titratable acidity ($r = 0.5800$), and total sugars ($r = 0.5982$). Consequently, based on the created calibration graphs, nondestructive measurements of tomato fruit quality parameters using near-infrared spectroscopy (NIR) can be carried out. The reliability assessment of the obtained results and comparison of nondestructive techniques and traditional methods showed that there is a strong correlation between them by measuring soluble solids, lycopene, β-carotene, ascorbic acid, skin firmness and strength of the flesh, and average correlation by determining dry matter, titratable acidity, and total sugar content of tomato fruits.

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Parameters	Coefficient of determination (R^2)	Coefficient of correlation (r)	Average values	
			NIR	Reference methods
Dry matter (%)	0.4200	0.6480	0.872	0.798
Soluble solids (%)	0.8559	0.9251	3.823	3.742
Titratable acidity (%)	0.3364	0.5800	0.701	0.618
Lycopene (mg 100 g^{-1})	0.7570	0.8701	4.41	4.61
β-Carotene (mg 100 g^{-1})	0.8998	0.9486	1.026	1.074
Ascorbic acid (mg 100 g^{-1})	0.6483	0.8052	17.8	21.5
Total sugar (%)	0.3579	0.5982	4.273	4.025
Skin firmness (N cm^{-2})	0.9814	0.9906	253.64	252.49
Flesh firmness (N cm^{-2})	0.8778	0.9369	33.46	46.88

Table 3. Reliability of investigated parameters

5. Tomato as the functional food ingredients

Fresh vegetables are an essential source of minerals, dietary fibres, and especially vitamins. Humans get 90% of their vitamin C from vegetables, which are also rich in B group vitamins. Vegetables stand out from other food products due to their high energy value. They are very important for human nutrition, and they supplement the human body with minerals, vitamins, proteins, fats, and carbohydrates [2, 81, 82]. Due to their biochemical composition, tomatoes are very valuable vegetables. Their fruit is prized for its good taste and its nutrition value. Tomato fruit contains soluble sugars, organic acids, fibre, pectins, proteins, fats, minerals (potassium, phosphorus, sulphur, magnesium, calcium, iron, copper, and sodium), many vitamins (B1, B2, B3, PP, C, A, I, and H), and the alkaloid tomatine with phytoncide properties. The greatest influences on both valuable and harmful substances in tomato fruits are environmental and growing conditions, fruit ripening stage, and cultivar characteristics [70, 83, 84]. Tomatoes can be eaten fresh, fried, boiled, or pickled in various salads and other dishes. In addition, processed tomatoes (paste, juice, sauce) retain all nutritional characteristics of the fresh fruit [2, 85].

Long ago, it was believed that tomatoes contained oxalic acid, which adversely affects metabolic processes; therefore, older people were advised not to eat them. It has been confirmed that the amount of oxalic acid in tomatoes is less than in lettuce, potatoes, or red beets, and the influence of purines (protein metabolic products that lead to gout (podagra)) is less than in many other plant products. Tomatoes can be eaten by children, adults, and the elderly. If someone's stomach is very sensitive, fresh tomatoes should be peeled first because the skin can stick to the stomach walls and cause inflammation. Vitamins and other valuable nutritional compounds found in tomatoes not only improve human nutrition but also prevent various diseases. Ascorbic acid directly removes free radicals of oxygen and superoxides. The human body does not synthesise ascorbic acid, so it must be obtained from food. Ascorbic acid is one

of the most important antioxidants found in tomato fruits [86, 87]. It is believed that carotenoids, found in tomato fruits (which can reach 3.67 mg 100 g^{-1}), may reduce the risk of human diseases, in particular cardiovascular diseases and prostate cancer [88, 89]. Epidemiological studies have shown the existence of an inverse relationship between lycopene intake and prostate cancer risk. Patients with prostate cancer had lower lycopene levels in their blood plasma than control patients [90]. The inverse relationship is also expressed in aggressive prostate cancer cases. Prostate cancer risk was lowered by 83% for the patient group with the highest lycopene plasma levels (0.40 μmol l^{-1}) compared to the lowest concentration (0.18 μmol l^{-1}) group [91]. Similar results were obtained in other studies, where it was found that two or more tomato dishes per day can reduce the risk of developing prostate cancer [89, 92].

There have been several epidemiological studies that have outlined the relationship between lycopene concentrations in the blood plasma and cardio vascular disease risk. One found that men who had coronary disorders had lower lycopene levels in their plasma compared to men without coronary disorders [35]. Alternatively, a study of the relationship between the lycopene level in fatty tissues and heart disease showed that an increased lycopene concentration had a protective effect against cardiac dysfunction [93, 94].

Lycopene consumption efficiency is determined by lycopene (the active principle compound of tomatoes, which acts as an antioxidant) bioavailability. Unfortunately, the mechanism of lycopene uptake remains unclear. It is known that absorption of consumed lycopene reaches only 10% (in some cases can increase up to 30%). Furthermore, lycopene absorption from fresh tomatoes is less than from the processed products (tomato paste or sauce) [7] because the mechanical and thermal treatment of tomatoes enhances lycopene uptake. There are other factors that affect the process of lycopene absorption. It has been found that the addition of oils in tomato dishes enhances carotenoid absorption [95], but the addition of various fibre substances can reduce absorption [96].

It is believed that processed fruits and vegetables are less valuable than fresh, but lycopene is better absorbed from processed tomatoes. Heat-treated tomatoes can have more bioavailable lycopene, and this justifies tomatoes as a functional food [2, 5, 6]. Undoubtedly, the effect will be negligible or absent, if the consumed amount of lycopene is 6-8 mg per day. It has been reported that 25-35 mg of lycopene should be consumed daily, that is, approximately 200 g tomatoes per day [97].

Thus, tomatoes, as a source of various antioxidants and vitamins, can increase the human's body resistance to the impact of radiation, reduce cholesterol accumulation, heal some skin diseases, and prevent cardio diseases and prostate cancer [8, 98].

6. Lycopene in tomatoes: Chemical and physical properties affected by food processing

The importance of lycopene is mainly due to its beneficial properties for human health. Lycopene protects humans from attack by pathogenic agents responsible for a number of

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chronic diseases, such as cardiovascular disease, different types of cancer (digestive tract, cervix, breast, skin, bladder, and prostate), hypertension, osteoporosis, neurodegenerative diseases, male infertility, and even the transmission of immunodeficiency syndrome from mothers to babies [99].

The availability of lycopene in food may depend on several factors. First, the carotenoid content of food may be increased by mechanical processing. Food processing may be beneficial because it disrupts food matrices, facilitating the release and solubilisation of carotenoids, resulting in increased carotenoid bioavailability, including lycopene bioavailability [100]. Within the plant, lycopene is part of the matrix in chloroplasts or chromoplasts, and the absorption of lycopene from raw tomatoes is low because it is occurs mostly in the *trans*-isoform and is tightly bound within the matrix [101]. Second, the bioavailability of lycopene is greatly increased by thermal (cooking or by commercial) processing, such as conversion to soups, sauces, and catsup [102]. Nevertheless, increased uptake or higher blood levels of lycopene have been achieved predominantly by the intake of tomatoes or tomato products rather than by the intake of purified lycopene [8, 103]. In synthetic nutritional supplements, lycopene is in the form of an oleoresin embedded in phospholipid complexes and oils. Third, the addition of lipids, such as vegetable oils, increases lycopene absorption [101]. For example, it has been reported that lycopene is more efficiently absorbed when tomato juice is warmed with a supplemental lipid. Moreover, lycopene is lipophilic, and the dissolution of carotenoids in a lipid phase occurs in the stomach and the duodenum. Roldan-Gutiérrez and de Castro [104] reported that, due to the action of bile salts and pancreatic lipases, carotenoids in a lipid phase (droplets) enter the duodenum and form multilamellar lipid vesicles. During intestinal absorption, carotenoids and lycopene incorporate into chylomicrons and interact with other carotenoids [104]. Interactions with other carotenoids are complex and have not been fully studied. For example, β-carotene in the same dish as lycopene causes an increase in the absorption of lycopene [102].

Moreover, during exposure to thermoenergy, oxygen, and light, lycopene can undergo isomerisation and degradation. Isomerisation converts all-*trans*-isomers to *cis*-isomers and results in a reduction of the biological properties of lycopene [99]. Red tomatoes normally contain 94%-96% all-*trans*-lycopene. All-*trans*-lycopene is thermodynamically the most stable form. Some authors have reported that the formation of *cis*-isomers of lycopene may increase biological activity. *Cis*-isomers are more soluble in bile acid micelles and may be preferentially incorporated into chylomicrons compared with *trans*-isomers [105]. *Cis*-isomers of lycopene have distinct physical characteristics and chemical behaviours from all-*trans*-isomers, including decreased colour intensity, greater polarity, lesser tendency to crystallise, and greater solubility in oil and hydrocarbon solvents. However, these physical characteristics have a direct impact on the sensory qualities and consumer health benefits of food. The determination of the degree of lycopene isomerisation during processing and storage would provide a measurement of the potential health benefits of tomato-based foods [99].

An overview of the observed results of Haymann and colleagues [106] during the isomerisation processes of lycopene is given in Fig. 16. The study demonstrated that various *cis*-isomers (predominantly *5-cis*- and *9-cis*-lycopene) were formed during energy-rich irradiation, whereas at the same time degradation of all-*trans*-, *15-cis*-, *13-cis*-, and *7-cis*-lycopene occurred

[108]. A theoretical study on the *cis–trans* isomerisation of lycopene revealed that *5-cis*- and *9-cis*-lycopene are more stable than other isomers since their rotational barrier to reisomerise the all-*trans* configuration is higher ($\Delta E_r^{\ddagger}$ = 35.2 kcal/mol and 23.1 kcal/mol, respectively) than that of all other isomers ($\Delta E_r^{\ddagger}$ = 16.8 to 19.9 kcal/mol) [106]. Furthermore, the stability of *5-cis*-lycopene and *9-cis*-lycopene is also induced by their much lower relative energy compared to other isomers. Those effects lead to the accumulation of the *5-cis*- and *9-cis*-isomers during irradiation with halogen lamp. In contrast, low rotational barrier ($\Delta E_r^{\ddagger}$ = 22.1 kcal/mol) and one of the highest potential energies of all mono-*cis*-isomers results in a dominant degradation of *7-cis*-lycopene during energy-rich irradiation [106, 107]. All-*trans*-lycopene underwent degradation, while the concentration of *cis*-isomers, mainly *13-cis* and *9-cis*, increased. The investigation showed that the *5-cis*-isomer changed distinctively during lycopene storage compared to the other lycopene isomers [106].

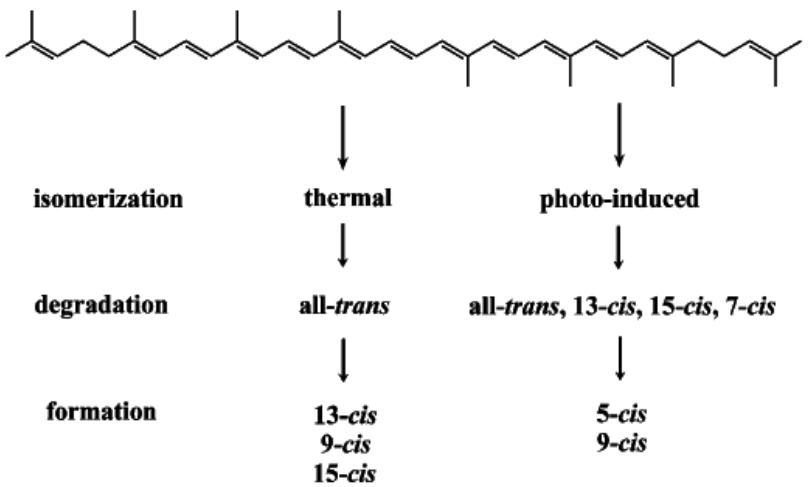

Figure 16. Thermal and photoinduced isomerization leads to degradation and formation of lycopene isomers in lycopene extract [106].

After intestinal absorption, carotenoids are carried to the blood stream by chylomicrons via the lymphatics. Concerning transport in the plasma, carotenoids are transported by lipoproteins, and transport depends on the carotenoid structure. Therefore, lycopene is found in the aqueous interface at the lipoprotein surface. For this reason, lycopene is transported in low-density lipoproteins, and oxygenated carotenoids are transported in both low-density and high-density lipoproteins [104].

It is important to develop more attractive ready-to-eat products to contribute to the increased consumption of fruit and vegetable products and their health benefits for consumers. Food processing should be adapted to enhance the bioavailability of nutrients [108]. Additional information needs to be collected on the thermal behaviour of lycopene before we can have

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definitive answers regarding its physical state and stability during processing and cooking. Little is known about the stability of lycopene in supplemental form [109].

7. Tomato plants in agricultural, industrial, and pharmaceutical applications

As was previously mentioned, tomatoes are one of the most widely produced and consumed 'vegetables' in the world, both for the fresh fruit market and the processed food industries. The tomato industry is one of the most globalised and advanced horticultural industries. Furthermore, tomato production has historically been located in temperate zones that have long summers and winter precipitation, but now, with new modern technologies (greenhouse structures, climate control, and crop protection), tomato production has expanded and is focused on the production of fresh tomatoes. However, cultivation practices, the ratio between production for processing or fresh consumption, and the organisation and structure of the industry and markets differ widely among countries. Further, tomatoes are harvested at different stages of ripeness for different purposes. Processing tomatoes are mechanically harvested red-ripe and immediately transported to a processing plant. Fruit destined for the fresh market is hand harvested at the mature green, partially ripe or fully ripe stages. Mature green fruits are picked because they are firm enough and have a sufficient shelf-life to survive the stress of being shipped considerable distances, and they are ripened to acceptable levels of quality at distant markets. Quality characteristics of fresh-market fruits are similar to those of processing tomatoes, but the characteristics that are readily apparent to the consumer (colour, size, shape, firmness, and aroma) dominate the others [110].

The industrial processing of tomato products produces waste such as tomato skins and seeds. Ripe tomato skins contain approximately five times more lycopene than the pulp. The largest portion of tomato waste is the peals which are the most abundant sources of lycopene. The lycopene content is over 90% in ripe tomato skin [106, 110]. Tomato waste is a potential natural source for lycopene extraction. One of the most important trends in the food industry is the demand for all-natural food ingredients that are free of toxic solvents and chemical additives. A unique process for the nontoxic, safe, and inexpensive extraction, separation, and concentration of lycopene is supercritical fluid extraction with carbon dioxide (SCF CO_2). SFE adds value to agricultural waste by extracting lycopene from tomato skins and using it for the fortification of foods and in pharmaceutical applications [110].

Studies have proposed that lycopene may work synergistically with other carotenoids, vitamins, and minerals present in the diet. Lycopene extracts and concentrates could be used not only in traditional food products but also as functional ingredients in specifically formulated foods and dietary supplements that enhance human health and wellbeing. Growth in consumer demand for healthier food products provides an opportunity for food industry to develop new functional foods enriched with natural lycopene, as well as for pharmaceutical industry to develop new nutraceutical products comprising pharmaceutical-grade lycopene [111, 112].

8. Conclusions

Tomato biochemical composition, nutritional value, colour, and flavour of tomato products depend mainly on lycopene, β-carotene, ascorbic acid, sugars, dry matter, and their ratios in fruits. The two most important carotenoids in tomato fruits are lycopene and β-carotene. Therefore, tomato products and their quality can be characterised by the contents of these elements. It has been established that tomato fruit quality varies due to species, stage of ripeness, farming system, climatic conditions, growing area, fertilisation, and other conditions of cultivation.

In additional to chemical composition, texture and fruit colour are also very important quality attributes of vegetables. Firmness is related to the susceptibility of fruit to physical damage during harvest and storage. For fresh tomatoes, two quality attributes, texture and skin colour, are very important to buyers and consumers. Texture is influenced by flesh firmness and skin strength, which can be used as an indication of fruit external quality.

To facilitate and simplify the determination of biochemical substances without tomato damage, it might be possible to use nondestructive methods, such as colour coordinate spectrophotometry and the near-infrared (NIR) spectroscopy method (based on the transmittance principle, using a near-infrared wavelength spectrophotometer). A reliability comparison of nondestructive techniques and traditional methods showed that there was a strong correlation between them for measuring soluble solids, lycopene, β-carotene, ascorbic acid, skin firmness, strength of the flesh, dry matter, titratable acidity, and total sugar content of tomato fruits.

Tomato fruits have positive health benefits and contain significant amounts of biologically active compounds, which are responsible for positive health effects. Epidemiological and other studies associated with the consumption of tomato products for the prevention of chronic diseases, such as cancer and cardiovascular disease, confirm that tomato products are a functional food and show that lycopene and β-carotene act as antioxidants. The growing interest and demand for healthy, environmentally safe, and cost-efficient products has driven the research of new technologies in the food, pharmaceutical, and cosmetic industries. Therefore, biocompounds, such as lycopene from tomato plant material, are important for agricultural, industrial, and pharmaceutical applications. However, the levels of lycopene and other biological compounds in plant material depend on the species, growing conditions, and climate trends.

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Chapter 4

Acid Phosphatase Kinetics as a Physiological Tool for Assessing Crop Adaptability to Phosphorus Deficiency

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Additional information is available at the end of the chapter

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Abstract

Acid phosphatases (APase) exuded from the roots is important in mobilizing organic phosphate in the soil . Enzyme kinetics can provide reliable physiological markers to detect the potential for superior plant performance under low P . Kinetic constants for the secreted APase could be used as an early physiological indicator for P stress tolerance in legumes, Desmodium tortuosum , Phaseolus vulgaris,Vigna unguiculata and Crotalaria juncea were grown from seed in +P and -P nutrient solutions and plants were harvested during the early vegetative phase in order to collect the root exudates in vivo and for dry biomass, leaves soluble Pi, and total P in the dry biomass. Root surface Na-soluble APase was extracted from +P and -P grown plants by incubating three intact plants in beakers with their roots immersed in a 0.1 M NaCl solution. Secreted APase was obtained with the roots of three plants individually immersed in a dialysis tube (12 kD) containing NaCl 100 mM and then transferred to a recipient containing 3L of the same solution. Kinetic constants Km and Vmax were determined using a range substrate (p-NPP)concentration (S). Activity (v) was expressed as μmoles PNP/h per g root fresh (FWr) or dry weight DWr. Graphical representations were used for the determination of the Km and Vmax: Linewaver-Burk double reciprocal plot 1/v vs. 1/S plot; Hanes-Wolf plot S/v vs. S and Woolf-Augustinsson-Hofstee plot v vs. v/S. The first visual indication of P deficiency was a reduction in leaf area and dry biomass and a higher soluble Pi in the leaves of +P plants. Activity was higher in -P plants at the beginning of the growth period and the proper timing for the onset of the P-stress was apparently crucial for the induction of APase. For Phaseolus vulgaris Km values apparently indicate the lack of phosphate starvation-inducible APase and a higher Vmax in -P plants; however, with the combination of a high Km with a high Vmax plant behaviour could be improved under P deficiency. In Vigna unguiculata the low Vmax in -P plants may be compensated for by its lower

Km. Crotalaria juncea showed considerably greater kinetic diversity, but Km was lower in -P plants. The practical implications of Km and Vmax are explained in terms of the potential for P-liberation under limiting Pi ; to be efficient an increase in Pi uptake is likely to occur if the APase released has a low Km (in the neighborhood of the soil P concentration) and a high Vmax as found for Desmodium, Phaseoulus and Vigna. The Km provided a means of comparing the enzyme from high or low-P plants indicating that Km is a reliable physiological tool for assessing plant adaptability to P-deficiency and it is suggested that Km, Vmax with total leaf area and relative growth rate (RGR).

Keywords: plant enzymes, root secretion, abiotic stress

1. Introduction

Phosphorus (P) is an essential element in the mineral nutrition of plants and under P starvation a reduction in biomass and leaf area among other physiological traits, is observed. Phosphorus occurs in soils not only as mineral phosphates but also as organic compounds that in order to be available to the roots they must be first hydrolyzed and several studies now indicate that acid phosphatase may have a role in mobilizing organic phosphate in the soil that in infertile tropical soils may contribute significantly to ameliorate P bioavailability from sparingly soluble P forms. Acid phosphatase (APase) from several plant species and genotypes has been shown to liberate P from soil thus decreasing organic phosphorus in the rhizosphere [8, 26, 27] and a large body of evidence now exists showing that P deficiency can trigger an increase in activity of root secreted APase in a variety of wild and cultivated plants and that the activity of the enzyme can vary among species along with the severity of P starvation [4, 24, 25]; however [28] showed that some tropical forage such as *Brachiaria* hybrids and *Arachis pintoi* have the ability to tolerate low-phosphorus stress without showing any increase in APase activity in root exudates. According to [29], the role of secreted APase in plant adaptation to low phosphorus availability is unclear. Enzyme kinetics can used to identify simple and reliable physiological markers, for screening purposes to detect the potential for superior plant performance under low P concentrations. Ascencio [4, 5] calculated the kinetics constants (Km and Vmax) for the exuded APase from different plants species grown under P- sufficient and P-deficient conditions. The numerical values of the Km for the substrate p-nitrophenyl-Phosphate (p-NPP) provided a means of comparing the enzyme from high or low P plants, so it is suggested that Km and enzyme activity (Vmax) may be used as physiological indicators to differentiate plants grown under P deficiency or sufficiency. Km values are related to the substrate concentration at which enzyme velocity is half of the maximum (Vmax), they are not strictly constants as those that are found with the purified enzyme as the activity is assayed using the crude root exudates as they might be released to the soil. Acid phosphatase enzymes are released to the soil as part of the pool of extracellular enzymes along with other products of root exudation; however, under laboratory conditions different forms of the enzyme are present depending on the procedure that is used to collect the enzyme. Collection of the

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enzyme *in vivo* can be performed directly from the intact plant by immersing their roots in the extracting solution which is assayed without purification (root surface Na-soluble APase) or by separating the enzyme from other exuded compounds by enclosing the roots of the intact plant inside a tube or a dialysis membrane (secreted APase). Collection of the enzyme *in vitro* from the root and other plant tissues (mostly leaves or seedlings) is achieved by grinding and collecting the resulting solution (extractable APase). Further purification of the enzyme in order to assess whether new APase isoenzymes are induced by P deficiency can be achieved using any of the extraction methods described above. The properties of the purified enzyme secreted in vivo by plant roots was first reported by Tadano [23], for the secreted enzyme from Lupinus roots under P-deficient conditions. Under P-deficient conditions new APase isoforms, which are different forms for the enzyme, are released. It has been shown [1,27] that APase isoforms were inducible under P deficient conditions but that for Lupinus, the major activity in the rhizosphere soil and in roots grown under hydroponic conditions corresponded to a previously purified APase secreted by the roots; thus in order to compare the potential of APase in the root exudates released by different plant species and genotypes that may increase plant performance under phosphorus deficient conditions, root secretions can be obtained *in vivo* and acid phosphatase activity measured in the secreted or exuded solution without further purification.

The objective of the present chapter is to give a broader picture, from an agronomical point of view, as to how kinetic constants for the APase secreted "in vivo" by the roots of leguminous plants grown under P-deficiency or sufficiency, could be used as an early physiological indicator for P stress tolerance.

1.1. Research methods

The leguminous plants species (*Desmodium tortuosum* (Sw.) DC, *Phaseolus vulgaris* L var Manuare, *Vigna unguiculata* (L) Walp cv Tuy and *Crotalaria juncea* L, were used in this study. *Desmodium tortuosum* (beggarweed) Fabaceae, is a slow growing tropical non-grain legume that grows wild, and on small farms, in association with other crop species as a nitrogen source, as a forage legume and green manure. *Phaseolus vulgaris* (common bean) and *Vigna unguiculata* (cowpea), -Fabaceae are very important grain crops used as a protein source worldwide; *Crotalaria juncea* (sunn hemp) Fabaceae is widely grown throughout the tropics and subtropics as a source of green manure, fodder and lignified fiber and has been recently looked at as a possible bio-fuel. Plants were grown from sterilized seeds and water culture experiments were performed in a highly ventilated greenhouse in plastic 950 ml pots with aerated Hoagland solutions containing either sufficient (+P) or deficient (-P) (mM P as KH_2PO_4 depending of the species). After establishment, plants were harvested during the early vegetative phase for each species and separated into groups in order to perform kinetic studies for the APase in the root exudates collected in vivo, for dry biomass determinations and to measure soluble Pi content in fresh leaves and total P in the dry biomass. The Soluble Pi content in leaves was measured using leaf discs (0.5-10 g fresh weight) macerated in cold 2% acetic acid a the extract diluted and centrifuged at 4 ^{o}C and the clear supernatant used for Pi concentration determinations using the colorimetric phosphomolybdate reaction. Total P in the dry biomass was measured using previously digested material with H_2SO_4:H_2O_2. Phosphorus efficiencies were calculated

(when indicated) as mg total P in plant in dry root biomass per mg P in the nutrient solution (phosphorus absorption efficiency PAE), and as g the total dry biomass per mg total P in the plant respectively (phosphorus use efficiency PUE).

1.2. Acid phosphatase activity and kinetic constants

The extraction experiment for the root surface Na-soluble acid phosphatase from +P and -P grown plants was performed by incubating three intact plants from each P treatment in 250 mL beakers wrapped in aluminum foil with their roots immersed in 100 mL of a 0.1 M NaCl solution inside a well lighted refrigerator at 4 C. After 6 h the solutions in the three flasks per P treatment were separated and filtered using Whatman paper # 1, and as the crystal-clear filtrate was obtained, used for the kinetic studies [5].The solution with the enzyme obtained in this experiment is referred to as extracted APase. The secretion experiment for the acid phosphatase enzyme was performed with the roots of +P and -P intact plants individually immersed in a dialysis tube (12 kD) containing NaCl 100 mM and then transferred to a recipient containing 3L of the same solution following the procedures reported [24].. After 24 h the solution inside the dialysis tubes of three plants was collected for kinetic studies when plants in the low P treatment showed moderate P deficiency symptoms as shown by growth inhibition. The solution with the enzyme obtained in this experiment is referred to as secreted APase. Root surface Na-soluble acid phosphatase (APase) activity (reaction velocity v) was assayed using aliquots of the extracted or secreted enzyme with the substrate p-nitrophenyl-1-phosphate, buffer Na-acetate 50 mM pH 5.0 in a water bath at 34C. Reaction was stopped after 30 min with a saturated Na_2CO_3 solution and the yellow p-nitrophenol (PNP) read at 410 nm in an spectrophotometer Varian DMS-90. Kinetic constants Km and Vmax were determined using a range substrate concentration (S) based on the Km value for the purified enzyme [1, 16]. Two different ranges depending on the species (0.2; 0.33; 0.50; 1,00;1,43; 2,00; 2,50; 3,33 and 5,0 or 1.0 ; 1.25; 1.43; 1.67; 2.0; 2.50; 3.30 and 5.50 mM p-NPP) were assayed as indicated in the enzyme activity plots using the root exudation or the secretion from different plants. APase activity (v) was expressed as μmoles PNP/h per g root fresh (FWr) or dry weight DWr. The first step was to examine the v vs. S curve that reflects the hyperbolic Michaelis- Menten to determine the degree of substrate saturation; in theory as the velocity of the enzyme reaction (v) responds in a characteristic way to increasing substrate concentration, but when crude extracts clear substrate saturation is not always observed, and in order to determine the affinity constant Km and maximal velocity of the enzyme reaction Vmax different graphical representations based on linear transformations of the data are used for the determination of the affinity constant (Km) and the maximal velocity of the enzyme reaction (Vmax). The most widely used graphical representations are: Linewaver-Burk double reciprocal plot 1/v *vs*. 1/S plot; Hanes-Wolf plot S/v *vs*. S and Woolf-Augustinsson-Hofstee plot v *vs*. v/S, where v represents initial enzyme velocity at any given substrate concentration (S). Under the conditions of this study, the Km refers to the apparent Km for the enzyme activity, or the concentration of substrate at which activity is one half the maximal velocity and Vmax refers to the apparent maximal velocity for enzyme activity, which is the maximum rate of P- hydrolysis. Enzyme kinetics data were analyzed using the Hyper32 free software.

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2. Results

The first visual indication of P deficiency as observed for the plants of this study, was a reduction in leaf area to different degrees in different species, which was reflected in lower biomass values. The large differences among -P and +P plants are explained in part due to soluble Pi concentration in leaves which remained higher in +P plants during the entire growth period. In spite of the large differences in growth and Pi content, P efficiencies were much higher in the low P plants where larger differences were found for PUE (indicating a superior ability of -P plants to convert phosphorus into biomass). The superior acquisition efficiency (PAE) for some plant species has been attributed to a more efficient mycorrhizae and/or root acid phosphatase activity in addition to other factors as superior uptake kinetic. For the plants of this study we focused the strategy on the kinetic constants for the secreted APase under P deficient conditions. Enzyme activity is induced under P deficient conditions which depends on the proper timing for the onset of the P stress; the low Pi content in leaves of -P plants on the other hand was compensated for by a 10 times higher phosphorus use efficiency (PUE) for the plants of this study, which is in agreement with the hypothesis that the efficient recycling of Pi inside the plant is an important mechanism for survival under conditions of P starvation.

2.1. Acid Phosphatase Kinetics of *Desmodium tortuosum* (beggar weed)

After 28 days of planting the roots of four plants grown under either P sufficiency (+P 1.0 mM P) or deficiency (-P 0.01 mM P) were immersed in the collection flasks or in the dialysis membrane and after the extraction period aliquots were taken to perform kinetic studies for (a) the extracted enzyme and (b) the secreted enzyme. For the secreted enzyme saturation kinetics was observed for the enzyme from both high and low grown plants (+P and -P) as substrate concentration was increased from 0.2 to 5.0 mM, as seen from the hyperbolic Michaelis-Menten plot in Figure 1a and Figure 2a Figure +P (a) and -P (a).

The apparent Km and Vmax values determined from Lineweaver-Burk, Hanes and Hofstee plots; Figures +P and -P. For the high P plant enzyme, Km values of 2.81, 1.54, 2.63 and 1.07 mM p-NPP and Vmax of 80.5, 57,3 80.85 and 51.6 μmol PNP/h/g FW roots were calculated from the plots depicted in Figure 1 (b, c and d) in Figure +P For the low-P plant enzyme values were 0.92, 0.56, 1.58 and 0.73 mM p-NPP and 105.1, 87.6, 138.4 and 101.3 μmol PNP/h/g FW roots (Figure 2 (b, c and d) (Figure -P). As seen from the results obtained in this investigation, lower Km a higher Vmax values were found for the enzyme from -P Desmodium plants with any of the plots used to transform the data. For the extracted enzyme Vmax values were similar for the +P and -P plant enzymes but a lower Km was found for -P as compared to +P grown plants: 2.00, 1.48, 1.78,1.61 mM p-NPP *vs* 3.23, 3.27, 2.96, and 2.69 mM p-NPP for +P, as calculated from the plots seen in Figures 3 and 4, for +P and –P treatments using flasks for the extraction of the enzyme. Besides enzyme velocity (Vmax) was lower for the extracted enzyme between 8.85 and 11.63 μmol PNP/h/g FW roots for +P and -P plants) as compared to the secreted enzyme.

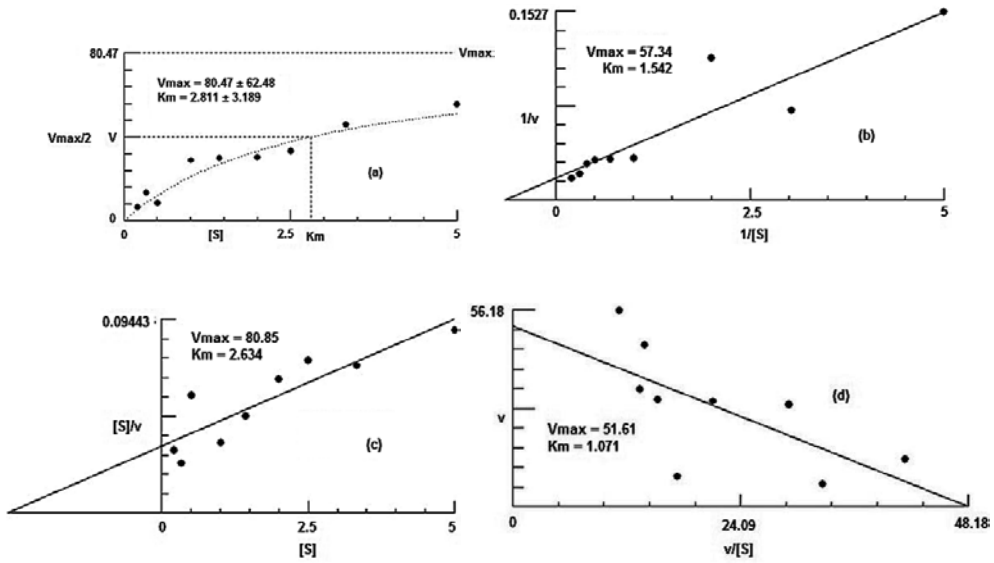

Figure 1. Enzyme kinetics plots of the acid phosphatase activity from *Desmodium tortuosum* roots with the substrate p-nitrophenylphosphate (p-NPP). The secreted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

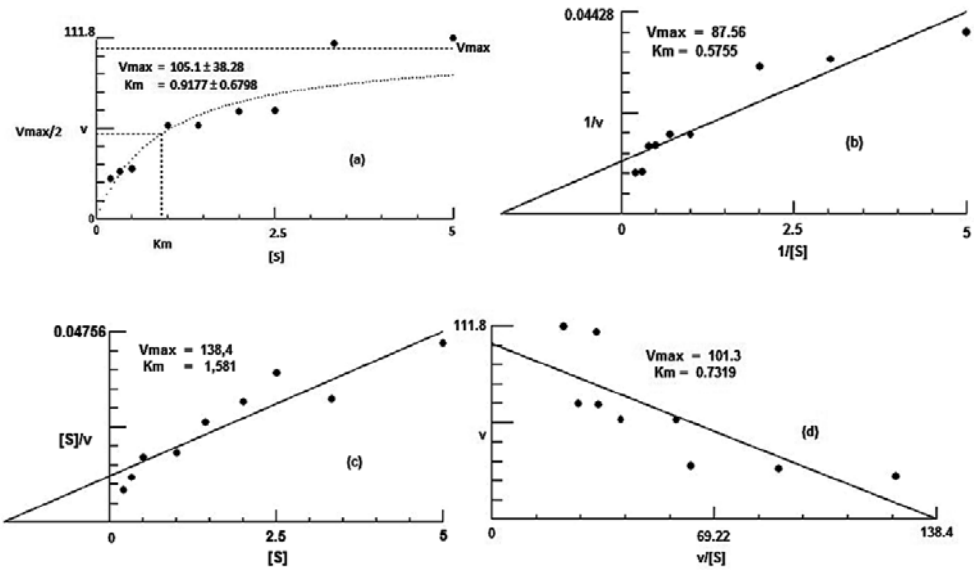

Figure 2. Enzyme kinetics plots of the acid phosphatase activity from *Desmodium tortuosum* roots with the substrate p-nitrophenylphosphae (p-NPP). The secreted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated

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2.2. Acid Phosphatase Kinetics in *Phaseoulus vulgaris* (common bean)

After 14 days of planting the roots of four plants grown under either P sufficiency (+P 1.0 mM P) or deficiency (-P 0.005 mM P) were immersed in the dialysis membrane

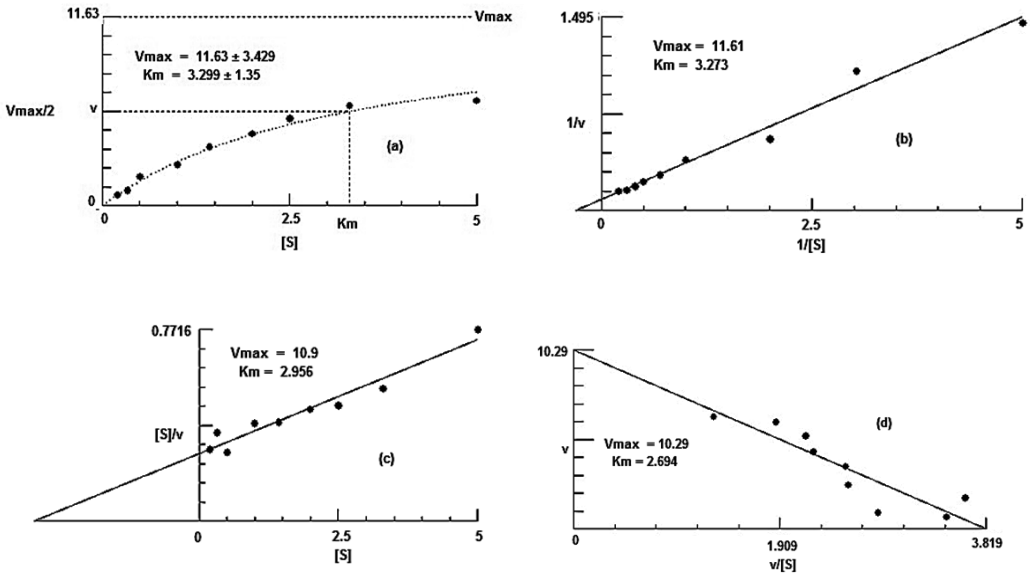

Figure 3. Enzyme kinetics plots of the acid phosphatase activity from *Desmodium tortuosum* roots with the substrate p-nitrophenylphosphate (p-NPP). The extracted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated

For the extracted enzyme the roots of 21 days old plants were immersed in the collection flasks and after the extraction period aliquots were taken to perform kinetic studies for (a) the extracted enzyme and (b) the secreted enzyme. For the secreted enzyme saturation kinetics was observed for the enzyme from both high and low grown plants (+P and -P) as substrate concentration was increased from 0.2 to 5.0 mM, as seen from the hyperbolic Michaelis-Menten plot in Figure 5a and Figure 6a Figure +P (a) and -P (a). The apparent Km and Vmax determined from Lineweaver-Burk, Hanes and Hofstee plots Figures +P and -P. for the high P plant enzyme Figure 5 (a, b, c and d) were Km values of 0.93, 0.73, 1.36, and 0.85 mM p-NPP and Vmax values of 8.5, 7.66, 10.13, and 8.34 μmol PNP/h/ g FW roots. For the low P grown plants Km values were similar to those found for the +P plant enzyme as calculated from plots in Figure 6 (a, b, c, and d) while the Vmax values were higher for the low P plants between 13.36 and 14.28 as calculated from Figure 6 (a, b c and d). For the extracted enzyme Km values were inconsistent and no clear differences were found between +P and -P plants (calculated from plots in Figure 7 and 8 respectively).

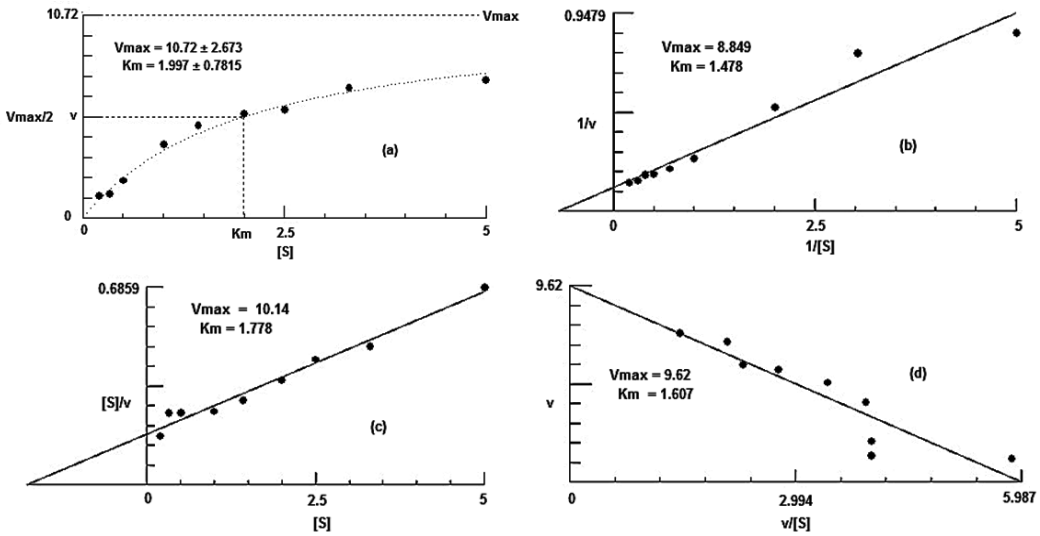

Figure 4. Enzyme kinetics plots of the acid phosphatase activity from *Desmodium tortuosum* roots with the substrate p-nitrophenylphosphate (p-NPP). The extracted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

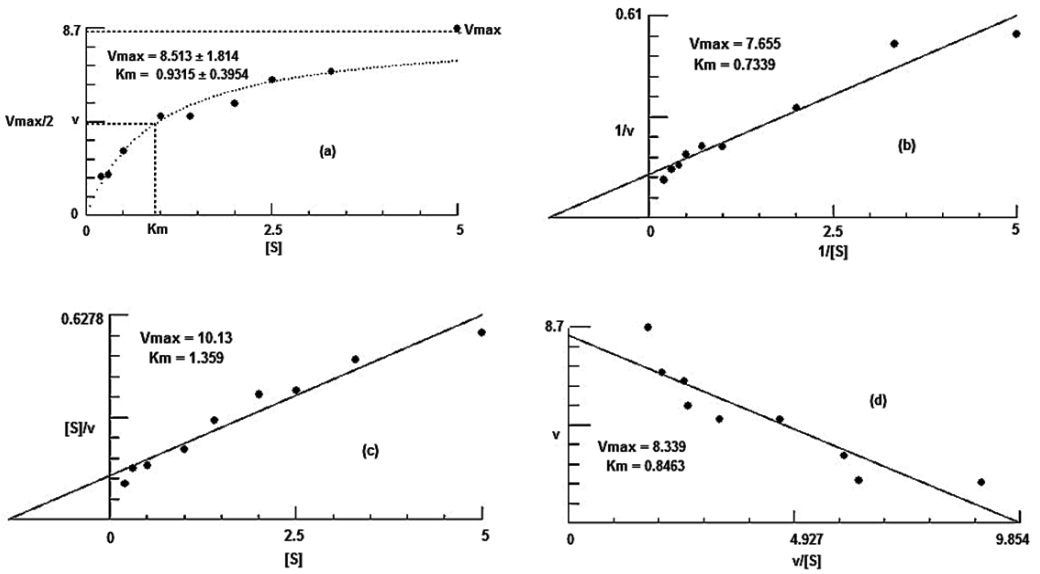

Figure 5. Enzyme kinetics plots of the acid phosphatase activity from *Phaseolus vulgaris* roots with the substrate p-nitrophenylphosphate (p-NPP). The secreted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

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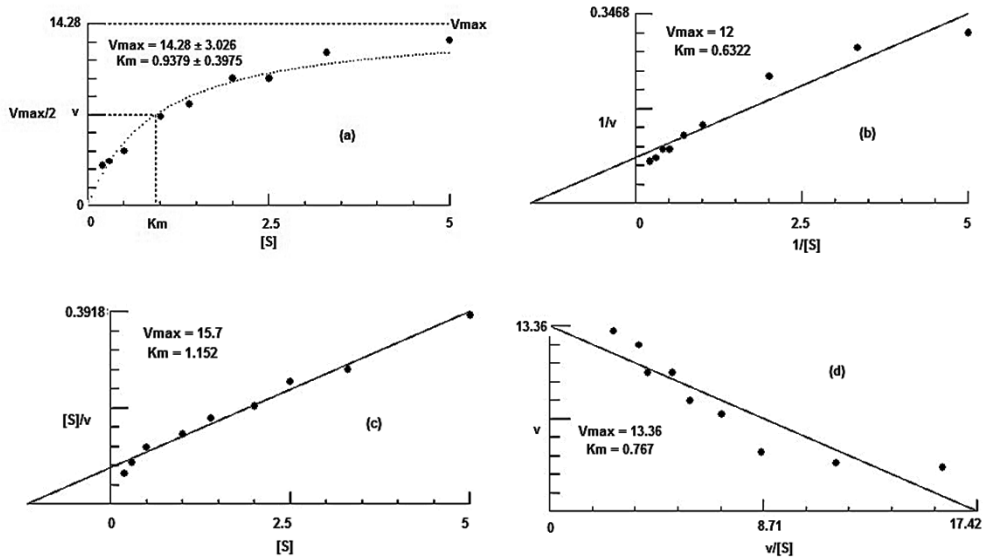

Figure 6. Enzyme kinetics plots of the acid phosphatase activity from *Phaseolus vulgaris* roots with the substrate p-nitrophenylphosphate (p-NPP). The secreted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

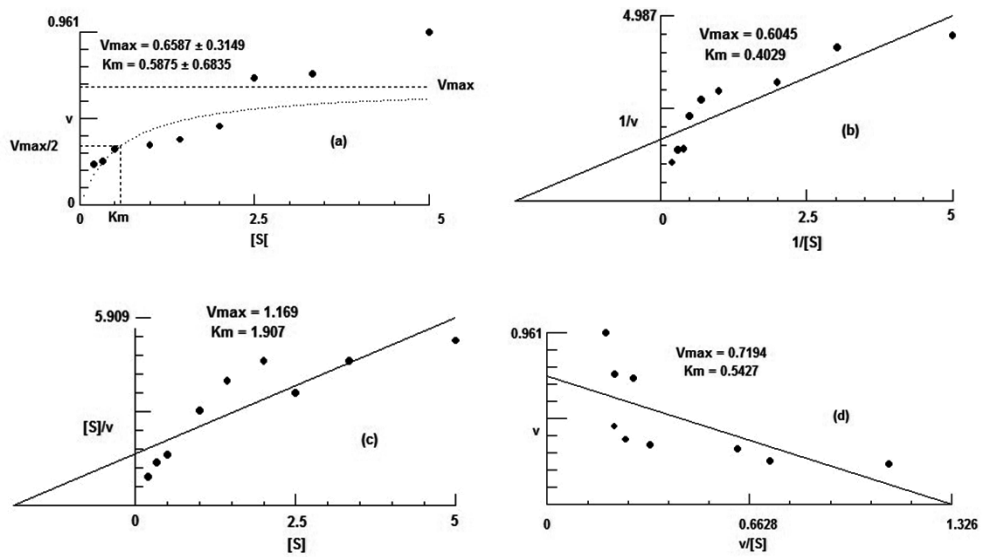

Figure 7. Enzyme kinetics plots of the acid phosphatase activity from *Phaseolus vulgaris* roots with the substrate p-nitrophenylphosphate (p-NPP). The extracted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated

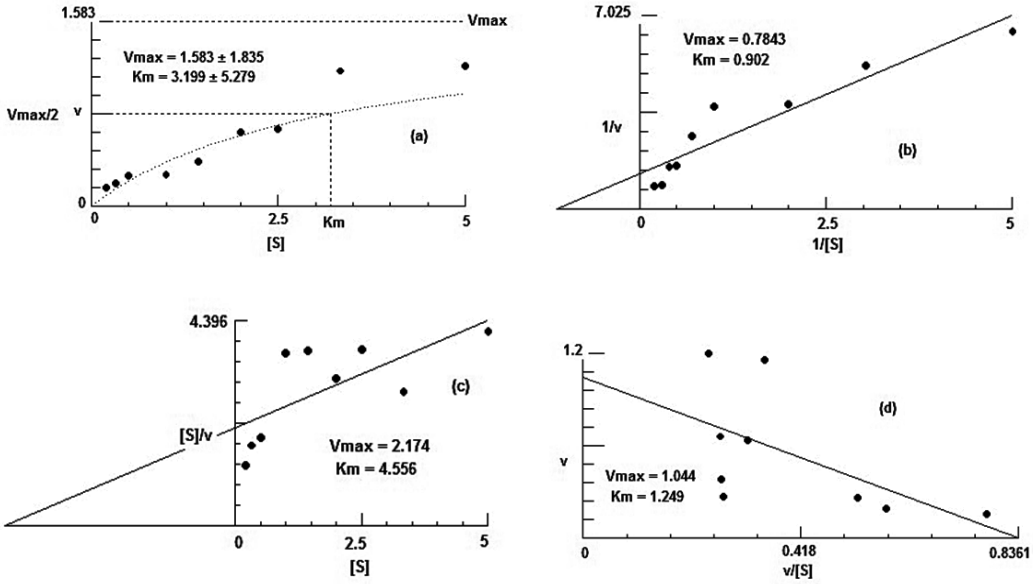

Figure 8. Enzyme kinetics plots of the acid phosphatase activity from *Phaseolus vulgaris* roots with the substrate p-nitrophenylphosphate (p-NPP). The extracted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

Vmax values were lower than those for the secreted enzyme although higher for -P plants, as found for the secreted enzyme. However, no clear tendency was found in the Km values for the extracted APase collected from root extracts.

2.3. Acid Phosphatase Kinetics in *Vigna unguiculata* (cowpea)

After 14 days of planting the roots of four plants grown under either P sufficiency (+P 1.0 mM P) or deficiency (-P 0.005 mM P) were immersed in the dialysis membrane and after the extraction period aliquots were taken to perform kinetic studies for the secreted enzyme. Data for the extracted enzyme are not presented as they were very low and similar for +P and -P plants and in some cases not detectable. For the secreted enzyme saturation kinetics was observed for the enzyme from both high and low grown plants (+P and -P) as substrate concentration was increased from 1.0 to 5.0 mM, as seen from the hyperbolic Michaelis-Menten plot in Figure 9a and Figure 10a Figure +P (a) and -P (a). The apparent Km and Vmax determined from Lineweaver-Burk, Hanes and Hofstee plots Figures +P and -P. for the high P plant enzyme Figure 9 (a, b, c and d) were Km values of 1.02, 1.31, 1.04 and 0.96 mM p-NPP and Vmax values of 4.11, 4.46, 4.12 and 4.03 μmol/h/ g FW roots. For the low P grown plants Km values were lower (0.72, 0.68, 0.98 and 0.71) to those found for the +P plant enzyme as calculated from plots in Figure 10 (a, b, c, and d) while the Vmax values were higher for the high P plants (4.11, 4.46, 4.12, and 4.03 as compared to 3.23, 3.19, 3,52 and 3.23 μmol PNP/h/g FW roots for -P enzyme as calculated from Figures 9 and 10.

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2.4. Acid Phosphatase Kinetics in *Crotalaria juncea* (sunn hemp)

After 20 days of planting the roots from +P (0.86 mM P) and -P (.004 mM P) intact plants were individually immersed in a dialysis membrane and after an extraction period of 24 h, aliquots from the solution inside the dialysis membrane were taken to perform the kinetics studies of root secreted acid phosphatase. Two separate experiments were performed using different plants. The following concentration range for the substrate was used: 1.0, 1.25, 1.43, 1.67, 2.0, 2.50, 3.30 and 5.50 mM p-NPP and as no clear substrate saturation was observed for either +P or -P enzymes using the hyperbolic Michaelis-Menten plot, the apparent Km and Vmax values for the two experiments were determined from Lineweaver-Burk, Hanes and Hofstee plots and linear transformations were adjusted to the best fit line.

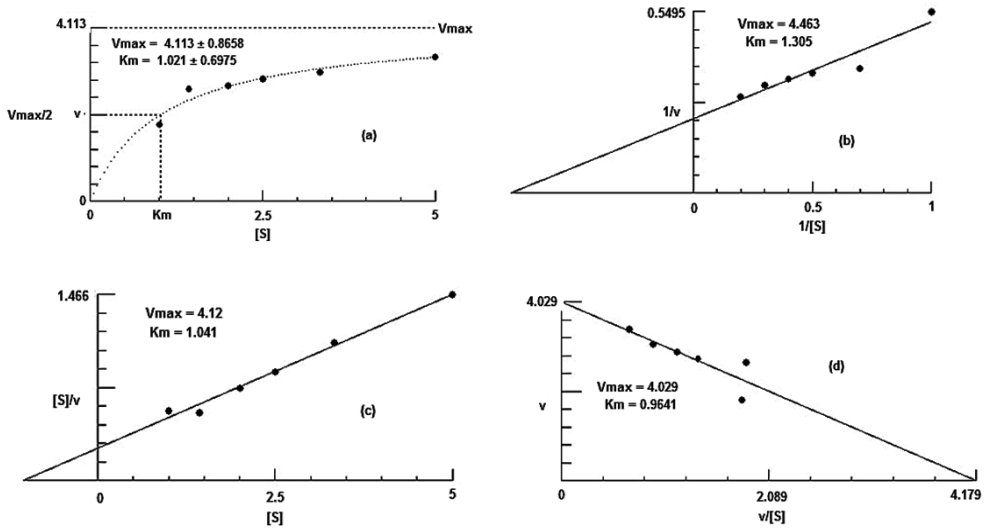

Figure 9. Enzyme kinetics plots of the acid phosphatase activity from *Vigna unguiculata* roots with the substrate p-nitrophenylphosphate (p-NPP). The secreted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated

It is important to note that a homogeneous preparation is by no means necessary for kinetic analyses, but the purer the enzyme the less complications from competing reactions that may use up the substrate or the product (23). As shown from the results in Table 1, apparent Km and Vmax values, showed considerable kinetic diversity but the degree of adjustment for the linear equations (r^2) was always higher than 0.70 except for the Hofstee plot for +P plants in experiment 1, where a low r^2 value of 0.50 was obtained. The apparent Km values determined from Lineweaver-Burk plots were, for the first and second experiments, 0.53 and 0.57 mM for -P and 0.82 and 0.76 mM p-NPP for +P plants respectively; from the Hanes plot 1.75 and 0.79 (-P) and 0.62 and 1.13 (+P) and from the Hofstee plot 0.53 and 0.59 (-P) and 0.86 and 0.78 (+P).These results show that the Km from low P plants was lower than that for +P plants (except when calculated from the Hanes plot) and varied from 0.53 to 0.59 mM in -P and 0.76 and 1.13

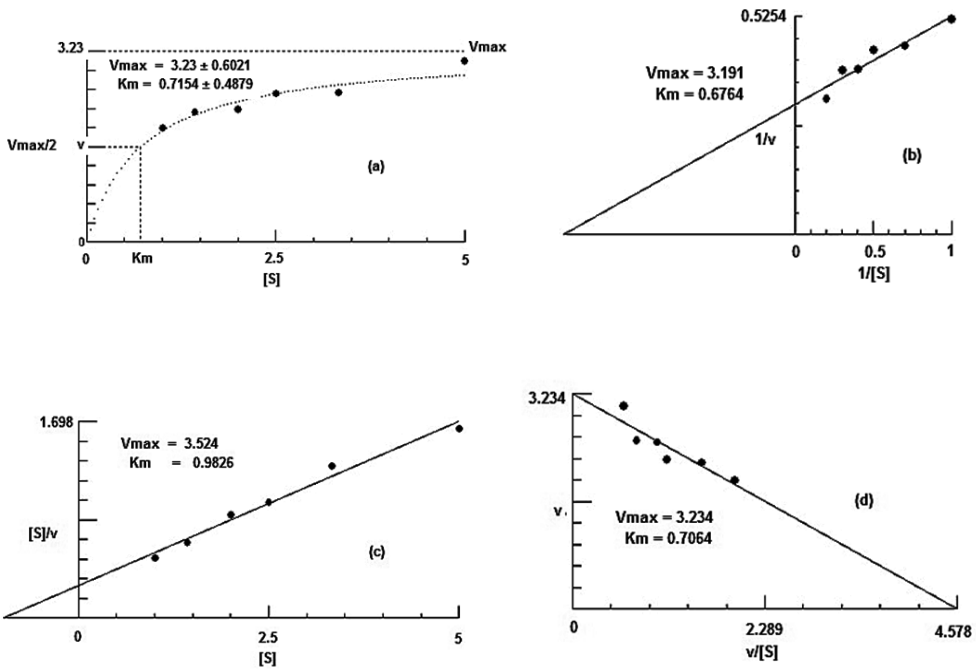

Figure 10. Enzyme kinetics plots of the acid phosphatase activity from *Vigna unguiculata* roots with the substrate p-nitrophenylphosphae (p-NPP). The secreted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

mM in +P plants. Thus for practical purposes and according to the results presented in this study, the Lineweaver-Burk and Hofstee plots are the best options to fit the data, if the objective of the study is to find out the differences in Km between -P and +P plant enzymes The apparent Vmax values determined from Lineweaver-Burk double reciprocal plot were 39.90 and 25.97 for -P and 25.44 and 25.38 μmol PNP/g root DW/h for experiments 1 and 2 respectively; from the Hanes plot these values were 33.78 and 28.08 (-P) and 38.04 and 28.73 (+P) and from the Hofstee plot 36.94 and 26.12 (-P) and 26.13 and 25.64 (+P).These results show that Vmax values were similar for the secreted enzyme from roots of low P and high P plants.

Plot	Experiment	Treatment	Equation	r^2	Km (mM p-NPP)	Vmax (μmol PNP/h/g DW r)
Lineweaver	I	-P	Y =0,0271+0,0144x	0,9209	0,53	36,90
		+P	Y= 0,0393+0,0321x	0,7144	0,82	25,44
	II	-P	Y=0,0385+0,0221x	0,8869	0,57	25,97
		+P	Y=0,0394+0,0299x	0,8542	0,76	25,38

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Plot	Experiment	Treatment	Equation	r^2	Km (mM p-NPP)	Vmax (μmol PNP/h/g DW r)
Hanes	I I	-P	Y=0,0520+ 0,0296x	0,9075	1,75	33,78
		+P	Y=0,0163+ 0,0262x	0,9980	0,62	38,04
	II	-P	Y=0,0282+0,0356x	0,9948	0,79	28,08
		+P	Y=0,0393+0,0348x	0,9888	1,13	28,73
Hoffstee	I	-P	Y=36,9396-0,5331x	0,8929	0,53	36,94
		+P	Y=26,1334-0,8593x	0,4997	0,86	26,13
	II	-P	Y=26,1232-0,5892x	0,8487	0,59	26,12
		+P	Y=25,6493-0,7819x	0,7837	0,78	25,64

Table 1. Apparent Km and Vmax values for the root secreted acid phosphatase in +P and -P grown plants of *Crotalaria juncea*. (Ascencio and Santana, unpublished) -P= 0,004 mM P; +P= 0,86 mM P

3. Discussion

Root acid phosphatase activity for the plants was higher in -P plants at the beginning of the growth period (depending on the species) and that the proper timing for the onset of the P-stress is apparently crucial for induction of APase in different species [2, 6]. It has been shown in this and many other reports in the literature, that P deficient conditions in the plant can trigger APase activity [5, 10, 23]; and that new isoenzymes could be activated under P deficiency in roots [12, 16], leaves [29] and seedlings [14].However for bean and cowpea APase activity appears not to be inducible when 0.02 mM P was used to grow the plants under P deficient conditions even though differences in P concentration in the dry matter were large enough to suspect that plants were suffering from mild P stress [9]. In the present study 0.005 mM P was used in the low P treatment and even though large differences were not found for enzyme activity between +P and -P plants larger differences in total dry matter, leaf area and soluble Pi concentration in leaves were found. However from an agronomic point of view and focusing on the APase role to dissolve organic-P in soils with low P bioavailability, the enzyme secreted with the root exudates to the soil is a major concern in plant adaptation, specially to phosphorus-limited tropical soils [21]. Like many other secreted proteins the APase is glycosylated, which protects the enzyme against proteolytic enzymes and contributes to its stability over a wide pH range. Secretory APase can liberate bound P from soil and have shown to deplete organic P in the rhizosphere of several plant species; however, mineral phosphorus is hardly available in tropical soils and for organic P to be used in agricultural soils, increased secretion of APase may be involved as part of the coordinated adaptive strategies to withstand P deficiency. However, under soil conditions where extracellular enzymes such as APase function are associated with soil colloids, a large fraction of the free enzyme may be immobi-

lized as extracellular enzymes such as APase primarily function associated with soil colloids [20]. Compared to the free enzyme, properties and kinetic behavior of such complexed enzymes had a different pH activity dependence and sensitivity to temperature and protein degradation [19]. According to reports in the literature, the kinetics of APase in synthetic enzyme complexes simulating those usually encountered in soil, showed Michaelis-Menten kinetics with a lower Vmax and higher Km values as compared to the free enzyme [13]. Many APase isozymes exist in the root and leaves but only one of them was secreted into the rhizosphere in a large amount [25]. When the enzyme was mixed with aqueous solutions extracted from a P-deficient soil its activity declined to 55% of its original activity after 14 days and to 9% after 21 days. We have performed experiments applying the secreted APase enzyme solution obtained from low P grown plants of *Centrosema rotundifolia* and *Crotalaria juncea* to low P soils [6]; according to the results APase activity in the soils showed significant differences depending on soil type and root secretion but was higher in soils with the secreted APase from *Crotalaria* plants. Under the conditions of a higher Km the enzyme will not efficiently function under P starvation as a higher substrate concentration is needed to achieve half the maximal velocity. Under these circumstances, in order to unbind APase to perform efficiently, besides having a lower Km value, the roots should have the ability to secrete larger amounts of the enzyme into the rhizosphere to compensate for the low Vmax. It has been shown that the APase secreted by white lupin roots is stable in soil solution and shows low substrate specificity which is important to improve their ability to use organic P [12]. According to our results, true saturation Michaelis-Menten kinetics was not observed for all the species, specially for the enzyme from +P plants; we have also found similar results with crude extracts from other wild and cultivated species, and as seen from the shape of the plots of enzyme velocity *versus* substrate concentration, the presence of several isoenzymes should not be discarded. In this context the Hofstee plot (v vs, v/S) is the best alternative in detecting the presence of multiple enzymes that catalyze the same reaction [23]. For agronomic purposes, it is better to assay the crude enzyme secretion or extract, without further purification, as it is the form that it is released from the roots to the environment. Differences in APase activity for *Phaseolus vulgaris* as seen from the Km values apparently indicate the lack of phosphate starvation-inducible APase, as it has been found in other crops, for example, see [14]; Vmax values on the other hand were higher in -P plants; however the combination of a high Km with a high Vmax could improve plant behaviour under P deficiency. The opposite was noted with *Vigna unguiculata* where a low Vmax in -P plants may be compensated by a lower Km. As compared to *Phaseoulus* and *Vigna,* the APase secreted from the roots of *Crotalaria juncea* showed considerably greater kinetic diversity depending on the methods of plotting enzyme kinetics data for the calculation of Km and Vmax values for -P and +P plants. For maximum efficiency it seems reasonable to expect that the enzyme from low P plants under the conditions of this study would have a low Km and a high Vmax; we have found for *Crotalaria* differences in the Km from -P and +P plants, but not for Vmax where the values were similar for the enzyme secreted from low P and high P plants, as found for *Phaseoulus and Vigna*. The less suited combination for the enzyme to perform efficiently under P deficient conditions is to have a high Km and a low Vmax (which means that the substrate concentration must be high and does not compensate for a low Vmax). From our results, it is seen that the enzyme from -P plants is better suited

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to cope with P deficiency, due to a consistent lower Km. In this context, favorable kinetic properties (low Km and highVmax) as well as the amount of secreted enzyme are important as one may compensate for each other; in this connection the best combination for the enzyme to perform efficiently under natural conditions, where a low P concentration exist in the soil, would be a low Km and a great amount of secreted enzyme to the environment. This might be the better strategy for plant species to perform efficiently under low P soils. We have shown from previous studies that leguminous plants have developed several growth strategies to withstand P limitations imposed by the soil; under P deficiency total leaf area, relative growth rate (RGR) and root length were reduced by 50% in severely stressed *Desmodium tortuosum* and other plant species [4,5], and that for tomato APAse activity was highly correlated to development and recovery from P stress and that total weight and average root diameter decreased under P stress while root surface area per unit dry weight increased, [10]. As large differences were found in Relative Growth Rate (RGR) between the high and low P plants and as these differences were consistent for all the species analyzed, RGR is an adequate physiological indicator of plant performance under P deficient conditions and a useful tool if used in screening purposes.

4. Conclusions

The practical implications of kinetic constants Km and Vmax for the enzyme exuded by the roots of several plant species were analyzed in this study in terms of the potential for P-liberation under limiting condition of Pi bioavailability in soil; an increased Pi uptake is likely to occur if the APase released by the roots has a low Km value (in the neighborhood of the soil P concentration) and a high Vmax, in order to be efficient in liberating Pi from the soil organic-P pool. It has been shown that one of the advantages of the APase secreted from the roots of some leguminous species such as lupinus, was a higher Vmax value as compared to the enzyme from other species. We have found similar results for enzyme from *Desmodium*, *Phaseoulus* and *Vigna*. The numerical value of the Km for the substrate p-nitrophenyl-P provided a means of comparing the enzyme from high or low-P plants.Our results showed that Km is a reliable physiological tool for assessing plant adaptability to P-deficiency and its is suggested that Km, enzyme activity (Vmax), total leaf area and relative growth rate (RGR) may be used as physiological indicators to differentiate plants grown under P deficiency or sufficiency.

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Section 3

Molecular Analysis

Chapter 5

Genetic Strategies to Enhance Plant Biomass Yield and Quality-Related Traits for Bio-Renewable Fuel and Chemical Productions

Massimiliano Lauria, Francesco Molinari and Mario Motto

Additional information is available at the end of the chapter

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Abstract

Owing to the increasing concerns on the environment, climate change, and limited natural resources, there are currently considerable efforts applied to produce chemicals and materials from renewable biomass. While initial emphasis has been placed on biofuel production from food plant sugars, the competition between crop usage for food and non-food applications has promoted research efforts to genetically improve yield and quality-related traits for biorefining applications. This chapter summarizes the potential of genetic and biotechnological strategies for improving plant biomass yields and quality-related traits and for breeding varieties more suitable to meet biorefining applications. Attempts were also made to provide a description on the genetic and molecular mechanisms affecting starch, cell wall composition and architecture, and oils synthesis and deposition, including genetic strategies to modify these traits. Similarly, the chapter covers the genetic strategies to improve yields by emphasizing the efforts done to identifying genetic variation and gene(s) governing critical morphological, structural, and physiological traits that in turn influence biomass yields. Finally, in the chapter it is suggested that knowledge of plant biosynthetic pathways will eventually provide valuable opportunities for metabolic engineering, as well as access to chemical transformations unique to plants for breeding varieties with built-in new traits.

Keywords: Starch biosynthesis, cell wall and compositions, gene regulation, signal transduction network, genome editing, yield genes, sink strength, transgenic plants, metabolic engineering

1. Introduction

Current society and economy are largely dependent on petroleum as a source of many industrial products ranging from fuels to commodity and specialty chemicals. However, petroleum feed-stocks are limited and nonrenewable, and their broad use is also deeply contributing to unwanted increases in atmospheric CO_2 concentrations [1]. Therefore, there is at present increasing demand to develop and implement strategies for production of chemical commodities or platform molecules (see Glossary) from biomass instead of using petroleum. The drive towards bio-based products (such as fuels, chemicals, and plastics), which seeks to replace the conventional petrochemical processes with new technologies, must be economically competitive, if not advantageous [2].

Advances in genetics, biotechnology, process chemistry, and engineering are leading to a new manufacturing concept to convert complex biomass into value-added products. In this context, emphasis has been placed upon the genetic improvement of plant biomass as a sustainable source of organic carbon (C) for the large-scale production of chemicals and materials. Accordingly, in this chapter we focus our attention on the potential of genetic and biotechnological strategies for improving plant biomass yields and quality-related traits to develop dedicated and highly specialized plant varieties that meet targeted applications and end-uses, maximizing the value throughout the whole bio-based value chain. Future perspectives in this field of research are also described.

2. Biomass feed-stocks for biorefinery applications

Notably, biomass—organic matter that has stored energy through the process of photosynthesis—accounts for over 10% of global primary energy supply and is the world's fourth largest source of energy [3]. Thus, biomass in a variety of forms (solid stock, herbaceous matter, seeds, algae, biowaste, and crop residues) represents an abundant C-neutral renewable resource for the production of bioenergy and biomaterials [4].

While initial emphasis on biomass for biorefinery applications has been placed on biofuel production from fermentable feed-stocks, such as starch and sugar, resulting in an increasing demand for agricultural crops, such as maize and sugarcane, the drive to reduce the competition between crop usage for food and non-food applications has promoted research efforts to access the less digestible saccharides in cell walls (lignocellulosics) [5]. This 'biorefining technology' of using cellulosic biomass as the feed-stock has not yet been fully commercialized because of high production cost. In addition, the bulky biomass harvested seasonally in rural areas poses a challenge to feed-stock logistics and storage. It is worth noting that the biorefinery technology has the same goals as today's petroleum refineries, namely the conversion of a raw material source (in this case biomass or bio-derived feed-stocks) into bio-based products, most commonly via microbial conversion of fermentable sugars derived from cellulose and, ideally, hemicelluloses [reviewed in 6]. Figure 1 outlines a scheme of the biorafinery concept.

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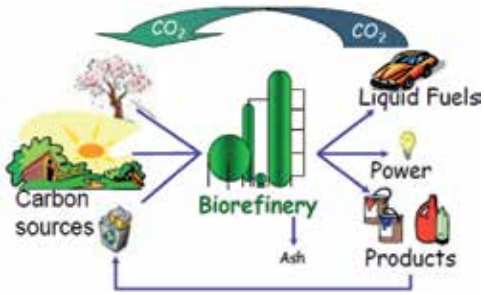

Figure 1. Illustration of the biorefinery concept.

3. Genetic strategies to modify plant biomass properties

The potential for improvement of plant biomass production has not yet been extensively explored because traditional breeding of crop plants (e.g., maize, rice, and soybean) has been focused on selection of the high grain-yield traits. Thus, to convert biomass effectively into fuels, fine chemicals, and commodity materials, a range of approaches using genetic strategies have been explored. This can be addressed from at least two points of view: modifying biomass properties to reduce processing costs or increasing biomass yield and reducing agricultural inputs. An example of these possibilities is given in Figure 2.

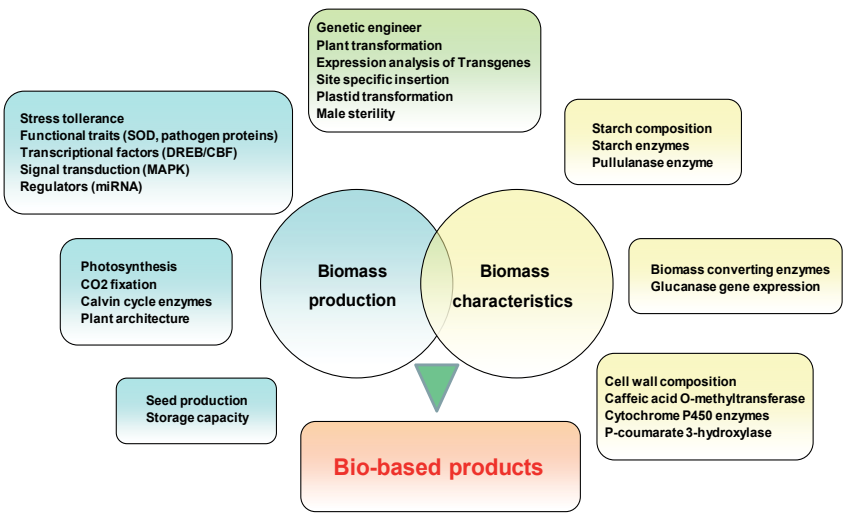

Figure 2. Possible approaches to enhance productivity of a crop biomass. Diagram indicating the two main routes for enhancing plant biomass yield and quality-related traits via genetic strategies. The first objectives to increase the biomass yield per land area (*i.e.* the biomass yield and its stability). The second objectives to modify biomass characteristics and composition to generate conversion process-friendly products for fuels and biochemicals.

3.1. Genetic strategies to modify biomass quality-related traits

The quality-related traits herein considered refer to sugar- and starch-based and lignocellulosic feed-stocks. In addition, we will also consider in this section plant oils. Bio-based polymers can be generated, besides from polysaccharides and lignin of biomass crops, also from lipids, oils, and fatty acids (FAs) synthesized in oil crops (e.g., oil palm, canola, soybean).

3.1.1. Sugar- and starch-based feedstocks

The prevalence of metabolic fluxes inside plant cells is focalized on the production and usage of sugars, the primary products of the photosynthetic process, and their conversion into storage and structural carbohydrates, namely starch and cellulose.

Starch is the dominant constituent of many harvestable organs (e.g., tubers or grain) and is the second-largest form of biomass produced by vascular plants. It is a versatile and useful biopolymer not only because it is a cheap, natural material, but because of the ease with which its physicochemical properties can be altered through chemical or enzyme modifications and/or physical treatments. Starch can be efficiently hydrolyzed by α-amylases that break starch mainly into oligosugars and glucoamylases that yield glucose monomers in biotechnological production processes [7].

3.1.1.1. Biosynthesis and genetics of starch

In recent years, our understanding on the nature and starch accumulation has largely increased, resulting in a vast body of published literature. The reader is referred to several reviews on these topics for detailed information [e.g., 8, 9]. The main findings emerging from these studies indicate that in chloroplasts, starch is accumulated for only short periods of time and thus is named 'transitory starch'; whereas in amyloplasts, starch is accumulated for long-term storage and therefore termed 'reserve starch'. In storage tissues starch is deposited as insoluble, semi-crystalline granules but also occurs to a lesser extent in most vegetative tissues of plants. It is composed of two distinct fractions: amylopectin (highly branched, 75–80% of starch dried mass) and amylose (mostly linear, 20–25% of dried mass). Both are made of α-1,4 glucosidic bond glucose residues branched via α-1,6 glucosidic linkages. The clustered nature of the α-1,6 branch points allows glucan side-chains to form double-helical structures, compacting large amounts of glucose. Consequently, extremely large structures can be synthesized and packaged in an insoluble state.

Advances in genetics and biochemistry have led to significant discoveries in how starch is synthesized in plants [10]. Three enzymes in this pathway, localized in the plastids, are playing a cardinal role in the synthesis of amylose and amylopectin: ADP-glucose pyrophosphorylase (AGPase, involved in the initiation of starch biosynthesis), starch synthase (SS, involved in elongation and granule formation), and branching enzyme (BE, involved in branching and granule formation). Further studies across plant species have indicated that these enzymes carrying out starch synthesis are encoded by well-conserved families of genes. Moreover, a number of mutations that cause defects in various steps in the pathway of starch biosynthesis were described and used to clone genes involved in this biosynthesis. Table 1 gives a list of

the enzymes and genetic loci of cloned genes involved in the pathway of starch synthesis in maize endosperm. Furthermore, these mutants have provided information to achieve modified natural starches by reshaping endogenous processes by using, for instance, antisense RNA technology, ectopic expression or mutant enzymes, or by introducing or modifying enzymes or molecules that are implicated indirectly in starch synthesis [12].

Enzyyme	Class	Mutation[a]	Mayor biochemical changes[b]	Structural changes in mutant
Sucrose synthase	SuSy-SH1	*sh1*	↑ Sugars, ↓ Starch	None/minimal
AGPP	Cytosol. SS	*bt2*	↑ Sugars, ↓ Starch	None/minimal
AGPP	Cytosol. LS	*sh2*	↑ Sugars, ↓ Starch	None/minimal
AGT	AGT	*bt1*	↑ Sugars, ↓ Starch	None/minimal
Starch synthase	GBSSI	*wx*	↑ 100% Amylopectin	Low-amylose
Starch synthase	SSI	-	-	-
Starch synthase	SSII	*su2*	↑ Sugars, ↓ Starch	Lacks intermediate chains in amylopectin
Starch synthase	SSIII	*du1*	↑ Apparent amylase	Lacks longer chains in amylopectin
Starch synthase	SSIV	-	-	-
Branching enzyme	BEI	*sbe1*	None/minimal	None/minimal
Branching enzyme	BEIIb	*ae*	↑ Apparent amylose ↑ Loosely branched polysaccharide 60%, 70%, 45%	High-amylose, long chain amylopectin
Debranching enzyme	ISAI	*su1*	↑ Sugars, ↓ Starch	Compound granules, phytoglycogen
Debranching enzyme	ISAII	-	-	-
Debranching enzyme	ISAIII	-	-	-
Debranching enzyme	ZPUI	*pu1*	None/minimal	None/minimal

a) ae = Amylose extender; bt1 = brittle1; bt2 = brittle2: du1 = dull1; sbe1= starch branching enzyme1; sh1= shrunken1; sh2= shrunken2; su1= sugary1; su2= sugary; wx = waxy. pu1= pullulanase1.

b) Changes relative to normal. ↑↓= increase or decrease, respectively. Sugars = the alcohol-soluble sugars.

Table 1. Summary of mutant effects in specific maize endosperm mutants where an associated enzyme lesion has been reported. [Modified from Motto et al., 2011, [11].

Notably, starch biosynthesis is a remarkable regulated process, both at transcriptional and post-transcriptional level; it is also interconnected with an ample variety of cellular processes

and metabolic pathways [10]. Its regulation involves a complex and an as yet not well clear assemblage of factors that are adjusted to the physiological status of the cell. For example, marked regulatory properties were found for enzymes involved in starch biosynthesis, especially for AGPases, which is subject to multilevel regulation. AGPases are heterotetramers that contains two large (51 kD) and two slightly smaller (50 kD) subunits that are both required for optimal enzyme activity but have nonequivalent roles in enzyme function; the large subunit plays more of a regulatory role, while the small subunit has both catalytic and regulatory properties.

Evidence from comprehensive expression profiling in various plant species has revealed a pathway-wide regulation of the expression of genes affecting sucrose-starch interactions [13]. Furthermore, the coordinated regulation of gene expression in source and sink tissues appears, to a large extent, orchestrated by the sugar status of the cell. Although the sensing and signaling mechanisms mediating these processes are largely unknown, studies have suggested the presence of members of the WRKY(term derived from the most prominent feature of these proteins the WRKY domain), or WRKY domain) and AP2/EREBP (APETALA 2/ethylene response element binding protein) families of transcription factors (TFs) [14,15] and of an ethylene receptor participating in source-sink communication and sucrose-mediated regulation of starch synthesis [16]. Additional research has shown that starch biosynthesis may also be regulated by post-translational protein modification, including allosteric regulation by metabolites, redox regulation, protein–protein interactions and reversible protein phosphorylation [17]. It is suggested that some of the newly discovered aspects of fine control of the starch biosynthetic pathway may apply to many other proteins that are directly and indirectly involved in polymer synthesis and degradation. Thus, to achieve a significant progress in the rate of starch synthesis, it would be important to increase the expression of a set of enzymes and transporters in the starch pathway.

3.1.1.2. Modification of starch-specific properties

Altering the quality of the starch by plant breeding and molecular biology has already been achieved via the commercial exploitation of some starch mutants that include types that cook to form clear colloidal solutions rather than opaque gels (e.g., waxy maize or wheat) or others that are useful industrially (e.g., amylose extender maize), and finally others that accumulate less starch and more sugar (e.g., sweet maize, sweet potato). However, the industrial applications of starch to formulate commercial products are yet limited due to poor reactivity of glucose, which is the elementary unit of starch. The addition, during starch biosynthesis, of glucose residues possessing reactive side-chains or charged groups would expand the number of commercially usable chemical alterations and, consequently, enlarge the future uses of starch industrial applications [12]. Thereby, the need for starch possessing specific properties by diverse industries is fueling starch biotechnology research [18]. Figure 3 gives examples of possible genetic and biochemical modifications directed for improving starch characteristics more adapted to industrial end uses.

Another strategy to improve the efficiency of starch as a feed-stock is to reduce the energy requirements for the starch in the biorefining conversion process of plant biomass to chemicals

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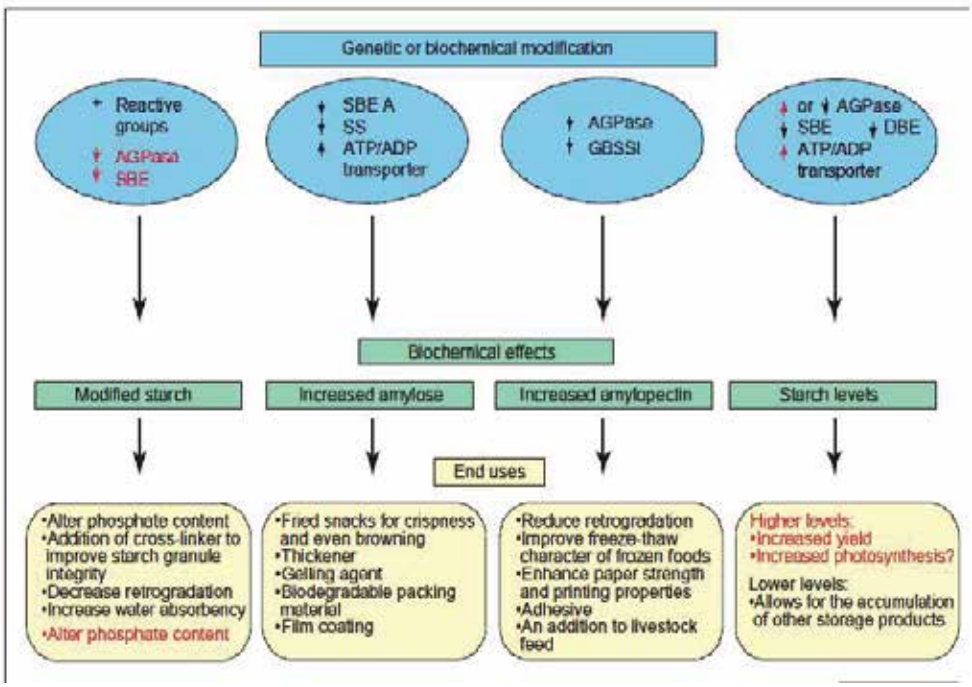

↑: an enhancement in the level of an enzyme;
↓: a decrease in the level of an enzyme;
+: addition of reactive groups.
DBE: debranching enzyme; GBSSI: granular-bound starch synthase I; SBE: starch branching enzyme; SBE A: class A SBE; SS: starch synthase.

Figure 3. Alteration of starch and their enduses. The makeup, modification and levels of starch can be modified through genetic and biochemical strategies. The resulting variations may modify the characteristics and applications of starch (Reproduced with permission from Slattery et al., 2000, [12]).

including *in planta* production of enzymes useful for starch degradation. For instance, gelatinization is the first passage for bioethanol production from starch. It is reliable that a modified starch possessing a lower gelatinization temperature might need a minor supply of energy in the biorefining conversion process. Rice research has highlighted that the expression of a recombinant amylo-pullulanase-formed starch that when heated to 85°C was perfectly transformed into soluble sugars [19].

In addition to altering starch quality, it is also possible to modify starch quantity via biotechnological approaches by increasing starch content, and thus yield, in storage organs of crops [10]. These strategies include enhancement of AGPase activity, extending the supply of starch precursors to the amyloplast, increasing the supply of sucrose to heterotrophic cells, expanding sucrose breakdown within the heterotrophic cell, enhancing the expression of starch synthase class IV, blocking starch breakdown, altering the expression of global regulators, enhancing trehalose-6-phosphate content, blocking the activity of ADPG breakdown enzymes, and increasing starch content in heterotrophic organs.

Altering contents of molecules or enzymes that are not directly affecting starch biosynthetic processes may also be a useful strategy to change positively starch quality and quantity. Production of starches possessing novel properties might permit to maintain natural starch properties that could be damaged by post-harvest operations. Surely, the generation of these starches might abolish the necessity for post-harvest changes [9].

Additional investigations are required to unveil how the different levels of regulation (e.g., transcriptional, allosteric, and post-translational) interact to control the subtle structure of starch and starch granules. Only when this level of knowledge will be achieved, the complete capacity for the comprehensive arrangement of starch molecules with specific functionality will be practicable.

3.1.2. Lignocellulose feed-stocks

Lignocellulosic biomass, derived from crops and agricultural residues, is a promising renewable source for the production of fuels and bio-based materials. It is estimated that there is an annual worldwide production of 10–50 billion tons of dry lignocellulose, accounting for about half of the global biomass yield [20]. Thus global availability and unsuitability for human nutrition have promoted lignocellulosic feed-stocks into the focus for biorefinery applications, as a fundamental source of fermentable carbohydrates for biofuel productions and for a broad array of chemicals and biodegradable compounds. Nevertheless, the generation of fermentable sugars from lignocellulose is one of the major constraints for the industrialization of lignocellulose biorefining. This is attributable to the compact and rigid structure of lignocellulose commonly referred as biomass recalcitrance [21], a distinguishing feature closely related to the chemical and physical characteristics of the cell walls that is crucial for plant survival [22].

Biomass crops, either woody species (e.g., pine, poplar, eucalyptus) or grasses (e.g., sugarcane, sorghum, miscanthus, maize stover) consist primarily of cell walls. These are formed by intricate assemblages of celluloses, hemicelluloses, pectins, lignins, and proteoglycans [23]. A diagrammatic illustration of the framework of lignocellulose structure forming cell walls can be seen in Figure 4.

3.1.2.1. Cell wall composition and architecture

The main component of cell walls is cellulose, which makes up 15–30% of the dry biomass of primary cell walls and up to 40% of the secondary cell walls. Cellulose is a β(1- 4)-linked chain of glucose molecules, with a degree of polymerization varying in length between 8000–15,000 residues [24]. Its building blocks, qualified as elementary fibrils, which are supposed to accommodate approximately 36 β-D-glucan chains, are covered with other non-cellulosic polysaccharides to form microfibrils; these are subsequently cross-linked with hemicellulose/pectin matrixes to generate macrofibrils that confer structural stability at the cell wall. Hemicelluloses (20–30%), the second most abundant constituent of lignocellulosic biomass, are heterogeneous polymers derived from varying combinations of both hexoses (D-glucose, D-mannose, D-galactose) and pentoses (D-xylose and L-arabinose), including heteromannans, xyloglucan, heterxylans, and mixed-linkage glucan [25]. In contrast to cellulose, the polymer

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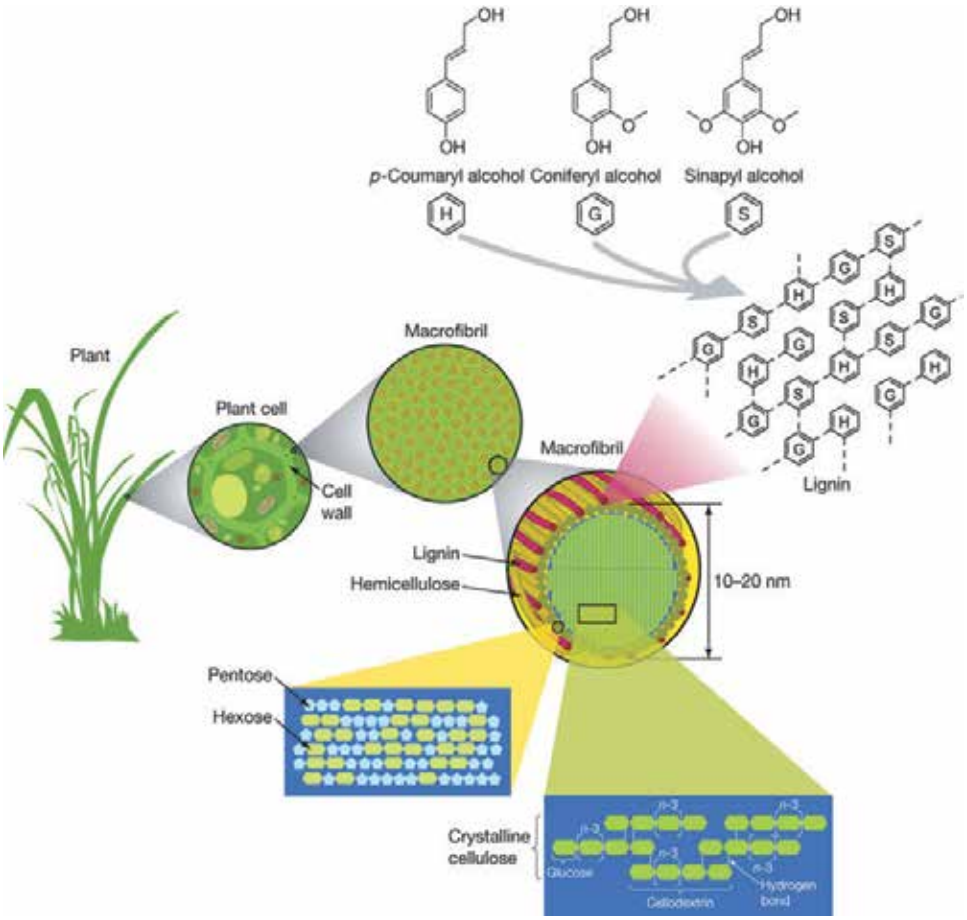

Figure 4. Structure of lignocellulose. Cellulose, hemicellulose, and lignin form structures called microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall. (Reproduced with permission from Rubin 2008, [23].

chains are branched and achieve comparatively short molecular lengths (500–3000 residues). The utilization of hemicellulose, in general, and its main component xylose, in particular, are at the center of research efforts in metabolic engineering to optimize lignocellulosic feed-stocks for biorefining technologies. Lignin (15–25%) is the third most-abundant biopolymer in cell walls and the largest available resource of natural aromatic polymers [26]. It is a heteropolymer mainly composed of three major phenolic monomers, namely p-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S). Combinations of these structural units or monolignols are incorporated into lignins with species, tissue, and developmental specificity. Lignin performs an important role in strengthening cell walls by cross-linking polysaccharides, thus providing support to structural elements in the overall plant body and conferring an exceptionally resistant to biological attacks, properties that interfere, however, with enzymatic conversion of polysaccharide components.

3.1.2.2. Biogenesis and genes involved in cell wall assembly

Genetic progress to improve cell wall composition and structure is a crucial objective for two motives: i) cell walls constrict cell size and shape and thus have a remarkable role in plant growth, affecting biomass production and ii) cell walls are recalcitrant to degradation by microorganisms to liberate sugars for fermentation, consequently affecting biomass quality [27].

According to Carpita and McCann [28], plants devote approximately 10% of their genome (i.e., ≈2,500 genes) to construction and dynamic rearrangement of their cell walls during growth. Specifically, the previous authors have grouped ≈1200 cell wall-related genes that are implicated in the synthesis, assembly, and disassembly of the plant cell walls into six categories/stages of cell wall biogenesis consisting of substrate generation, polysaccharide synthesis, membrane trafficking, assembling and turnover, secondary cell wall formation, and signaling. What emerges from this study is that the differences among angiosperms in cell wall compositions are reflected in the structure of these gene families.

In higher plants, cellulose is synthesized by large multimeric plasma membrane-associated cellulose synthase rosettes, termed CESA [29]. The subunits are encoded by the *CESA* genes represented by multiple, usually 10 or more, members, the majority of which appear to be involved in primary wall formation as judged from mutational genetic studies and gene expression profiling. In addition to *CESA* genes, chemical and genetic screens have also identified various genes that indirectly contribute to cellulose biosynthesis, such as *COBRA* (encoding a protein anchored to the membrane through glycophosphatidylinositol, GPI), *KOBITO (*encoding a membrane associated protein of unknown function), and *KORRIGAN* (encoding a membrane-anchored β-glucanase) [29]. A member of the *COBRA* gene family, *CobL4* from Arabidopsis, and its orthologs *Brittle culm-1* (*Bc1*) from rice, and *Brittle stalk-2 (Bk2*) from maize, have been shown to specifically affect cellulose formation in secondary cell walls.

The complexity of events contributing to the activation of *CESA* at the plasma membrane and its motility suggest that the list of players in this biosynthesis is far from complete and might include accessory proteins and cell wall-sensing mechanisms that appear to affect cell wall biogenesis [29]. Furthermore, gene expression studies have revealed that CESA proteins are expressed spatially and temporally throughout plant development, indicating that specific transcription factors belonging to NAC (no apical meristem), MYB (myeloblastosis), WRKY, and leucine zipper families, play a role in cell wall biogenesis. Therefore, a better understanding of the regulation, activation, and assembly of the CESA complex as well as discovery and characterization of CESA accessory proteins and plant-specific TFs will further clarify targets for genetic manipulation.

The biosynthesis of hemicelluloses requires the coordinated expression of several glycan synthases and glycosyltransferases (GTs) for polymer backbone and side-chain formation, respectively [25]. In this context, it was found that several *CELLULOSE SYNTHASE-LIKE F* (*CSLF*) genes encoding Golgi-localized GLs, are involved in hemicellulose biosynthesis. More specifically, evidence indicates that 25 *xylem-specific GT* genes from 7 GT families support this

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biosynthesis [30]. Furthermore, it was clarified that several *GT* gene families (i.e., *GT43*, *GT47*, and *GT61*) cooperate in xylan biosynthesis. Additionally, the identification of Arabidopsis *IRX* mutants has implicated that *GT8*, *GT43*, and *GT47* families as potential *glucuronoxylan* (*GX*) biosynthetic genes. Further research has documented that the biosynthesis of GAXs would require at least three GTs: xylosyl-transferase (XylT), arabinosyl-transferase (AraT), and glucuronosyl-transferase (GlcAT) [31]. Similarly, it was reported that in xylan synthesis glycosyl hydrolases may also play a role, as well as a number of transcription factors [32]. These include, in particular, master switches such as Secondary Cell Wall Associated NAC Domain 1 (SND1) and other TFs directly downstream, such as multiple MYB factors and a KNOTTED1-like homeodomain protein. Furthermore, in maize, *UDP-glucose 6-dehydrogenase* genes were found to encode central enzymes of hemicellulose biosynthesis and appear essential for cell wall formation in young organs. Although, the information reported above has pointed out the importance of the hemicelluloses in plant growth and development, future research is required to combine these single components and their assemble. This will permit to improve our understanding on the biosynthesis of this important class of plant cell wall components. It is expected that altering the expression of those genes will very likely change the amounts and properties of hemicellulose, which in turn, may lead to decrease recalcitrance.

Lignin is one of the most relevant objectives for genetic improvement of cellulosic biomass adapted for biofuel production: modifications in its chemical composition and quantity directly influence the pretreatment costs presently used in biofuel production starting from cellulosic biomass as feed-stock [33]. Biosynthesis of lignin involves two major processes: the monolignol pathway (via the phenylpropanoid pathway) in the cytosol and polymerization of the monomers into the cell wall [34]. The enzymes needed for monolignol biosynthesis are well described and comprise ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-(hydroxy)cinnamoyl CoA ligase (4CL), hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT), p-coumaroylshikimate 3′-hydroxylase (C3′H), caffeoyl CoA O-methyltransferase (CCoAOMT), (hydroxy)cinnamoyl CoA reductase (CCR), ferulic acid 5-hydroxylase (F5H), caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT), and (hydroxy) cinnamyl alcohol dehydrogenase (CAD) [35]. Similarly, the genes involved in the synthesis from phenylalanine to hydroxycinnamates and monolignol substrates of lignin biosynthesis are well established [30]. Furthermore, it was found that many of the genes encoding key lignin biosynthetic enzymes belong to multigene families [36]. Thus, specific isoforms may be expressed in different cell types at different developmental stages or in response to changing environmental conditions, complicating attempts to alter lignin accumulation.

3.1.2.3. Genetic strategies to improve lignocellulosic components

In recent years, genetic modification of the lignin biosynthesis pathway has received great attention because of the use of model plants to dissect the biosynthetic pathway and because its content in biomass is inversely correlated with its forage digestibility and quality value in the pulping industry [33]. Moreover, these findings indicate that lignin cannot be simply removed from growing plants without causing negatives developmental effects. In several plant species (e.g., maize, switchgrass, poplar, and pine), efforts using natural mutants or

silencing (RNAi) strategies directed at the down-regulation of a number of genes encoding lignin biosynthesis enzymes were not successful. This is likely due to the fact that those interventions drastically reduced lignin content in a non-selective way. Nevertheless, there are cases in which mild genetic manipulations have been used to moderately reduce lignin content or modify its composition in biomass, modestly improving saccharification efficiency, forage digestibility, and pulping yield [37].

The recent development of targeted genome-editing technologies, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered, regularly interspaced, short palindromic repeats (CRISPR) or CRISPR-associated (CAS) systems, offers exciting potential to resolve the issues of highly specific genome modifications with great efficiency and specificity [38,39]. These technologies make use of sequence-specific designer nucleases that cleave targeted loci, enabling creation of small insertions and deletions, insertion of novel DNA, or even replacement of individual alleles. A simplified model summarizing the emerging techniques for plant engineering of lignin proposed by Eudes and coworkers [33] is reported in Figure 5. According to the previous authors, this strategy will eventually offer the opportunity to design crops with optimized lignin composition and distribution while retaining all other traits related to the phenylpropanoid pathway.

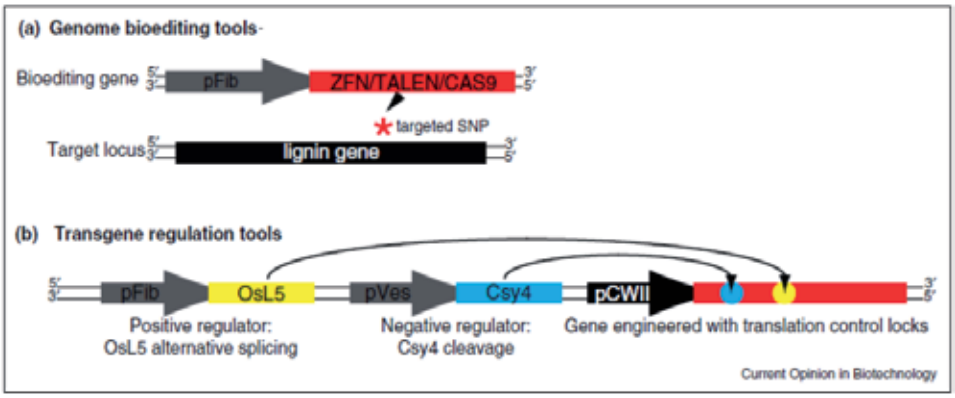

Figure 5. Cases of novel strategies for multifaceted genetic engineering of plants. (a) Genome bioediting techniques. Black box, endogenous lignin locus (target of editing); grey arrow, fiber specific promoter used to drive the expression of the bioediting gene; red box, bioediting gene: ZFNs, TALENs or CRISPR/CAS9; red star, SNP generated when the genome bioediting gene is expressed. (b) Transgene regulation techniques. Grey arrow, fiber (pFib) or vessel (pVes) specific promoter; yellow box, gene encoding the OsL5 protein with the alternative splicing cassette shown in the same color inserted in transgenes (yellow circle); blue box, gene encoding the Cys4 protein with its cognition sequence shown in the same color inserted in transgenes (blue circle); black arrow, secondary cell wall promoter (pCWII); red box, engineered gene: gene used to manipulate lignin composition which has been engineered with transgene regulation tool (yellow circle, OsL5 alternative splicing cassette; blue circle, Cys4 cognition sequence) (Reproduced with permission from Eudes et al., 2014, [23]).

Besides traditional lignin reduction methods that directly target genes from the lignin biosynthetic pathway, novel dominant approaches are currently in development. This new trend for lignin engineering focuses on the redirection of C flux to the production of related

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phenolic compounds and on the replacement of monolignols with novel lignin monomers to improve biophysical and chemical properties of lignins such as recalcitrance, or industrial uses [33]. Alternatively, although lignocellulosic feedstocks might be employed for conversion to biomaterials, two principal drawbacks in the producing systems are the costs of transport and processing of biomass. A solution to this problem is to produce directly in the plant cells the microbial cellulase enzymes. This will promote directly *in planta* the conversion of fermentable sugars during the biomass transportation to bioraffineries [40]. In maize, the expression of the catalytic domain of the thermostable 1,4-b-endoglucanase of *Acidothermus cellulolyticu*s [41] corroborates the idea that plant may be used as a biofactory for cellulose-degrading enzymes. Alternatively, although lignocellulosic feed-stocks might be used for conversion to biomaterials, two major limitations for this process are the costs of transport and biomass processing. A solution is to produce microbial cellulase enzymes in the plant cells to facilitate the conversion of fermentable sugars *in planta* during the biomass to biorefinery conversion process [40]. Expression of the catalytic domain of the thermostable 1,4-b-endoglucanase of *Acidothermus cellulolyticu*s in maize [41] supports the opinion that maize can be used as a biofactory for cellulose-degrading enzymes.

3.1.3. Lipids

Oil from crop plants represents the bioproduct that is chemically more similar to petrol and consequently has the highest aptitude to substitute it in the chemical industry. Petrol is considered to be originated from ancient, lipid-rich organic compounds, namely spores and planktonic algae (sedimented and transformed) under high pressure and temperature, throughout millions of years [42].

3.1.3.1. Biogenesis and genes involved in oil production

Virtually, all plant seeds contain storage lipids in the form of triacylglycerol esters (TAGs) containing three FAs with chain lengths of C8–C24, with C16 and C18 (i.e., palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid) being the most commons [43]. A number of comprehensive reviews on characteristics of structure and enzymes that are involved in oil biosynthesis and deposition in seeds have been recently published. Again the reader is referred to several excellent in-depth reviews and books for detailed information [e.g., 44, 45]. Briefly, studies in this field have indicated that plant oil is synthesized from glycerol-3-phosphate and fatty acyl-CoA in the endoplasmic reticulum as TAGs, and esters of FAs acids and glycerol. Moreover, FAs are synthesized in plastid from acetyl-CoA and then transported to the cytoplasm in the form of fatty acyl-CoA. In the ER, FAs are employed for the acylation of the glycerol-3-phosphate backbone either by the Kennedy pathway or by acyl exchange between lipids. Then, the resulting TAGs are deposited in specialized structures termed oil bodies. A schematic outline of the biosynthesis of storage lipids in seeds and in vegetative tissues is depicted in Figure 6.

Additional studies have indicated that some plants can produce, besides the common five FAs above-cited, also an array of unusual or 'novel' FAs exhibiting a wide diversity of FA structures, including various functionalities such as hydroxylation, epoxidation, acetylation, and

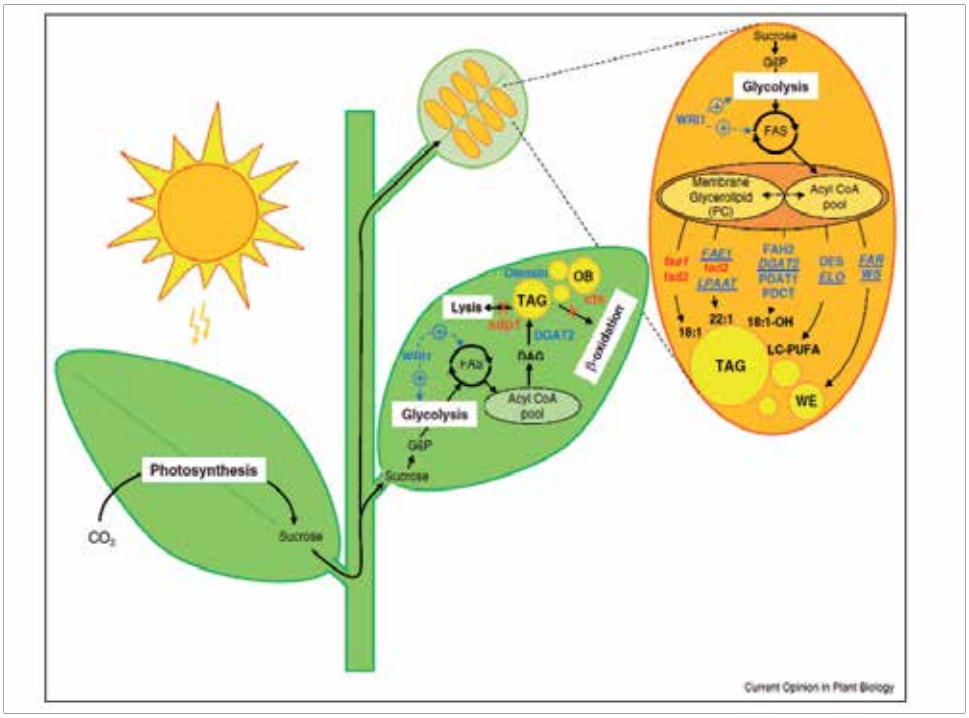

Figure 6. Schematic representation of metabolic engineering strategies for manipulation of oil content and composition in vegetative and seed tissues. Blue: target genes suitable for overexpression; Red: target genes for inactivation by mutation or RNAi constructs. Genes encoding enzymes using acyl-CoA substrates are underlined. FAS = plastid localised fatty acid synthase (Reproduced with permission from Napier et al., 2014, [46]).

conjugation that impart properties required for specific industrial uses [46, 47]. Furthermore, studies in this field have discovered a large diversity occur in the function of various FA-modifying enzymes, such as FA desaturase2 (FAD2), thioesterases, fatty-acid elongases, cytochrome P450s, acyl-CoA desaturases, acyl-ACP desaturases, sphingolipid-Δ8 desaturases, and S-adenosylmethionine methyltransferases [48]. The properties of many of these enzymes showing new functionality have recently been reviewed in detail by Voelker and Kinney [49], while for gene required for lipids synthesis visit http://www.canr.msu.edu/lgc/.

3.1.3.2. Genetic strategies to modify oil content and composition

At present, the industrial value of the current seed oils is limited by their FA composition that is not well suited for the manufacture of specialty chemicals and polymers. Therefore, due to complex inheritance of oil content and composition in seeds, several metabolic engineering approaches have been employed. Although, several examples of transgenic plants with altered oil composition were obtained, none of these plants had a high level of an unusual FAs necessary for industrial application (≈ 90–95% of the total FAs) [48]. These results suggest that plants vary considerably in the ability of their background metabolic machinery to handle the newly synthesized FAs. It is probable that further research will uncover genes for specialized

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forms of the various acyltransferase and TAG assembly enzymes capable of efficiently handling the unusual FAs. It is suggested that co-expression of such genes along with the previously introduced FAs acid biosynthetic pathways should contribute to further increases in accumulation of novel FAs transgenic plants in the future and lead to the development of economically viable crop sources of industrial raw materials.

In addition, to search for naturally occurring enzymes that can be used for the transgenic production of industrially useful FAs, other strategies have been considered such as diverting carbon flow from starch to TAG, up-regulating FA synthesis, modifying expression of individual TAG biosynthetic enzymes, and the use of TFs [43, 46]. In this context, genetic investigations have found various seed-specific regulatory genes that may play an important role by controlling oil deposition in the seed. For instance, the *WRINKLED1 (WRI1*), an AP2/EREBP transcription factor initially described in Arabidopsis, plays a regulatory function [50]. These studies have also identified several motifs that are important for WRI1 binding and transactivation. This is further highlighted by the study of Shen and co-workers [51] in which the maize ortholog of *LEAFY COTYLEDON1 (LEC1*) (another TF involved in the regulation of oil accumulation, upstream of *WRI1*) was expressed in transgenic maize under the control of an oleosin promoter. The resulting *ZmLEC1* overexpression lines of maize manifested outstanding levels of seed oil (up to 48% greater than null segregants), mainly due to extended storage lipids in the embryo. Although this high-oil phenotype was stable over various generations, it was also unfortunately associated with detrimental agronomic traits, such as reduced seed germination and plant growth, suggesting unacceptable and unpredictable pleiotropic effects caused from impaired seed-specific expression of *ZmLEC1*. To overcome these negative effects, the previous workers performed analogous experiments by overexpressing ZmWRI1. This research resulted in a high oil phenotype (≈ 31% higher than nulls) without the negative side effect on germination and growth. Notably, the strength of the promoter adopted to drive their expression of *ZmWRI*1 gave a favorable impact on the enhancement in seed oil deposition. Alternatively, the use of a weaker promoter produced only an accumulation in the seed approaching 17%.

The possibility of producing TAGs in leaves and other vegetative tissues has recently attracted considerable attention [46]. A schematic representation of metabolic engineering strategies for manipulation of oil content and composition in vegetative and seed tissues is given in Figure 6. A number of reports have documented that TAG accumulation can be increased by ectopic expression of individual biosynthetic enzymes, TFs that control seed development and maturation, or by mutating genes involved in TAGs and FAs turnover [46]. However, in the majority of these investigations enhancement of TAGs concentration in leaf was low and/or dependent on carbohydrates supply. Because key enzymes for both oil synthesis and breakdown are expressed in vegetative tissues, it was suggested that attaining significant amounts of storage lipid in the leaf will be essential in the re-orientation of C flux into TAGs [46]. Nevertheless, a remarkable increase in TAG levels (exceeding 15% of dry weight in vegetative tissue) has only been realized by integrated metabolic strategies directed to improve FAs and TAGs synthesis while inhibiting lipolysis. Additionally, the detection of non-seed proteins affecting the binding and stabilization of lipid-rich molecules in the cytosol of plant cells has

highlighted a new angle of the cellular machinery influencing TAGs packaging in plant vegetative tissue. It will be attractive to clarify if oil accumulation in green biomass can be further improved without severely impairing photosynthesis and plant development. A possibility for achieving this target is the use of senescence-induced promoters to engineer plants in which TAG accumulations will be initiated only after leaves have reached their maximum size [52]. Another strategy that might be devised is to directly connect C fixation to FAs biosynthesis by introducing a functional glycolytic pathway that is efficient to transform 3-phosphoglycerate to phosphenolpyruvate. Independently from the strategy that will be employed, the challenge of using photosynthetic cells to accumulate very high amounts of oil is an attractive objective. However, reaching levels of oil accumulation exceeding those currently found in seed oil crops, namely, superior to 35% of TAGs (% dw), remains a noteworthy metabolic engineering goal.

3.2. Genetic strategies to improve biomass yield

Plant breeding is driven by the need to continually increase sustainable yield and quality of crop plants and by meeting projected increases in global food demand. Targeted genetic improvement in yield for developing new varieties suitable as biorefinery feed-stocks will depend on identifying genetic variation in critical morphological, structural, and physiological traits affecting biomass production. This involves manipulating complex traits, such as those associated with plant growth and development. Biomass yield can also be enhanced by manipulation of additional pathways such abiotic and biotic stress. These topics are outside the scope of this chapter and have been reviewed elsewhere [e.g. 53, 54].

3.2.1. Yield genes

To accomplish maximal biomass yield in the development of new biomass crops, it is relevant to: i) identify genes and genetic pathways that are crucial to biomass production, ii) recognize the selective forces that have molded the frequencies of these genes in current varieties, and iii) establish which morphological and physiological traits may eventually lead to more efficient plants in yield performance. Although there is genetic variability in yield traits, many of them (yield, yield stability, nutrient, and water use) that are important in crop productivity are multigenic traits and are often difficult to breed for. Quantitative trait loci (QTLs) mapping approaches are common genomic tools to dissect the genetic architecture underlying complex traits and to identify QTLs [55]. Furthermore, the development of high-throughput sequencing and genotyping technologies has greatly improved the accuracy of QTL analysis. In this respect, biomass QTL mapping has been conducted in several crops with the purpose of identifying genomic regions and genetic loci underlying biomass feedstock yield (e.g., poplar, maize, switchgrass, perennial ryegrass) or sugar yield in sugarcane [56]. These studies carried out in different species and population types have manifested the prevalence of additive main effects, digenic epistasis, QTL x environment interactions, multiple minor effects, and QTL distributed over several genomic regions; moreover it was shown that both parents were contributing favorable and unfavorable alleles irrespective of their biomass yield potential.

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3.2.2. Molecular biology approaches for increasing biomass yield

Although genetic dissection of yield components, such as those affecting biomass productions, can help to elucidate the physiological route from gene to phenotype, current progresses in our knowledge of how plants function and develop can expand potential and efficiency of plant breeding programs devoted to yield improvement. Insights into gene and genome sequences, the regulation of gene expression and the molecular and cellular mechanisms and pathways underlying plant architecture, development, and function, may offer new options to plant geneticists to comprehensively devise novel breeding programs.

These strategies include molecular approaches to increasing biomass yield and transgenic research directed toward increasing biomass yield through genetic modification of different plant traits.

3.2.2.1. Photosynthesis

Photosynthesis provides the primary energy and C input for plant growth. Improving photosynthesis has been identified as a key strategy for the production of crop plants with higher biomass yield [57]. Molecular targets were identified by the study of bottlenecks of photosynthesis) and approaches to overcome these bottlenecks were mostly based on the up-regulation or down-regulation of single genes [58]. In some instances, synthetic pathways were used to overcome limitations of the endogenous pathways. A list of potential targets to improve photosynthesis is given in Table 2.

1. Improving Rubisco function
i. Improving Rubisco catalytic activity
ii. Altering Rubisco amount per leaf area
2. Increasing thermostability of Rubisco activase to sustain Rubisco activity at high temperature
3. Enhancing CO_2 concentration around Rubisco to maximize catalytic rate and minimize photorespiration
i. Turning C3 plants into C4 plants
ii. Installing algal or cyanobacterial CCM into C3 plants
iii. Redesigning photorespiratory metabolism
iv. Improving CO2 transfer pathways via stomata and/or mesophyll cells
4. Enhancing chloroplast electron transport rate
i. Improving whole chain electron transport
ii. Modifying light-harvesting systems
5. Enhancing enzyme activity of Calvin cycle (*e.g.*, FBPase, SBPase)
6. Enhancing the capacity of metabolite transport processes and C utilization

Table 2. Potential targets for improving plant photosynthesis.

An obvious target for increasing the source strength is the production of photoassimilates during photosynthesis. In this respect, research has been undertaken to introduce a more efficient, C4-like photosynthesis in C3 plants [58]. These last workers indicated that the introduction of single C4 enzymes (i.e., phosphoenol pyruvate carboxylase, PEPC, and pyruvate orthophosphate dikinase, PPDK) in C3 plants has until now not generated improvement in photoassimilate accumulation. This is probably attributable to disturbances in the fluxes of C4 intermediates for metabolic pathways excluding the C4 cycle. Notably in rice, the joined expression of two C4 cycle enzymes was found to raise photosynthetic ability up to 35% and grain yield up to 22%. In this research, the maize genes were transferred to the rice genome together with their corresponding promoters, which might have turned out in a superior spatial and temporal expression of the C4 cycle enzymes than the more expected expression of transgenes by using constitutive promoters. Additionally, work in transgenic tobacco has also showed that increased levels of fructose-1, 6-bisphosphatase (FBPase) and sedoheptulose-1,7-bisphosphatase (SBPase), two Calvin cycle enzymes, significantly increased dry weight [59].

Besides the above strategies based on C4 photosynthesis, other approaches have been taken to improve the efficiency of photosynthetic C assimilation. One of these strategies is focused on the enzyme RUBISCO ACTIVASE, a key regulator of RUBISCO (Ribulose 1,5-bisphosphate carboxylase/oxygenase essential component of the photosynthetic process of fixing CO_2 into organic C) activity [60]. Transgenic Arabidopsis plants expressing a heat-tolerant version of RUBISCO ACTIVASE showed a significant improvement in photosynthesis and leaf growth when exposed to heat. Other efforts to improve the photosynthetic efficiency of plants have been focused on: i) increasing electron flow by overexpressed CYTOCHROME C6 (CYTC6), a protein involved in the photosynthetic electron transport chain [61]; ii) engineering new pathways into the chloroplast that bypass photorespiration and release CO_2 directly into the chloroplast stroma [62] ; and iii) using various 'add-ons' or CO_2-concentrating mechanisms (CCMs) to elevate CO_2 levels in the vicinity of RUBISCO. These CCMs were pursued by improving mesophyll CO_2 conductance via overexpressing aquaporin [63] or stomatal CO_2 conductance via manipulating stomatal characteristics [64]. In this respect, more recently Lin and co-workers [65] have successfully engineered tobacco plants containing a functioning RUBISCO from a cyanobacterium. The cyanobacterial enzyme has a greater catalytic rate than any 'C3' plant. The lines generated in this research open the way for future addition of the remaining components of the cyanobacterial CCM, an important step towards enhancing photosynthetic efficiency and improving crop yields. Furthermore, it has been shown that the triose-phosphate/phosphate translocator (TPT) strongly limits photosynthesis under high CO_2 conditions [65]. The TPT provides a regulatory link between CO_2 assimilation and cytosolic C metabolism. In this context an approach for increasing plant yield was performed by overexpressing sucrose transporters in sink cells, thereby enhancing sink demand and inducing an increase in photosynthesis and assimilates export. When overexpresssing a potato sucrose symporter (*StSUT1*) in storage parenchyma cells of pea seeds, there was enhanced sucrose influx into cotyledons and greater cotyledon growth rates [67]. In addition, it has been shown that enhancement of sucrose synthase activity represents a useful strategy for increasing starch accumulation and yield in potato tubers. In a future world of higher CO_2 concen-

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tration, enhancing the capacity for sucrose export and carbon utilization would be an important component for maximizing photosynthesis and yield. While altering transport capacity alone is unlikely to change photosynthetic capacity, enhancing photosynthetic capacity as well as transport capacity could lead to improved plant growth and yield.

Technology for nucleus or chloroplast genome transformation has been advancing and it would enable easier and more precise manipulation of the photosynthesis process. It is expected that such plants could exhibit more efficient photosynthesis under controlled conditions; the plant factory in which plants are produced in an optimized growth environment would have potential advantages of high productivity. In the future, the combined uses of several strategies would greatly help to improve photosynthetic capacity and thus plant growth and ultimately yield.

3.2.2.2. Plant architecture

Considerable progress has been realized over the past decade in revealing the molecular mechanisms that underlie the formation of plant architecture. Dissection of plant architecture with plant morphological mutants enabled plant scientists to gain insides into various aspects affecting plant architecture as exemplified by the shot apical meristem (SAM) maintenance and differentiation, the initiation of axillary meristems (AMs), the formation and outgrowth of axillary buds, the elongation of stems, and the number or angle of branches (tillers) [68].

Studies carried out in model plants (i.e., Arabidopsis and petunia) and crop plants (e.g., tomato, maize, and rice) served as platforms for understanding the molecular basis of plant architecture. In this respect, RNA interference (RNAi) technology was used to improve crop productivity by modifying plant architecture. For example, in rice over-expression or suppression of the *OsPIN1* (a member of the gene family of auxin transporters in plants) expression through a transgenic approach resulted in changes of tiller numbers and shoot/root ratio, suggesting that *OsPIN1* played a role in auxin-dependent adventitious root emergence and tillering [69]. In general, these studies have provided insights on the molecular mechanisms affecting each specific aspect that collectively specializes a particular type of plant architecture, among which the signaling pathways that regulate plant height and control the maintenance and differentiation of the SAM have been particularly well analyzed. Although our current understanding of how a particular plant specializes its architecture is still poor, the findings obtained so far in studying plant architecture have already allowed us to open a way for optimizing the plant architecture of crops by molecular design and improving biomass productivity.

3.2.2.3. Regulation of plant biomass production

In plants, the potential to improve biomass production has not yet been largely investigated. This is because the conventional breeding of crop plants (e.g., maize, rice, wheat, and soybean) has been centered on the selection of the superior grain-yield traits. However, current molecular and genetic research has singled out a number of regulators affecting plant biomass production. A diagram depicting various factors affecting plant biomass production reported by Demura and Ye [70] is given in Figure 7. It is evident from this graphical representation

that an increase in biomass yield could be achieved by an enhancement of the vegetative apical meristem activity by KNOX proteins (i.e., KNOX are homeodomain TFs) and of the vascular cambium activity by KNOX and cytokinin, an inhibition of the transition into reproductive growth by activating FLC (protein encoded by the *FLOWERING LOCUS C,FLC,* affecting flowering time) and by repressing SOC1/FUL/FT (encoded respectively by *FLOWERING LOCUS T, FT*, and *SUPPRESSOR OF OVEREXPRESSION OF CONSTAxlink (SOC1*), both involved in regulation of the indeterminacy of meristems and the longevity of plants), an increased cell elongation by gibberellin, an elevated photosynthetic efficiency, and an increase in secondary cell wall biosynthesis. An improved cellulose digestibility by modifying cell wall properties could contribute to increased biofuel production. Therefore, flowering-time genes could potentially be manipulated for generation of grass crops with increased biomass yield. Overexpression of some members of NAC family (a plant-specific family of TFs) also induces increased biomass production. Arabidopsis transgenic lines overexpressing *NAC1* are bigger, with larger leaves, thicker stems, and more abundant roots compared with control plants [71]. Similarly, overexpression in Arabidopsis of another NAC-domain transcription factor, ATAF2, leads to increased biomass, mainly by production of bigger leaves with larger cells [72].

Additionally it is obvious that genes regulating cell number and organ size in plants could potentially contribute to yield increases. For example in maize, Guo et al. [73] have isolated and described a family of genes termed *Cell Number Regulators (CNRs)*. Insight into their function was achieved by ectopically expressing one of this members, namely *CNR1.* It was shown that *CNR1* reduced overall plant size when ectopically overexpressed, while plant and organ size increased when its expression was co-suppressed or silenced. Leaf epidermal cell counts showed that the increased or decreased transgenic plant and organ size was due to changes in cell number, not cell size supporting the idea that *CNR*s function as cell number regulators in maize. This suggests the potential for application to crop improvement via generation of more vigorous and productive plants.

3.2.2.4. Phytohormones

Phytohormones are known to play important roles in plant growth and development, including the regulation of meristematic activities and cell elongation, both of which are crucial for biomass yield. For example, the plant hormones auxin and brassinosteroids (BRs) are important regulators of plant growth, stimulating both cell division and cell elongation. Arabidopsis plants that are unable to synthesize or perceive BRs are dwarfs with rounded leaves and reduced pollen fertility that show sizeable delayed flowering time and leaf senescence [74]. STEROID 22a HYDROXYLASE, encoded by the *DWF4* gene, is a key enzyme for BR biosynthesis. Overexpression of *DWF4* in *A. thaliana* produced plants that grew 35–47% taller and produced 33% more seed. The rice mutant *dwf4-1* had depressed levels of BRs and an ideotype characterized by slight dwarfism and erect leaves. Although individual *dwf4-1* plants had reduced biomass yield, their ideotype allowed high-density planting that led to increased grain yield per unit area [75].

A way by which auxin controls final plant organ size is through the transcription factor *ARGOS (AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE*) which acts upstream of *AINTE-*

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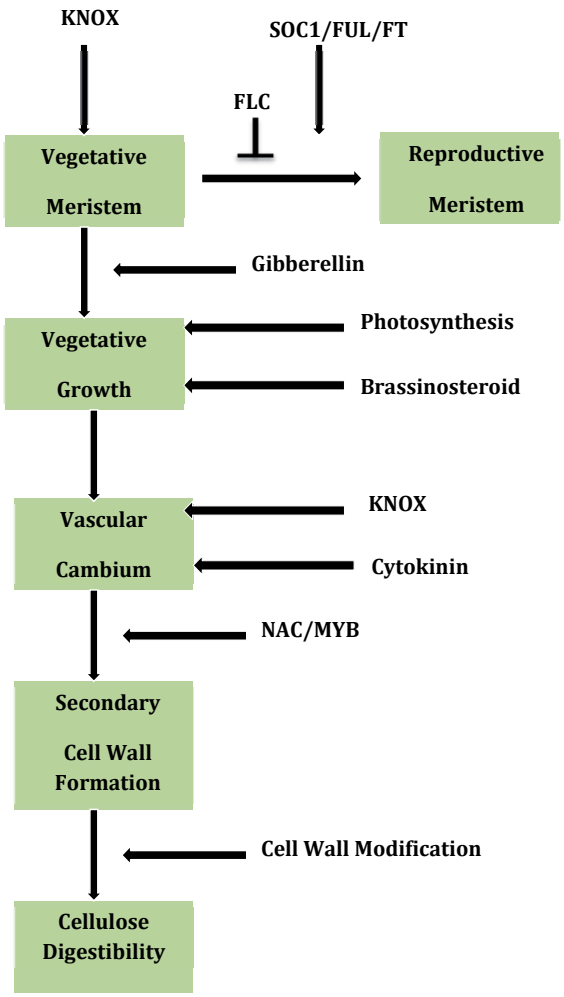

Figure 7. Schematic representation indicating several factors influencing plant biomass yield (Redrawn from Demura and Ye, 2010, [70]).

GUMENTA (ANT), a member of the AP2/ERF family of TFs [76]. For both *ANT* and *ARGOS*, transgenic plants overexpression resulted in an increased plant and organ size. These last genes promote growth through prolonging the cell proliferation period (but not the rate), and the final organ size change is mainly due to an increase in cell number, not cell size. Notably, Arabidopsis *ARGOS-LIKE*, another growth promoting factor, enlarges organ size by enlarging cell size not cell number. *EBP1*, a putative Arabidopsis ortholog of human *ErbB-3* epidermal growth factor receptor binding protein, is another key regulator promoting plant and organ growth. In contrast with ARGOS and ANT, transgenically overexpressing *EBP1* accelerates plant growth and development by simultaneously stimulating cell growth, proliferation, and development, resulting in both increased cell number and cell size [77]. For all three of these growth-promoting genes, not only does transgenically increasing expression promote plant

and organ growth, but also down-regulation of gene expression or loss-of-function via mutation reduces the plant and organ growth. Among these key players are growth repressors, an example is Arabidopsis *AUXIN RESPONSE FACTOR2* (*ARF2*). Loss of *ARF2* function causes enhanced organ size, such as thicker stems and larger seeds, floral organs, and leaves [78]. Physiological studies have suggested synergistic roles for auxin and BRs in cell elongation and genome-wide microarray expression studies identified a large number of genes that respond to both auxin and BRs [79]. Integration of auxin and BR signaling has been suggested to occur at the level of ARFs because the auxin-response element (TGTCTC) is enriched in the promoters of BR-responsive genes [80].

There is information indicating that cytokinins also promote organ growth by stimulating cell proliferation with cytokinin depletion or overproduction resulting in smaller or larger leaves and flowers, respectively [76]. Likewise, gibberellins promote growth through expansion and/or proliferation, acting to repress the activity of the growth-restraining DELLA proteins; DELLA factors may be important in adjusting growth in response to environmental influences. In addition to these classical phytohormones, a novel mobile signal whose synthesis depends on the related cytochrome P450 enzymes KLUH/CYP78A5 and CYP78A7 promotes leaf and floral-organ growth. Investigations on chimaeric plants indicate that the presumed signal is integrated across flowers, suggesting that it may be used to coordinate growth within and between individual organs [76].

In conclusion, it is worth noting that a better understanding of genes affecting hormonal metabolism and signaling should help to design strategies to increase plant growth and organ size and, ultimately, crop yield.

3.2.2.5. Enhancing sink strength

To increase sink strength, credible biochemical targets are represented by enzymes directly or indirectly involved in the conversion of sucrose into starch. One approach to increase sink strength has been devoted to modify the adenylate pools in potatoes; adenylate levels was found to be relevant for starch content in their tubers [81]. In this respect, it was shown that down-regulation of the plastidial isoform of adenylate kinase induced a 60% upgrade of starch in potatoes and, interestingly, a 39% gain in tuber production [82].

Other approaches for yield improvement are more specifically directed towards starch synthesis. Many of these attempts showed only limited success [83]. On the contrary, positive results were obtained in different plant species by acting on AGPase that catalyses a rate-limiting step in the starch biosynthetic pathway. As mentioned above, these enzymes, subject to allosteric control by orthophosphate (P_i) and 3-phosphoglycerate (PG) effectors, have received the most attention [84]. For example, in maize overexpression of wild-type *Sh2* (that encodes the endosperm large subunit of AGPase suscettible to P_i inhibition) and *Bt-2* (*Bt2*, which encodes the endosperm small subunit of AGPase stimulated by PG) increased individual seed weight to 15% by increasing starch content [85]. More recently, Hannah and co-workers [86], by studying the expression of a transgenic form (*HS33*/*Rev6 Sh2*) of AGPase with enhanced heat stability and reduced P_i inhibition, reported an increased maize yield up to 64%. Interestingly, as previously found in wheat and rice, this transgene increases maize yield by

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increasing seed number. Moreover. genetic, physiological, and molecular data provided by Hannah et al. [86] point to *HS33/Rev6 Sh2* expression in maternal tissue, rather than in the seed, as the cause of enhanced seed number. Therefore, these workers concluded that the transgene does not increase ovary number; rather, it increases the probability that a seed will develop. Furthermore, the addition of allosterically altered AGPases and increased Arabidopsis leaf transitory starch turnover improved its growth characteristics [87] and increased fresh weights of aerial parts of lettuce plants ([88].

3.2.2.6. Other strategies

An approach to remodel plant growth is to vary the expression of cell cycle-related genes. Cockcroft et al. [89], by overexpressing the Arabidopsis *CYCD2* gene in tobacco, obtained plants that were 35% higher in comparison to controls. These transgenic plants also manifested normal cell and meristem size associated with an exalted overall growth rates, an enhanced rate of leaf initiation, and a faster rate of growth at all stages of plant development considered in the experiment. Similarly, the overexpression of another Arabidopsis D-type cyclin, *CYCD3;1*, a rate-limiting gene affecting the G1/S transition phase, was found to promote ectopic cell divisions, generating leaves with more but smaller cells [90]). On the contrary, the ablation of *ABAP1*, an Arabidopsis gene that controls the cell proliferation rate by limiting mitotic DNA replication, resulted in larger leaves containing more cells [91]. Further studies on developing leaves revealed that during early leaf development, cell division rates were smaller in *ABAP1*-overexpressing plants in comparison with controls. On the other hand, cell division was faster in plants possessing a defective copy of *ABAP1* gene. Another gene whose variation increases biomass is *CDC27a* [92]. This gene encodes a protein that is a component of the ligase Anaphase-promoting Complex (APC). Overexpression of Arabidopsis *CDC27a* in tobacco produced plants that were up to 30% higher at flowering time, with slight alterations of apical meristems [92]. These last authors concluded that it is likely that the growth promotion mechanism observed in *CDC27a* overexpressing plants is due to the APC, rather than CDC27a protein itself, because the overexpression of at least two other subunits of the APC displays similar enhanced growth.

Another mechanism promoting cell proliferation and ultimately organ growth involves TFs of the *TCP* (*TEOSINTE BRANCHED1, CYCLOIDEA, PCFs) and GROWTH-REGULATING FACTOR (GRF*) classes, two redundant multi-gene families in Arabidopsis. The importance of the TCP family in growth control became evident from the *cincinnata (cin*) mutant in snapdragon (Antirrhinum) and the jaw-D activation-tagged mutant of Arabidopsis [93,94]. In these mutants, leaves overgrow to a highly-wrinkled shape because of excess cell proliferation particularly at the leaf margins. It was found that *CIN* encodes a member of the TCP family and in *cin* mutants, the size of the proliferative region at the leaf base appears enlarged and its distal boundary is concave. In this respect cells at the leaf margin still proliferate at positions where cells in the center have already arrested proliferation. In the *jaw-D* mutant, overexpression of the microRNA miR319a down-regulates five genes of the TCP family. Down-regulation of another three gene family members causes even more severe phenotypes of overproliferation in leaves, while miR319-resistant versions of TCPs and loss-of-function mutations in

miR319a reduce organ size and cause premature cell differentiation [93, 94]. In fact, promoting cell differentiation has been proposed as the primary function of TCPs, rather than directly arresting proliferation [95].

A novel gene regulation mechanism was recently discovered in metazoans, the RNA silencing or RNAi, or microRNA (miRNAs) [96]. Rodriguez et al. [97] found that overexpression of miR396 in Arabidopsis had a negative impact on cell proliferation in developing leaves through the repression of GRF activity and a decrease in the expression of cell cycle genes. Accordingly, disruption of the recognition of *GRF2* by miR396 resulted in larger leaves with more cells than control plants. Similarly in Arabidopsis, it was reported that over-expression of miR156 promotes a vegetative-phase transition delay and an enhancement in leaf initiation rate [98], while over-expression of miR172 induces adult leaf traits and flowering [99]. The regulation of both miRNAs is closely connected as their expression is influenced by age, temperature, and light acting in a contrasting way [100]. At a molecular level, miR156 acts through the down regulation of genes coding for the *SQUAMOSA PROMOTER BINDING-LIKE (SPL*) and the *AP2-like transcription factor*, respectively, are subjected to feedback regulation by their targets [101].

Recently, miRNAs have also emerged as key regulators of phytohormone response pathways *in planta* by affecting their metabolism, distribution, and perception [100,102] have demonstrated that gibberellic acid (GA) promotes flowering in Arabidopsis through a miR156-dependent pathway, indicating a connection between miRNA and phytohormone signaling pathways in the control of shoot development. In general, it appears evident from this data that miRNAs as growth regulators are interesting targets for gene manipulation for improving biomass yield.

4. Conclusions and future perspectives

Due to the increasing concerns on the environment, climate change, and limited natural resources, there is considerable effort applied to produce chemicals and materials from renewable biomass. While initial emphasis was reversed on biofuel production from food plant sugars, the competition between crop usage for food and non-food applications has stimulated research efforts to genetically improve yield and quality-related traits of plants for biorefining applications.

Targeted genetic improvement of biomass for biorefining applications depends on identifying genetic variation in critical morphological, structural, biochemical, and physiological traits affecting biomass yield and its chemical composition. This involves manipulating complex traits, such as those associated with plant growth and development or tolerances to abiotic and biotic stresses, usually in production environments that are highly variable and unpredictable, as well as to gain insights into the less digestible carbohydrates in cell walls components. This last aspect, in turn, has promoted inspections on the use of other plant-derived metabolites for chemical productions embracing the high-value specialty segments via platform intermediates needed for bulk production.

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Although there is genetic variability in the traits sustaining biomass yield, many of them important in crop productivity and sustainability are complex multigene traits, often difficult to breed for. Nevertheless, advances in plant genetics and genomic technologies are contributing to the acceleration of gene discovery for product development. In this respect, several new genomics technologies such as next-generation sequencing and high-throughput marker genotyping can be used not only for developing high-density genetic and physical maps but also for generating transcriptome or sequence data [55]. These approaches together with –omics technologies (e.g., transcriptomics, genetical genomics, metabolomics, and proteomics) have emerged as powerful tools for understanding genome variation in crop species at DNA, RNA, as well as protein level and for identifying genomic regions or genes affecting the expression of trait(s) that are of interest to improving plants for biorefining applications or for breeding varieties with in-built new traits such as creating higher value-added products. Furthermore, a range of novel genetic techniques, particularly techniques collectively referred to as 'genome editing', have been developed that allow targeted changes to be made to genomes [103]. Changes can include adding or removing DNA at a specified location in the genome or replacing a specified segment of DNA with a different one. It is also expected to make epigenetic changes (histone modifications and DNA methylation), where the DNA sequence remains unchanged but gene expression is altered because of chromatin changes that may be heritable [104].

Knowledge of plant biosynthetic pathways will also provide valuable opportunities for metabolic engineering, as well as access to chemical transformations unique to plants. It is expected that genomics will corroborate plant biochemistry as researchers seek to understand the metabolic pathways for the synthesis of high value-added products. Identifying rate-limiting steps in their synthesis could provide targets for genetically engineered biochemical pathways to produce augmented amounts of useful compounds and new compounds in plant cells. Although, plant-based production of novel compounds such as biopolymers (e.g., polyhydroxyalkanoates) in plant tissues has been documented [105], plant metabolic pathways are complex and often feature multiple levels of regulation suggesting that it will be difficult to target the best intervention points and accurately predict the outcome [106]. However, recent developments in knowledge-based metabolic engineering strategies, that is the storing and mining of genomic, transcriptomic, proteomic, and metabolomic data, might permit to generate models of metabolic pathways useful to define and refine optimal intervention strategies for synthesis of specific chemicals for industrial applications [107].

The recent emergence of synthetic biology as the basis for metabolic engineering in plants promises to positively change the way in which different intervention strategies are selected and implemented, as is already the case in microbes [108,109]. In this context, metabolic engineering has contributed successfully in the last two decades towards broadening the product portfolio containing various value-added and commodity chemicals and materials from renewable resources [108,110]. Obviously, the attempts of engineering plants using the strategies employed for microbes are definitively more cumbersome. However, plant

scientists have already established efficient methods for the routine genetic transformation of the majority of our principal crops. Using these technologies, they have also achieved substantial progress in understanding and manipulating plant primary and secondary metabolism. Moreover, further research on metabolic regulation and genetic information and on novel tools for genetic modifications may help to overcome the limitations for breeding varieties that meet targeted applications and end-uses. This will certainly help maximize their value throughout the whole bio-based value chain for biorefining applications or eventually breeding varieties with in-built new traits that are creating higher value-added products.

5. Glossary

Biofuel: Fuel produced from crop-derived carbohydrates. Includes bioethanol produced from fermentable sugars and biodiesel produced from plant oil.

Biomass: Biological materials used for fuel or industrial production. Here, we refer to the sum of plant harvestable tissues.

Bio-based (or bio-derived) platform molecule: A chemical compound whose constituent elements originate wholly from biomass (material of biological origin. excluding fossil C sources), and that can be utilized as a building block for the production of other chemicals.

Biorefinery feedstocks/Platform chemical feedstocks: Biorafineries are typically differentiated into polvsaccharides, lignin, protein, and extractives (e.g., triglycerides and terpenes) as all are found as constituent parts within typical biomass in varying amounts.

Building block chemicals: Molecules with multiple functional groups that possess the potential to be transformed into new families of useful molecules. According to the US Department of Energy the top 12 sugar-based building blocks are: 1,4-diacids (succinic, fumaric and malic), 2,5-furan dicarboxylic acid, 3-hydroxy propionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, glycerol, sorbitol, and xylitol/arabinitol.

Metabolic engineering: Describes the targeted modification of endogenous metabolism to control the accumulation of one or more specific products.

Sink strength: Ability of a sink organ (any organ, e.g., roots, developing seeds, or immature leaves, that imports photosynthetic assimilates) to competitively mobilize assimilates toward itself.

Synthetic biology: Aims at creating novel functional parts, modules, circuits, and/or organisms using synthetic DNA and mathematical/logical methodologies, and has been shown to be practical and useful in various biotechnological applications.

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Chapter 6

Nod-Factor Signaling in Legume-Rhizobial Symbiosis

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Abstract

Leguminous plants (or Legumes, family Fabaceae) are known to form symbioses with extremely broad range of beneficial soil microorganisms (BSM), representing examples of almost all plant-microbe mutualistic systems. One of the most ecologically important and well-studied legume beneficial symbioses is root nodule (RN) symbiosis (symbiotic association with nitrogen-fixing bacteria). Compared with other interactions of legumes with BSM, RN symbioses demonstrate high level of genetic and metabolic integrity, which implies, *inter alia*, highly specific mutual recognition of partners. In this chapter, we describe the mechanisms of plant-microbe recognition during initial steps of RN symbiosis using the interaction of model legumes - pea (*Pisum sativum* L.), barrel medic (*Medicago truncatula* Gaertn.) and *Lotus japonicus* (Regel.) K. Larsen - with rhizobia as an example. We paid particular attention to symbiotic system of *P. sativum* since pea, besides its importance as a model object of genetics, is also a valuable crop plant. Hence, in conclusion, we discuss the potential to use obtained knowledge for optimizing the broad spectrum of plant adaptive functions and to improve the sustainability of legume crop production.

Keywords: legume-rhizobial symbiosis, Nod factor, plant signaling, genetic control

1. Introduction

Plants are attached immobile organisms and thus have to adapt to their environment in order to survive and reproduce successfully. Usually, plants experience multiple simultaneous

impacts from different sources, so they developed complex signaling pathways to effectively detect these impacts and adequately respond to them [1]. Various microorganisms, which are constantly present in the environment, form one of the major factors affecting the life cycle of plants [2, 3]. Although many plant-associated microbes are pathogens that impair plant growth and reproduction, there are also a lot of beneficial (mutualistic) microorganisms able to provide plants with nutrition and additional defense mechanisms. Cooperation with such microorganisms constitutes the universal and highly effective strategy of plants' ecological adaptation, so they tend to form long-lasting associations, which sometimes grow into highly integrated symbioses where one or both partners can develop novel features useful for their survival. Establishing of such symbiotic relationships involves the complicated developmental programs implemented under the joint control by plant and microbial partners and based on the cross-regulation of their genes.

Leguminous plants (or Legumes, family Fabaceae) are known to form symbioses with extremely broad range of beneficial soil microorganisms (BSM), representing examples of almost all plant-microbe mutualistic systems. One of the most ecologically important and well-studied legume-beneficial symbioses is root nodule (RN) symbiosis (symbiotic association with nitrogen-fixing bacteria). Compared with other interactions of legumes with BSM, RN symbioses demonstrate high level of genetic and metabolic integrity, which implies, *inter alia*, highly specific mutual recognition of partners. As legume plant plays a central role in establishing of RN symbiosis, performing functions of initiation, coordination, and regulation of all developmental processes, it possesses complex receptor system capable of accurate identification of microsymbiotic partner. In this chapter, we describe the mechanisms of plant-microbe recognition during initial steps of RN symbiosis using the interaction of model legumes – pea (*Pisum sativum* L.), barrel medic (*Medicago truncatula* Gaertn.), and *Lotus japonicus* (Regel.) K. Larsen – with rhizobia as an example. We pay particular attention to symbiotic system of *P. sativum* since pea, besides its importance as a model object of genetics, is also a valuable crop plant. Hence, in conclusion, we discuss the potential to use obtained knowledge for optimizing the broad spectrum of plant adaptive functions and to improve the sustainability of legume crop production.

2. Legume-rhizobial symbiosis: An example of highly integrated plant-microbe system

Nitrogen is an essential component of all living systems, since it is part of the most important biological molecules – DNA and proteins. Molecular nitrogen (N_2) in the atmosphere, despite being abundant, is extremely chemically inert and thus cannot be used by the majority of organisms, causing them to compete for more accessible nitrogen sources. Leguminous plants are able to grow in the soil/substrate without any combined nitrogen due to the fixation of atmospheric nitrogen by their symbiotic nodule bacteria (collectively called rhizobia) [4]. Nitrogen fixation occurs within special plant organs – root nodules (or, in some associations, also stem nodules). Development of these organs represents a well-organized process based on the tightly coordinated expression of specialized symbiotic plant and bacterial genes. The

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legume nodules provide an ecological niche for bacteria, as well as structure for metabolic/signal exchange between the partners and for the control of symbionts by the hosts [5].

Family Fabaceae contains about 19,000 species divided between three subfamilies (Caesalpinioideae, Mimosoideae, and Papilionoideae), with more than 700 genera of worldwide distribution [6]. With a single exception (*Parasponia*, family Ulmaceae), the ability for symbioses with rhizobia is restricted to Fabaceae, although in eight related dicotyledonous families (Rosid I clade) an ability to form nodules with the nitrogen-fixing actinomycete *Frankia* is known [7].

By contrast to legumes, their nitrogen-fixing microsymbionts do not constitute a taxonomically coherent group of organisms. The majority of rhizobia belong to the α-proteobacteria previously assigned to the *Rhizobiaceae* family solely on the basis of their ability to nodulate the legumes (e.g., *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Sinorhizobium*). In the last years, several non-rhizobial symbionts capable of forming nodules and fixing nitrogen in legume roots have been documented. According to modern conception, bacteria that can form RN symbiotic associations (about 44 species of 12 genera) are not clustered in any common lineage, instead being distributed in the classes α-proteobacteria and β-proteobacteria (close to *Burkholderia*, *Cupriavidus* and *Ralstonia*) and dispersed over nine monophyletic groups along with taxa that do not contain legume symbionts [8]. Recently, some γ-proteobacteria (belonging to *Escherichia*, *Enterobacter*, and *Pseudomonas* genera) have been discovered that can also form nitrogen-fixing nodules with the legumes [9]. All these bacteria (collectively still referred to as rhizobia) vary significantly in their overall genome structure, location of "symbiotic" (*sym*) genes, their molecular organization and regulation [10, 11]. However, a particular legume plant can find the appropriate rhizobial partner (species, or even strain) due to the fine-tuned mechanism of molecular interaction.

The development of nitrogen-fixing nodule is complex process that is traditionally divided into three major stages: preinfection, root colonization/nodule morphogenesis, and nitrogen fixation. On the first stage, the mutual recognition of partners occurs. The interaction between micro- and macrosymbiont begins with the activation of bacterial *nod*-genes under the influence of flavonoid molecules secreted by the plant root [12, 13]. *nod*-genes provide the synthesis of the main bacterial signaling molecule called Nod-factor (NF), which is crucial for identification of microsymbiont [14-16]. After the proper reception of Nod-factor, plant activates two parallel processes: bacterial penetration into root hair cells via so-called infection thread (IT), and differentiation of nodule from the root cortex. IT is a special structure generated by invagination of plant cell membrane, covered with plant-derived cell wall and filled with matrix produced by both plant and bacteria. It grows into root hair cell and then to the cortex where nodule tissues are formed (Figure 1) [17].

The key stage of nodule development is conversion of bacteria into the form of intracellular symbionts through endocytosis-like process. Herein, the distal area of IT transforms into structure called infection droplet (ID), which releases membrane vesicles containing bacteria into plant cytoplasm. After leaving IT, rhizobia for some time retain their size and shape, subsequently differentiating into a specific form called bacteroids [18]. Compared to free-living bacteria, bacteroids have significantly (about 3-7 times) increased size and more complex

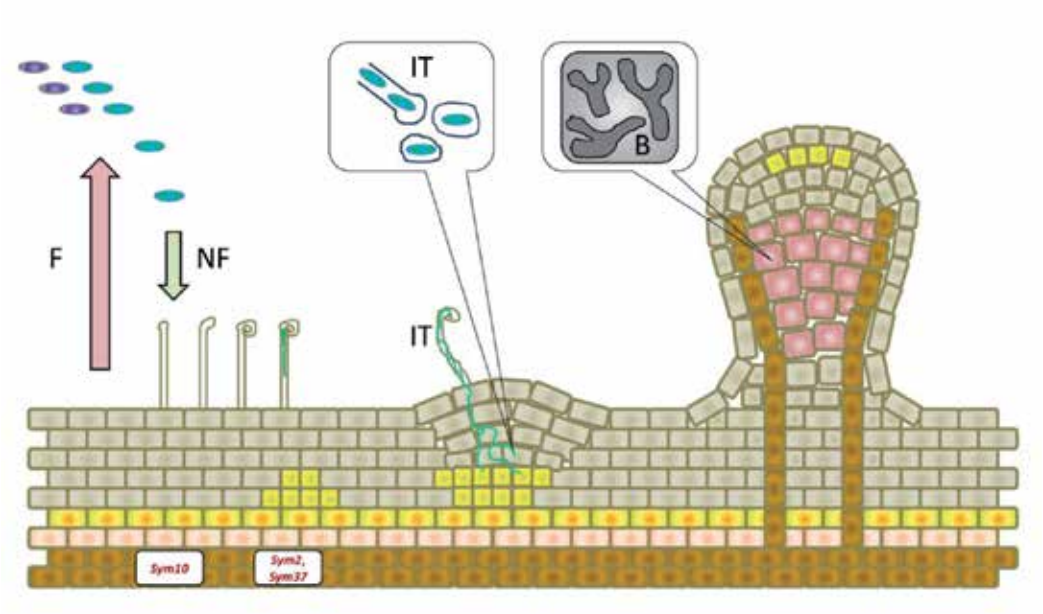

Figure 1. General scheme of RN symbiosis formation and functioning in pea. From left to right: three major stages of symbiosis, namely preinfection, root colonization/nodule morphogenesis, and nitrogen fixation. F – flavonoids excreted by the plant, NF – Nod-factors excreted by nodule bacteria, IT – infection thread, B – bacteroids. *Sym10* and *Sym2*, *Sym37* – stages on which symbiosis is blocked in case of corresponding pea mutants/genotypes.

shape, which can be round, pear-shaped, Y- or X-like, depending on specific symbiotic system. After the aforesaid differentiation, the synthesis of nitrogenase (the enzyme catalyzing reduction of N_2 into NH_4^+) and other proteins involved in nitrogen fixation is activated in bacterial cells [19].

Bacteroids are embedded into a membrane structure named symbiosome, which are derived from membrane vesicle originating from ID. They are organelle-like units of plant cell responsible for nitrogen fixation. Symbiosome formation as well as bacteroid differentiation is induced by plant. Peri-bacteroid membrane (PBM) that surrounds bacteroids is an active interface of RN symbiosis where exchange of metabolites between symbionts occurs [19, 20]. Plant cells containing symbiosomes also undergo the deep differentiation, increasing the amount of their membrane structures (endoplasmic reticulum and the Golgi complex), which participate in the development of PBM and biosynthetic processes. Many proteins associated with nitrogen fixation appear in these cells *de novo*.

The developmental program described above is typical only for evolutionary advanced legumes belonging to the inverted repeat–lacking clade (IRLC) of Papilionoideae, such as *Medicago*, *Pisum*, or *Trifolium* (clover). They form so-called "indeterminate" nodules which are characterized by stable apical meristem and division into histological zones with constantly renewed N_2-fixing zone. Rhizobia in these nodules undergo terminal bacteroid differentiation and cannot revert to free-living form [21, 22]. Other legumes such as *Lotus* or *Phaseolus* (bean),

however, form morphologically more simple "determinate" nodules, where apical meristem exists only for several days, nitrogen-fixing zone is not strongly expressed, and infected (N_2-fixing) cells intermingle with noninfected ones [21]. Bacteroids in determinate nodules show no sign of terminal differentiation as they usually maintain their normal bacterial size, genome content, and reproductive capacity lacking from those in indeterminate nodules [22].

Several Papilionoideae members, such as *Arachis* and *Stylosanthes*, demonstrate the reductive scheme of nodule development: rhizobia invade roots through the cracks of epidermis, and instead of IT they are brought into cytoplasm by the direct endocytosis from intercellular space [6, 23]. Even more primitive morphology of symbiosis is typical for members of Caesalpinioideae subfamily, as they lack endocytosis step, and nitrogen fixation occurs within modified persistent ITs called "fixation threads" [24]. This is also relevant for evolutionary primitive woody plants from Papilionoideae: *Andira* and *Hymenolobium*, and for *Parasponia*.

Such a complicated system of biological nitrogen fixation will work properly only when suitable partners meet each other in soil. This rendezvous becomes possible owing to reciprocal molecular signal exchange, which is not exhaustively studied to date.

2.1. Specificity of legume-rhizobial symbiosis

Root-nodule symbiosis is well known as highly specific plant-microbe interaction. According to the early surveys of symbiotic specificity [25], legumes were suggested to comprise a range of taxonomically restricted cross-inoculation groups (CIG) within which the free cross-inoculation occurs, while the species from different groups do not cross-inoculate.

The best studied examples of this classification are represented by four CIG: "*Trifolium* – *Rhizobium leguminosarum* bv. *trifolii*," "*Pisum*, *Vicia*, *Lathyrus*, *Lens* – *R. leguminosarum* bv. *viciae*," "*Galega* – *R. galegae*," "*Medicago*, *Melilotus*, *Trigonella* – *Sinorhizobium meliloti*, *S. medicae*." However, it was demonstrated later that such strictly defined specificity is limited to the herbage papilionoid legumes growing in temperate zones and representing the so-called Galegoid complex [26, 27]. Other legumes, including the majority of tropical species, tend to broad their symbiotic specificity, where cross-inoculation is possible between tribes, subfamilies, and even with non-legume plant *Parasponia* [28].

The analysis of CIG structure for both strictly and broadly specific legumes has shown that plant specificity towards rhizobia has good correlation with plant taxonomy on the genus or tribe level. It was also revealed that specificity of nodule formation does not correlate with symbiotic efficiency, i.e., efficiency of nitrogen fixation: several bacterial strains form normal nodules with one plant species, and are inactive (not able to fix nitrogen, Fix^-) with another [26]. This could be due to the fact that nodulation is an early stage of symbiosis similar (and supposedly related) to pathogenic interaction, and is based on strict cross-activation of plant and bacterial genes ("gene-for-gene" interaction), while nitrogen fixation occurs on the later stages for which "gene-for-gene" interaction is not typical.

Moreover, it is specificity that makes possible the natural selection of effective **symbiotic pairs**, but not the single "symbiotically effective" plant or single "symbiotically effective" microorganism. On the other side, specificity of legume-rhizobial symbiosis should be

somewhat associated with nitrogen-fixing intensity, upon which is based the ecological efficiency of cooperation; otherwise it would not be an evolutionary advantage. The majority of "Galegoid complex" members have both narrow specificity and effective nitrogen fixation, suggesting that these two features are connected, though specificity of recognition is obviously not the only condition required for effective symbiosis.

It is also important to note that the range of potential symbiotic partners can vary for both bacteria and plants. Symbiotic pair *Trifolium–Rhizobium leguminosarum* bv. *trifolii* represents one side of this continuum, as they are the only possible partners for each other. On the opposite side are *Phaseolus vulgaris* and *Vigna unguiculata*, which are able to exchange their symbionts with many unrelated legume species [25]. In bacteria, the *Sinorhizobium fredii* strain NGR234 was shown to interact with more than 120 plant species from all three Fabaceae subfamilies, as well as with *Parasponia*, thus being the most "unscrupulous" strain known so far [29]. The most striking feature of this strain is that its genome, although not particularly large (6.9 Mbp), encodes more different secretion systems than any other known rhizobia and probably most known bacteria [30]. These, among others, include type III and type IV secretion systems which allow bacteria to direct effector proteins or DNA into the cytoplasm of their eukaryotic hosts. There seems to be a correlation between the host range of rhizobia and the number of specialized protein secretion systems they have, as "classic" narrow-host-range rhizobia such as *S. meliloti* and *R. leguminosarum* carry neither type III nor type IV secretion systems. Furthermore, NGR234 is shown to secrete a large family of NFs that are variously 3-O, 4-O, or 6-O carbamoylated, which are N-methylated, and which carry a 2-O-methyl-fucose residue that may be either 3-O sulfated or 4-O acetylated (see below) [29]. Since no other rhizobia synthesize such a large family of NFs, it should be proposed as one of the main aspects contributing to the broad host range of NGR234 [17, 31]. Another possible aspect is that NGR234 not only treats the legume root to a large palette of NFs, but that their concentration is much higher than in even very closely related rhizobia [32].

2.2. Initial steps of rhizobium-legume symbiosis

The specificity of legume-rhizobia interactions is expressed mostly during the preinfection stage when rhizobia recognize the roots of appropriate host plants and colonize their surfaces. When the root-excreted signals (in particular, flavonoids) are perceived by bacteria, they activate the bacterial nodulation genes (*nod*/*nol*/*noe*) [13]. These genes control the synthesis of lipo-chito-oligosaccharidic (LCO) nodulation factors (Nod-factors) which induce the early stages of RN symbiosis development. NFs represent the unique group of bacterial signal molecules not known outside legume-rhizobia symbiosis. They are among the most potent developmental regulators: their effect is expressed at concentrations merely of 10^{-8} – 10^{-12} M. The core structure of these molecules, common for all rhizobia species, consists of 3-6 residues of N-acetylglucosamine and of a fatty acid (acyl) chain (Figure 2). The type of symbiotic specificity is dependent mainly on the chemical modifications in NF structures [14-16]. However, a sufficient impact to the host specificity of RN symbiosis can also be made by the interactions between bacterial surface molecules (some polysaccharides and proteins) [33, 34] and the lectins located on the root hair surfaces, as well as by means of NFs secretion [35].

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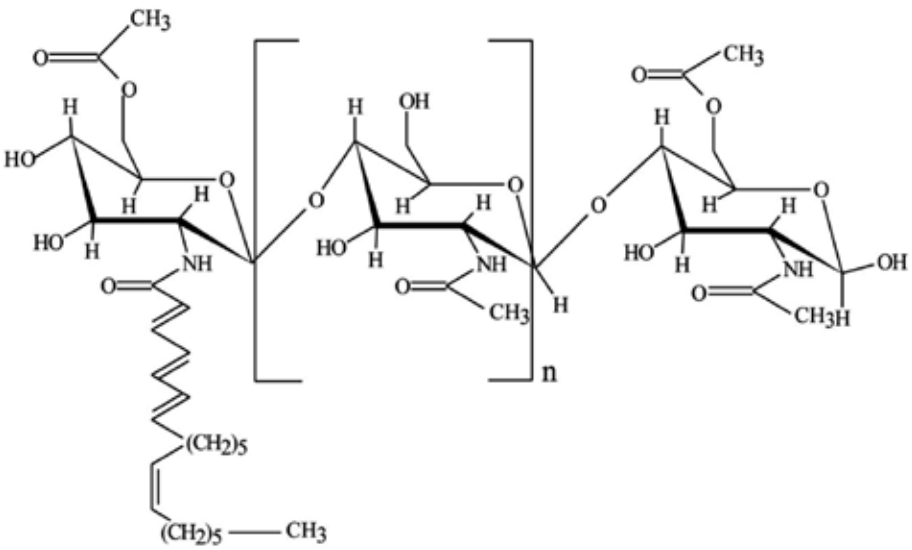


n =2 or 3; regarding Afghan peas, see below (3.2).

Figure 2. Example of Nod-factor excreted by *Rhizobium leguminosarum* bv. *viciae* strain TOM nodulating Afghan peas.

Rhizobia possess a wide range of genes involved in the early stages of nodulation, i.e., the NFs production [36]. Genes which are common to all rhizobia – *nodA*, *nodB*, *nodC*, and their regulator *nodD* – are responsible for NF core structure synthesis [37]. The other genes specific for particular species or strains control various modifications of signaling molecule. The difference in the spectrum of hosts possible for microsymbiont to interact with is based on the variety of combinations of different *nod*-genes. For example, presence of gene *nodE*, which encodes protein similar to fatty acid synthase in several genera of rhizobia, provides modification of fatty acid moiety on the nonreducing end of NF molecule, thereby affecting the ability of bacteria to nodulate certain plant species [38, 39]. Genes *nodH, nodP*, and *nodQ* found in *Sinorhizobium meliloti* control the specific NF modification – the O-sulfation of reducing end – which makes it recognizable for *Medicago* receptors [40]. Overall, each strain of rhizobia is characterized by specific set of *nod*-genes, which together form the "molecular key" suitable for plant receptor. It is significant to note that most rhizobia secrete an assortment of NFs varying in their structure instead of just one particular kind [41, 42]. Thereby, the symbiotic success of bacteria could be directly connected with diversity of NFs they are able to produce, and "molecular key" rather becomes the "set of lock picks," with secretion systems and surface molecules being additional tools in it (see above).

By perceiving the NF, plant starts various processes in root tissues. In particular, signaling molecule is required for the activation of plant genes in the epidermis cells and pericycle, as well as for mitotic reactivation of cortical cells and the formation of IT. Genes responsible for proper NF reception were first discovered in mutants of *Lotus japonicus* lacking any response

to NFs [43, 44]. These genes were named *NFR1* and *NFR5*, for Nod-Factor Receptor. Cloning of these genes revealed that they encode receptor-like kinases comprising LysM domains (LysM-RLK). LysM domains occur in a variety of proteins in bacteria and eukaryotes and have been shown to bind glycan-containing ligands (such as chitin) [45]. They consist of a repetition of a small motif typically containing from 44 to 65 amino acid residues – the LysM sequence, or LysM module [46, 47]. One LysM sequence has a $\beta\alpha\alpha\beta$ secondary structure with the two helices packing onto the same side of an antiparallel β sheet. Multiple LysM modules in a protein are often separated by small Ser-, Thr-, and Asn-rich intervening sequences [48].

Only in plants are LysM domains associated with a kinase-like domain [49] forming two main LysM-RLK gene families: the LYK family and the LYR family. All the LysM-RLKs are predicted to contain three LysM modules, although these modules exhibit a high degree of divergence, both within a protein and between proteins. It is considered that the initial function of LysM-RLKs has been recognition of chitin-based signal molecules produced by hostile microbes (termed as MAMPs ("microbe-associated molecular patterns") or PAMPs ("pathogen-associated molecular patterns")), similar to the function of CERK1 receptor-like kinase from *Arabidopsis thaliana* [2]. Based on microsyntenies between genomic regions around LysM-RLK genes in legumes and non-legumes (*A.thaliana*, rice) plants, it has been speculated that these genes are the descendants of a common ancestor [50]. Zhang et al. (2007) [51] proposed that in Leguminosae LysM-RLKs have undergone further duplication and diversification, with some LysM-RLKs acquiring the ability to perceive bacterial NFs, leading to mutually beneficial endosymbiosis with rhizobia. One aspect of this diversification is the adaptation of extracellular LysM domains to recognize specific structures of NFs, while another being evolution of the intracellular kinase domains to switch the signals from cascades inducing defense responses to symbiotic gene cascades. Recently, the function of NFRs as NF receptors was confirmed by demonstration of their ability to directly bind NF molecule *in vitro* [52].

In *Medicago* and pea, which belong to IRLC (see above), NF perception seems to be more complicated than in *Lotus*. Genes orthologous to *NFR1* and *NFR5* were identified in *Medicago truncatula* (*LYK3* and *NFP*) and in *Pisum sativum* (*Sym37* and *Sym10*), with careful description of corresponding mutant phenotypes [44, 53-55]. While phenotype of *nfp* and *sym10* mutants (in *Medicago* and pea, respectively) coincided with that of *nfr5* mutants in *Lotus*, mutations in genes *lyk3* and *sym37* (orthologs of *NFR1*) led to significantly different phenotype – successful penetration of bacteria into root hair with subsequent block of IT progress, instead of complete absence of responses to rhizobia [55, 56]. These data support the "two-receptor" model of Nod-factor perception proposed more than 20 years ago [40]. According to this model, which was developed on the base of the infection phenotype of several *S. meliloti nod* mutants, there are two different types of NF receptors – the "recognition" (or "signaling") receptor inducing early responses with high affinity for Nod-factor and low requirements toward its structure, and the "entry" receptor that controls penetration of bacteria into plant cell and has more stringent demands [40].

It is significant to note that NFR5 (and its homologs, NFP in *Medicago* and Sym10 in *Pisum*) lacks the independent kinase activity and thus can function properly only in complex with active kinase (which is suggested to be NFR1) [52]. It can be assumed, based on the above, that in general the "recognition" receptor (NFR5, NFP or Sym10) perceives NF and afterwards

forms complex with another receptor possessing kinase activity (NFR1, LYK3 or Sym37, respectively), thus constituting the "entry" receptor. Still, results of genome and transcriptome sequencing in *Lotus*, *Medicago* and pea show that legumes possess more than 10 genes of receptor kinases similar by structure to the aforementioned ones. So, the system of NF receptors could be actually much more complicated, suggesting that the overall mechanism of NF perception is probably even more intricate than was thought before.

3. Molecular genetics of Nod-factor signaling in legumes

As reviewed in our recent publication [57], plant genes involved in development of RN symbiosis may be divided into two groups, according to approach which was used for the gene identification. The first group, *Sym*-genes, had been identified with the use of formal genetic analysis (started from selection of plant mutants defective in nodule development). The other group of genes called nodulins was identified by molecular genetic methods, through identification of proteins and/or RNAs synthesized *de novo* in root nodules.

The large sizes of genomes of crop legumes (e.g., soybean or pea) in which the formal genetics of symbioses was initially developed, as well as low capability for genetic transformation, complicate greatly the cloning of symbiotic genes, analysis of their primary structures, and gene manipulations. Therefore, in the early 1990s, *Lotus japonicus* [58] and *Medicago truncatula* [59, 60] have been introduced in symbiogenetic studies as model plants. These species are characterized by relatively small genomes (470-500 Mb; [61]) and can be easily genetically transformed [60, 62-64]. In addition, the short life cycle and high seed productivity made them attractive and convenient model objects for studying molecular bases of RN symbioses, as well as other types of plant-microbial symbioses.

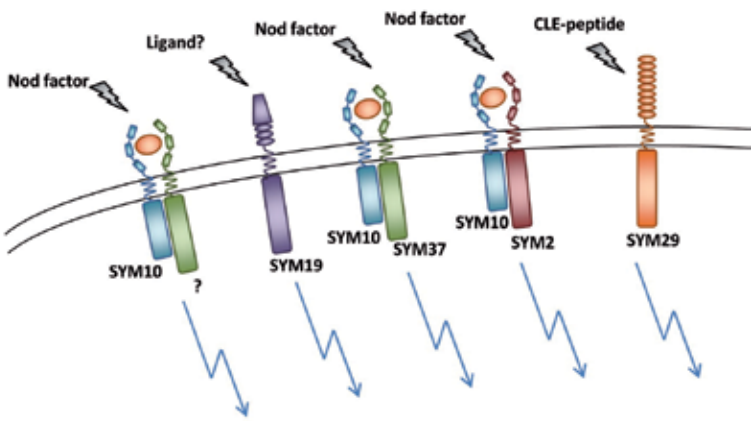
The analysis of signaling pathway in RN symbiosis was started with experimental mutagenesis. Large-scale programs of insertion, chemical and X-rays mutagenesis, performed by different research groups, resulted in generation of numerous symbiotic mutants in *L. japonicus* and *M. truncatula* [65, 66] which allowed researchers to identify and characterize a series of *Sym*-genes. The genes involved at the initial stages of nitrogen-fixing symbiosis (named "early *Sym*-genes") were of primary interest, allowing dissection of the mechanisms by which the NF signal is perceived and transduced by host plants.

3.1. Nod-factor signaling in model legumes

After the first step of NF reception implemented by LysM-receptor kinases (described above), the symbiotic signal is transmitted to the pathway named Common Symbiosis Pathway (CSP), for it shares components with another interaction – arbuscular mycorrhiza (AM) symbiosis, the association with obligate biotrophic fungi of phylum *Glomeromycota*. Arbuscular mycorrhiza is formed by at least 80% of contemporary land plants and is believed to be the most ancient plant-microbe symbiosis which has played a decisive role in plants adaptation for terrestrial life [67-69]. AM is the main source of plants' phosphoric nutrition, although in many temperate and boreal species it is supplemented or even completely replaced by other forms

of mycorrhiza (ectotrophic, ericoid) with various representatives of the *Ascomycota* and *Basidiomycota*, and for some plants (orchids) fungi supply not only mineral nutrition, but also organic carbon compounds [69, 70]. Being the first beneficial association with microorganisms known for plants (occurred approximately 400 million years ago), AM is considered as an ancestor for other mutualistic plant-microbe interactions, such as RN symbiosis. Therefore, it is supposed that NF signaling evolved on the base of previously existing AM signaling. Intriguingly, arbuscular mycorrhizal fungi excrete a set of chitin-derived Myc-factors structurally similar to Nod-factors [71], which also serve as the signaling molecules. It still remains unknown, however, how exactly the Myc-factors are percepted by plants.

The first player in the CSP was identified more than 10 years ago. It is LRR-receptor kinase, or SymRK (symbiotic receptor kinase) described for *Lotus* as SymRK (Symbiotic Receptor Kinase) and for *Medicago* as NORK (Nodulation Receptor Kinase) [72, 73]. In pea, the gene *Sym19* is orthologous to *SymRK* in *Lotus* and *NORK* (also known as *DMI2*, for Doesn't Make Infections) in *Medicago* [72]. Ligand of this receptor kinase is not known as yet (Figure 3). Interestingly, the activity of SymRK is also required for proper progression of late symbiotic stages, at least for rhizobial infection [74]. SymRK kinase domain has been shown to interact with 3-hydroxy-3-methylglutaryl CoA reductase 1 (HMGR1) from *M. truncatula* [75], and an ARID-type DNA-binding protein [76]. These results suggest that SymRK may form complex with key regulatory proteins of downstream cellular responses. Symbiotic Remorin 1 (SYMREM1) from *M. truncatula* and SymRK-interacting E3 ligase (SIE3) from *L. japonicus* have also been shown to interact with SymRK [77, 78].



From left to right: stages of symbiosis.

Figure 3. Receptor kinases of pea participating in nodulation signaling.

The symbiosis receptor kinase SymRK acts upstream of the NF-induced Ca^{2+} spiking in the perinuclear region of root hairs within a few minutes after NF application [79]. Perinuclear calcium spiking involves the release of calcium from a storage compartment (probably the

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nuclear envelope) through as-yet-unidentified calcium channels. To date, it is known that the potassium-permeable channels might compensate for the resulting charge imbalance and could regulate the calcium channels in plants [80-84]. Also, nucleoporins NUP85 and NUP133 (described only in *Lotus* so far) are required for calcium spiking, although their mode of involvement is currently unknown. Probably, they might be a part of specific nuclear pore subcomplex that plays a crucial role in the signal process requiring interaction at the cell plasma membrane and at nuclear and plastid organelle membranes to induce a Ca^{2+} spiking [85-86]. Recently, the third constituent of a conserved subcomplex of the nuclear pore scaffold, NENA, was identified as indispensable component of RN endosymbiotic development [87].

Ca^{2+} spikes are supposed to activate a calcium- and calmodulin-dependent protein kinase (CCaMK). This kinase contains an autoinhibition domain which, when removed, leads to a spontaneous activation of downstream transcription events and induction of nodule formation even in the absence of rhizobia [88]. Thus, CCaMK appears to be a general "manager" for both RN and AM symbioses and the last member of Common Symbiosis Pathway, because the next steps of nodulation signaling are independent from those of AM: the mutations in downstream *Sym*-genes do not affect the AM symbiotic properties of legume. Interestingly, mutations in any *Sym*-genes do not influence the defense reactions, suggesting that signaling pathways of mutualistic symbioses and pathogenesis are sufficiently different.

The CCaMK is known to form a complex with CYCLOPS, a phosphorylation substrate, within the nucleus [89]. *cyclops* mutants of *Lotus* severely impair the infection process induced by the bacterial or fungal symbionts. During RN symbiosis, *cyclops* mutants exhibit the specific defects in IT initiation, but not in the nodule organogenesis [90], indicating that CYCLOPS acts in an infection-specific branch of the symbiotic signaling network [35]. *Cyclops* encodes a protein with no overall sequence similarity to proteins with known function, but containing a functional nuclear localization signal and a carboxy-terminal coiled-coil domain.

It is supposed that CCaMK with help of CYCLOPS probably phosphorylates the specific transcription factors already present in cell, NSP1 and NSP2, which influence the changes of expression in several genes related to the symbiosis development [91, 92]. The activity of these proteins leads to the transcriptional changes in root tissues, for instance, increasing the level of early nodulins ENOD40, ENOD11, ENOD12, ENOD5, which are known to be the potential regulators of IT growth and nodule primordium formation [93-95]. Also, the changes in cytokinin status of plant are detected, followed by up-regulation of genes encoding for RN symbiosis-specific cytokinin receptors [96-98]. Moreover, transcription regulators NIN and ERN are to be induced specifically downstream of the early NF signaling pathway in order to coordinate and regulate the correct temporal and spatial formation of root nodules [99-102].

The presented genes are responsible for the signal cascade which is aimed to induce the nodulin genes involved in building the symbiotic structures and implementing their biochemical functions. It is supposed that this signaling pathway did not appear *de novo* in legumes when they become able to form nodules, but was developed from already existing system of AM formation into which the novel, nodule-specific genes were recruited. Still, new genes had been involved in RN symbiosis development, especially those encoding the receptors recognizing hormones (e.g., cytokinins) and hormone-like molecules (Nod-factors).

Another important signaling process in RN symbiosis is an autoregulation of nodule formation. It takes place after successful mutual partners' recognition and signal exchange. It is considered that legume host controls the root nodule numbers by sensing the external and internal cues. A major external cue is the concentration of soil nitrate, whereas a feedback regulatory system where nodules formed earlier suppress further nodulation through shoot-root communication is an important internal cue. The latter is known as the autoregulation of nodulation (AON), and is believed to consist of two long-distance signals: a root-derived signal that is generated in infected roots and transmitted to the shoot; and a shoot-derived signal that inhibits nodulation systemically [103-104]. Therefore, AON represents a strategy through which the host plant can balance the symbiotrophic N nutrition with the energetically more "cheap" combined N nutrition.

Recent findings on autoregulation of nodulation suggest that the root-derived ascending signals to the shoot are short peptides belonging to the CLE peptide family [105] [106]. The leucine-rich repeat receptor-like kinase HAR1 of *Lotus* and its homologues in *M. truncatula* and *P. sativum* (SUNN and Sym29, respectively) mediate AON and also the nitrate inhibition of nodulation, presumably by recognizing the root-derived signal [107-110] (Figure 3).

It was suggested that NF signaling induces expression or posttranslation processing of CLE peptides, which likely function as ascending long-distance signals to the shoot [110]. Thus, NF signaling is related to autoregulation as well, but in some indirect way. It is also worth noting that NF signaling pathway appears to work in mature nodules, since aforementioned "early nodulation genes" belonging to CSP, as well as NF receptor kinase genes, are highly expressed in nodule tissues (76, 111). Perhaps the active NF signaling is needed to prevent the induction of defense-like responses and/or to restrict the release of rhizobia into precise cell layers, thus regulating the formation of symbiotic interface [112].

3.2. Pea (*Pisum sativum* L.) as a unique example of increased specificity in plant-microbe interaction

Being one of the most ancient crops known to humanity, nowadays garden pea (*Pisum sativum* L.) is widely distributed in the world. According to the recent data, pea is a third most important legume for food industry, following beans and soybeans [113]. It is also the popular model for various genetic and physiological researches, including the studying of symbiosis with nodule bacteria. Despite the fact that work with pea is complicated by the presence of some negative properties, such as relatively large (about 4000 Mb) genome, low seed productivity, and poor transformation capability, the use of this object in study of symbiotic relationships continues and brings significant results.

There are several pea genes known to participate in NFs' reception, with the most interesting of them being *Sym2*. This gene was first described in the 1970s as determinant of "resistance" to nodulation in pea cultivars from Afghanistan and Iran [114, 115]. While being unable to form nodules with the majority of natural *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) strains obtained from European soils, these cultivars have demonstrated the ability to interact normally with strains from the Middle East, such as strain *Rlv* TOM [115]. This feature is controlled by specific recessive allele of *Sym2* named "Afghan allele" ($Sym2^{A}$). Presence of

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Sym2^{A} in homozygous state leads to block of infection thread progression in the root hair, similarly to phenotype of *sym37* mutants [55]. Later it was shown that *Rlv* strains able to nodulate "Afghan" cultivars have special gene called *nodX*, which is involved in the modification of NF structure [116, 117]. *NodX* encodes the acetyltransferase providing O-acetylation on reducing end of NF sugar backbone. Thus, only *nodX*-modified NFs can be recognized by plants with *Sym2*^{A} allele, although Ovtsyna et al. (2000) [118] show that fucosylation on the same position controlled by *nodZ* gene can also induce nodulation of "Afghan" peas.

More than 20 years ago, *Sym2* was localized on the pea genetic map. Using RAPD (Random Amplification of Polymorphic DNA) markers, Kozik and colleagues [119] created the detailed map of pea I linkage group fragment including *Sym2* and a few other symbiotic genes (such as *Nod3* and *PsENOD7*). Based on the fact that plants with *Sym2*^{A} allele show the "Afghan" phenotype then exposed to NF with specific structure, it was suggested that Sym2 protein could act as an "entry" receptor during preinfection stage (similar to NFR1 in *Lotus* or LYK3 in *Medicago*).

When *Pisum* gene *Sym37* was shown to be orthologous for *NFR1* [55], it was at first proposed as a candidate for *Sym2.* This was strongly supported by the fact that the missense mutation in *Sym37* carried by *Pisum* mutant line RisNod4 led to Nod^{-} phenotype (the absence of nodulation), which could be suppressed by *Rlv* strain A1 known to produce broad specter of NFs, including *nodX*-modified one [55]. However, the paralogue of *Sym37*, gene *K1*, was discovered shortly after, the similar structure of which indicated a possible involvement in the reception of NF, although the purpose of this additional NF receptor remained unclear.

The comparison of *Sym37* and *K1* nucleotide sequences obtained from "Afghan" (*Sym2*^{A}) and "European" pea varieties, as well as amino acid sequences of their corresponding proteins, shows that neither of these genes possesses any features correlating with "Afghan" phenotype [55]. Thus, there must be another determinant corresponding to *Sym2*. Recently, the promising candidate was found – the gene named *LykX* by the authors, which is the second paralogue of *Sym37* localized in the same region of the pea genome (Sulima et al., 2015, in preparation). Analysis of the LykX protein sequences revealed that there are amino acid substitutions within first LysM module of receptor domain typical for plants with "Afghan" phenotype [120]. Simultaneously, Li and colleagues [121] compared the sequence of *Sym37* from series of pea genotypes that differ in interaction with rhizobia mutant on *nodE* gene determining the structure of fatty acid on nonreducing end of NF. It was shown that the efficiency of interaction with mutant strain strictly correlates with particular variation of *Sym37*. Similar situation was observed for interaction between *nodX* and *Sym2* (*LykX*) genes: "Afghan" pea varieties requiring NF with additional acetyl group on reducing end of molecule also display characteristic features in structure of receptor protein LykX.

We proposed a model, based on the above, according to which the less specific "recognition" receptor (Sym10, perhaps in complex with other proteins) perceives the NF signal *per se* and "anchors" NF molecule on the membrane, subsequently "presenting" it to other components of reception complex, with reducing end being tested by Sym2 (LykX), and nonreducing by Sym37 [122]. Only if all participants in the process react positively will the signal be considered as adequate, and symbiogenesis will start properly (see Figure 4). So, in pea not only one

ortholog of *Lotus NFR1*, but two closely related paralogs – *Sym37* and *Sym2* – are involved in genetic control of Nod-factor reception. This is not surprising, if we take into account the complexity of Nod-factor molecule and the importance of its proper recognition for successful development of symbiosis.

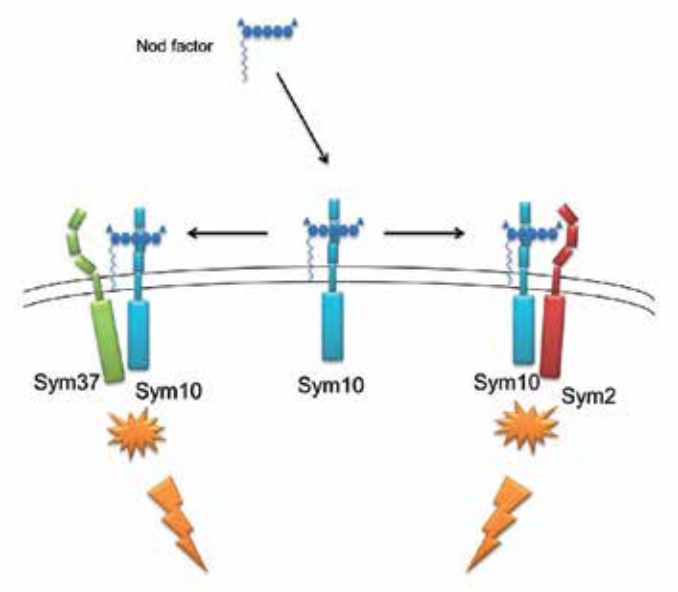

Figure 4. Hypothetical model for precise recognition of Nod-factor structure by receptor kinases in pea. The model is proposed by Dr. V.A. Zhukov (ARRIAM, St. Petersburg, Russia). At first step, less specific receptor (probably, Sym10) anchors NF molecule onto the membrane; then it presents it to Sym37, which tests the structure of the nonreducing end, and to Sym2, which tests the structure of reducing end. When both Sym37 and Sym2 bind NF, they activate downstream components of signal transduction pathway.

4. Conclusion

Among all the multicellular eukaryotes, plants have the greatest need for the beneficial interaction with microorganisms, as they lack active movement and therefore cannot choose more advantageous habitat. That kind of restriction can be compensated by the ability of photosynthesis, as carbon compounds produced by plants are a significant stimulus for various microbes to cooperate with them. As a result of such cooperation, plant acquires an access to the adaptations of microsymbiont, and *vice versa*, according to a principle of genome complementarity that was recently formulated by Prof. I.A. Tikhonovich and Dr. N.A. Provorov (ARRIAM, Russia) [122]. It means that, in spite of lacking the nitrogenase genes in its own genome, plant "exploits" corresponding part of microorganism's genome in order to implement biological nitrogen fixation, while rhizobia "exploit" plant genes controlling

http://dx.doi.org/10.5772/61165

photosynthetic apparatus, and so forth. Thereby the plant-microbe system acquires an advantage over plants and microbes that compete for survival separately.

The role of symbioses in the evolution of life, and plants in particular, cannot be underestimated. One can state that the tendency to establish mutually beneficial associations with microorganisms is an essential feature of plants, which has a wide variety of manifestations through a long coevolution of symbiotic partners. Photosynthesis itself, the main distinctive feature of plants, is provided by chloroplasts – the descendants of ancient symbiotic cyanobacteria. According to modern conception, plant colonization of land was possible primarily due to the symbiotic association with arbuscular mycorrhiza fungi. AM, in turn, is considered as a basis for the development of highly specific root-nodule symbiosis characteristic for legume plants. The possible path of the AM origin and its connection with RN was largely understood by studying *Geosiphon pyriformis* – the only representative of the phylum *Glomeromycota* that does not form symbiotic association with higher plants.. Instead, it contains intracellular symbiotic nitrogen-fixing cyanobacteria of the *Nostoc* genus which are essential for its proper nutrition and development [123, 124]. The intensive exchange of products of nitrogen, carbon, and phosphorus metabolism between partners indicates that mechanisms of reciprocal nutrients' transport probably emerged in symbiotic systems formed by *Geosiphon* and *Nostoc* ancestors and lately have been recruited in the evolution of AM [124, 125]. The transition from *Geosiphon-Nostoc*-type association to AM could occur through an intermediate "triple" symbiosis including plant, common ancestor of AM fungi, and *Geosiphon*, and ancestral forms of *Nostoc*, with subsequent loss of cyanobiont. It should be noted that ancient symbiotic fungi presumably carried additional bacterial symbionts both on the surface and in the cytoplasm. In the cells of modern *Glomeromycota*, including *Geosiphon*, various symbiotic bacteria are found, including those capable of nitrogen-fixation (close to β-proteobacteria of *Burkholderia* genus, some members of which were shown to form the RN symbiosis with legumes; see above) [126]. Thus, the AM symbiosis could be the direct "gateway" for introducing symbiotic bacteria, including the ascendants of modern rhizobia, into plant tissues. This suggestion is also supported by the existence of CSP and the similarity of rhizobial and fungal signal molecules.

Emergence of Nod-factor signaling was among the most important factors that determined the evolutionary success of legume-rhizobial symbiosis. The wide variety of Nod-factors as well as finely tuned receptor system in plants ensure that only specific partners will meet each other in the soil and consequently form a superorganism with high level of genetic and metabolic integration. This appears to be a basis for evolution of the efficiency of symbiotic pairs, instead of single organisms – the results we now observe.

Legumes provide both an important food source for humanity and a unique model for investigation of the evolution and the underlying genetic mechanisms of mutualistic plant-microbe symbioses. Further studies of the genetic bases of signal interactions between plants and microbes can provide more information about evolution of such a mutually beneficial association, as well as about spreading of the legumes across the world. Discovery of genes involved in recognition of partners, transduction of symbiotic signals and overall "management" of symbiosis will also provide a useful tool for agriculture, as the knowledge obtained

from this studying will facilitate the creation of highly-effective specific symbiotic pairs between crop plants and nitrogen-fixing bacteria in field..

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Chapter 7

Evolutionary Analysis of Basic RNase Genes from Rosaceous Species — S-RNase and Non-SRNase Genes

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Additional information is available at the end of the chapter

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Abstract

Over the past two and half decades there has been an explosion of progress in a growing number of model self incompatibility (SI) systems on our understanding of the molecular, biochemical and cellular processes underlying the recognition of self pollen and the initiation of a cascade of biochemical and cellular events that prevent self fertilization. These studies are unrevealing the complexity of a trait (SI) whose sole purpose, as far as we know, is to exert a strong influence on the breeding system of plants. Evolutionary interest in floral traits that influence the breeding system and in the forces that shape these traits began with Darwin who devoted one complete book to the subject (Darwin 1876) and significant portions of a second book. The evolution of plant breeding systems is often viewed as the interplay between the advantages and disadvantages of selfing. Evolutionary biologists have long noted that there are three primary advantages to selfing. First, there is an inherent genetic transmission advantage to selfing because a plant donates two haploid sets of chromosomes to each selfed seed and can still donate pollen to conspecifics. Second, selfing can provide reproductive assurance when pollinators are scarce or and third, it often costs less, in terms of energy and other resources, to produce selfed seed (e.g. fewer resources are expended to attract and reward pollinators. Some major questions remain unanswered concerning the evolution of stylar SRNases. Most pressing is the apparent disparity in patterns of diversification seen in the Solanaceae and Plantaginaceae relative to what is observed in the Rosaceae. Thus, we reviewing current publication regarding the evolutionary analysis basic RNases towards comprehensive view.

Keywords: S-ribonuclease, Transmitting tract specific glycoprotein, Evolutionary, Rosaceae

1. Introduction

All angiosperms make indiscriminate bunches, and their regenerative organs are in close partition. This makes a genuine inclination toward inbreeding. Inbreeding often results to decreased offspring wellness (e.g. more susceptible to diseases). With advance blooming, plantsare not able to utilizephrase different systems to prevent self-fertilization and thereby to generate and maintain genetic diversity within a species thus, the profoundly genuine and distinct plant kingdom, which is composed of >80% angiosperms would not exist [102, 110, 118].

Self-incompatibility (SI) is among the most important techniques utilized by many flowering crops to counteract self-fertilization and thus, generate and support genetic range inside a species. Common ancestral reports argue that SI virtually in most species may be managed by a simple polymorphic locus, the true self-incompatibility S-locus. At present, at the very least, there are two gene loci: pistil S and pollen S, therefore, the term haplotype is used to describe variants of the S-locus. Pollen inhibition occurs if the same S-haplotype is expressed equally by pollen and pistil [18].

Most *Prunus* fruit trees exhibit homeomorphic gametophytic self-incompatibility (GSI) where self/non-self-recognition can be controlled by a single multi-allelic locus, termed the S locus. SI reaction is activated if the same ''S allele'' specificity can be expressed in the pollen and pistil (Fig. 1). Thus, the growth of pollen tube bearing either one of two ''S allele'' specificities carried with the recipient pistil can be arrested inside the style. Exactly the same type of GSI is found not only in other genera in Rosaceae, but also in *Malus*, *Pyrus*, Solanaceae and Plantaginaceae [18, 56, 86, 117, 141].

As fruit trees of the *Prunus* kind are not able to bear fruits parthenocarpically, fertilization and seed formation are important for excellent fruit generation in SI *Prunus* fruit trees. In industrial orchards, appropriate cross cultivars that are part of different pollen-incompatibility communities and that bloom simultaneously are inter-planted; and beehives are also often placed throughout orchards to make certain fruit set [135]. Hence, determination of pollen incompatibility groups and assignment of cultivars to these groups are fundamental. Expectedly, this has been proficient by controlled fertilizations and minute assessments of pollen tube development, which is tedious and affected by natural elements. To maintain a strategic distance from the downsides identified with SI, the formation of impeccable creation of self-good (SC) cultivars with extraordinary pomological qualities is among the critical expansion focuses on that identify with SI Prunus trees' natural items [52].

In the last two years, genes for those two proteins controlling the real allele specificity of GSI acknowledgement in *Prunus* have been actually identified. It is now known that two separate genes at the S locus handle male (pollen) and female (pistil) specificities (Fig. 1).

The advancement of vegetable reproducing frameworks is often seen as the exchange involving the focal items and hindrances of selfing. Evolutionary professionals have since noted three favorable and crucial outcomes of selfing. To start with, there is usually a characteristic genetic transmission place of attention to selfing throughout light that the a vegetable gives a couple

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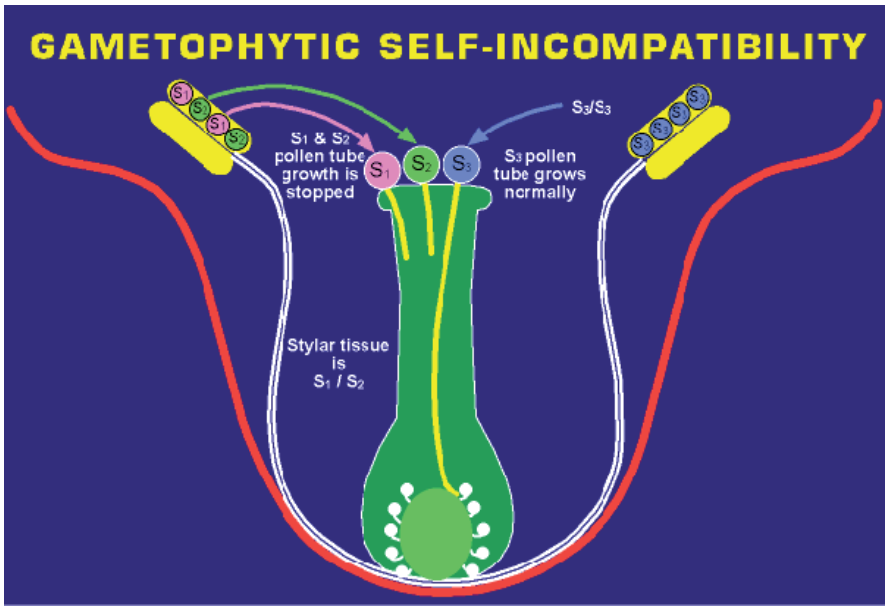

Figure 1. Genetic base gametophytic self-incompatibility

haploid sets of chromosomes to each one of these selfed seedling regardless may give dust to nonspecific [24]. Subsequently, selfing may cause regenerative affirmations when pollinators are rare (i.e. selfed descendants are better than no offspring) (e.g. Stebbins 1957; Schoen et al. 1996).Third, it often costs less, where vitality and various assets, to supply selfed seedling (e.g. less assets are utilized to pull and reward pollinators) [107, 108, 133].

Ribonuclease (RNase) and F-box genes were referred to as the pistil S and pollen S determinant genes, respectively (see review by [56]). Upon this discovery, the term 'S haplotype'is used to describe the real variants in the S locus, while the term 'allele'is used to describe the real variants in the S locus genes, pistil S and pollen S. On the real practical facet, these findings resulted to the advancement of new molecular approaches for S genotyping and SC screening process [120, 140, 141]. Molecular S genotyping and a number of marker-assisted SC offsprings are increasingly being successfully integrated in *Prunus* propagation programs worldwide.

Brewbaker (1959), in an expansive discussion of angiosperms, noted that SI has happened in no less than 71 families, and as of now, has been recorded all through around 250 to 600 genera that were explored; and the evaluation was that between 33% and another half off the blooming vegetation are self-contradictory. By and large, SI appears to have advanced no less than 21 exceptional times amid the development of blossoming vegetation [116] and a few one of a kind sorts may be recognized relying upon morphology, inherited genes, and molecular mechanism. In SI frameworks that are controlled by a single genetic locus, the locus has for every situation been termed the S-locus. It is obvious in any case, that various genes live inside of every S-locus, and the allelic complex of genes has been termed the S-haplotype.Nonetheless, it is apparent that many genes dwell within just about every S-locus, as well as the

complicated allelic genes continue to be termed the real S-haplotype. Although in most of these methods, the ancestral locus has long been termed S, a variety of biochemically unique mechanisms are engaged, at very least at the degree of recognition of self- and non-self-pollen. Molecular information can be obtained for simply three types, the single-locus sporophytic and also two distinct types of single-locus gametophytic SI. In the actual sporophytic SI system of *Brassica*, both pollen and pistil S-genes are actually identified, and the stylar result is mediated by means of protein receptor kinases (for review, see [54]). The molecules mediating pistil S-specificity are actually identified and also cloned in two unique single-locus gametophytic methods. In the real Papaveraceae, SI consists of a complex number of events such as changes throughout calcium ion attentiveness, phosphorylation of specific meats, and transcription of pollen genes and DNA fragmentation of nuclei [53, 114],and the pistil S-gene has no significant homology to any gene of known purpose. In probably the most phylogenetically widespread way of gametophytic SI [116], the pistil S-gene product is usually a glycoprotein [59] together with ribonuclease action [83], and these molecules are actually termed S-RNases. The DNA sequences in the genes curbing SI systems can be quite a treasure chest of molecular data; and research of self-incompatibility gene sequences can offer data not just on the actual development in the systems themselves, but also the individual structure and demographic record of species [92]. Our emphasis with this particular article is going to be on solanaceous variety SI.

To date, some valid inquiries on the development of stylar SRNases remain unanswered. The most pressing may be the clear originality in degrees of expansion affecting the Solanaceae and also Plantaginaceae regarding what exactly is seen inside Rosaceae. We review data concerning the structure, functions, and molecular physiology of S-RNases; attempt to integrate these results with evolutionary studies, provide new analyses of domain structure and conservation; and present new analyses of selection/recombination in S-RNases.

2. Self-incompatibility

S-RNase-based SI genetically classified as gametophytic locus, the pistil differentiates between self and non-self-pollen based on the S-allele in the haploid pollen and meets either in the two S-alleles in the diploid pistil. The SI phenotype of pollen is determined by its own S-genotype. The rejection based on matching of S-alleles in pollen and pistil.

Pistil S-allele products were initially called basic polymorphic glycoproteins whose genetic abundance weight and isoelectric spot ranged from ~22 to 35 kDa and from ~8–10, respectively, and then further isolated together with S-alleles. These proteins are extracellular, largely confined to the upper third of the stylar transmitting tract-the site of self-pollen tube inhibition-and are developmentally correlated with the onset of SI, being absent 1 day prior to anthesis (immature pistil are self-compatible) and present at 1–10% of total protein at pollen release. S-RNase occurs at a truly high focus in completely created pistils and it has been approximated at 10–50 mg/ml inside the extracellular network of the stylar exchange tract with regard to the solanaceous type [51]. The primary quality encoding of one of these brilliant basic proteins has

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been cloned through *Nicotiana alata* by Anderson et al. in 1986 and more than 50 S-RNase arrangements have been reported since then. S-RNase arrangements are exceptionally disparate together with an amino p character from 38% to 98% sequence identities. Despite the fact that essential confirmation for that inclusion about S-RNases all through SI has been correlative, direct affirmation is attained by transgenic experimental tests similar to those done for *Petunia* and *Nicotiana* [67, 88]. Experiments showed that the S-specificity of pistils of transgenic plants can be altered through expression of a sense or antisense S-RNase transgene, leading to a gain or loss of S-specificity, respectively. These experimental tests likewise showed to some extent that high levels of S-RNase inside wild-type pistils are vital for pollen rejection to be complete. This information is once in a little while translated on the grounds that showing the genuine S-RNases is key and abundant for irregularity toward oneself inside pistil. It is not entirely genuine as different qualities less living at the S-locus has been demonstrated to influence the genuine SI result [85], subsequently, S-RNases are fundamental for SI and encode the genuine pistil S-specificity, yet are not general.

3. S-RNase function and structure

S-RNases are highly divergent, with allelic amino p sequence identities of about 30% to over 90% [125, 86]. Regardless of the excessive allelic string diversity, the real analysis of solanaceous S-RNase alleles exposed five conserved areas, from C1 to C5 (Fig. 2). C2 and C3 areas contain conserved catalytic histidine residues. Apart from C4, these regions are conserved throughout *Prunus* and also plantaginaceous S-RNases [127]. The 4th conserved rosaceous, called RC4, differs in placement and amino p sequence from C4 in the solanaceous S-RNase. There is a single (RHV) hypervariable region in rosaceous S-RNase, while a hypervariable couple (HVa and HVb) were within solanaceous and plantaginaceous SRNases [139]. Although hypervariable region(s) can be positively chosen and thought to play an essential role in self/non-self-recognition, recent conclusions suggested that other regions are important for that specificity in recognition [97, 133, 146].

Structural and phylogenetic analyses indicated that S-RNases in the three families share a typical origin, and so, the S-RNase-based GSI system evolved just once in eudicots [45, 131]. A single intron that is certainly common within the T2 form plant RNases is usually present inside the coding sequence in the hypervariable place of S-RNases of Solanaceae and *Pyrus* and *Malus* of Rosaceae (Fig. 2). With *Prunus*, also intron, there can be another intron inside the S-RNase code sequence at the junction involving the signal peptide and the start of the fully developed protein [45, 120]. It is intriguing that a stylar-expressed non-S-RNase within *Prunus*, that includes a single intron with no role in GSI, continues to be identified [5, 140]. Although biological and evolutionary significance in the other intron inside *Prunus* S-RNase can be unclear, the occurrence of a couple of introns varying in proportions has already been successfully useful in developing molecular solutions to distinguish one of several *Prunus* S-RNase alleles. A couple of PCRs are usually enough for S-RNase genotyping in *Prunus*, while allele-specific PCR as well as cleaved amplified polymorphic string (CAPS) markers are essential for genotyping in *Pyrus* and *Malus* [141].

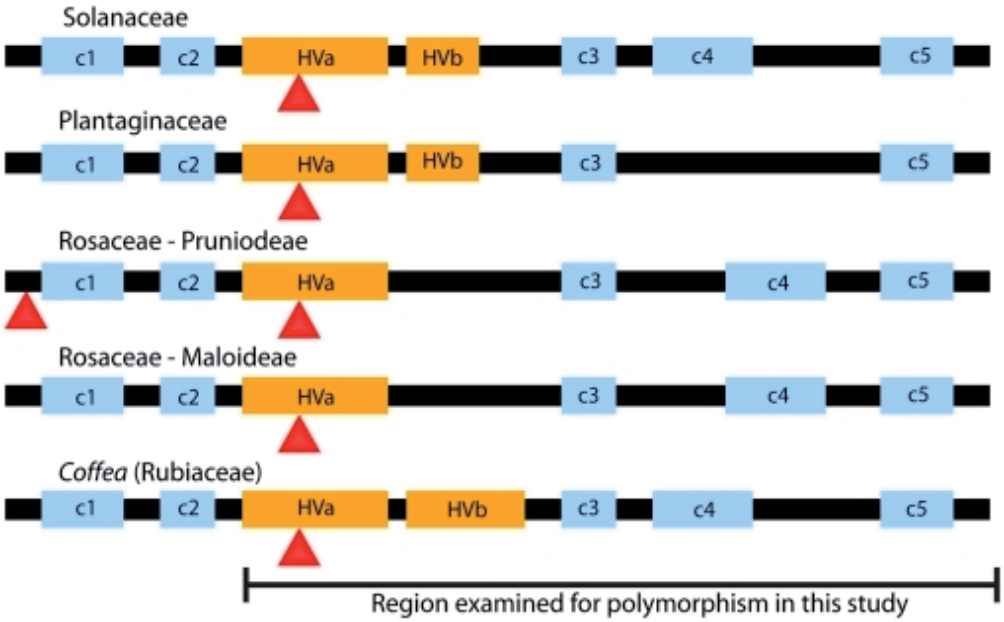

Figure 2. S-RNase structure and positions of intron groupings in S-RNase DNA succession. Solanaceous and rosaceous S-RNase structures are schematically represented. Intron arrangements are ordinarily found amid the coding successions for HVa and RHV of solanaceous and rosaceous S-RNases, separately. Notwithstanding this intron, there is another intron in Prunus S-RNase, yet not in *Malus* and *Pyrus* S-RNase. SP, signal peptide; C1 to C5, rationed locales 1–5; RC4, rosaceous preserved district 4; HVa and HVb, hypervariable areas a and b; RHV, rosaceous hypervariable area [119].

Several experiments of Solanaceae indicated that S-RNase exerts its cytotoxic effects inside the pollen tube through RNase action. Huang et al. (1994) confirmed that RNase action was essential for the pollen rejection response in *P. inflata*. Kowyama et al. (1994) looked into an SC *Lycopersicon peruvianum* variant and found that its SC lacks S-RNase action. Pollen rRNA was proved to be degraded right after SI, but not compatible pollination in *N. alata* [82]. Though every one of these fresh data was obtained with solanaceous vegetable species, it has been typically acknowledged that the RNase function is important for the real pollen rejection response in Rosaceae given that most functional S-RNases noted up to now have equally conserved catalytic histidine remains, which can be situated inside C2 and C3 areas. Gatekeeper and inhibitor types (Fig. 3) were proposed while using cytotoxicity in the S-RNase [86, 123].

The gatekeeper design assumed any recognition mechanism that helped only cognate S-RNase to enter the pollen tube to to exert its cytotoxicity, while the inhibitor design assumed the real presence of an inhibitor that inactivated the cognate S-RNase. Since immunocytochemical studies with Solanum chacoense showed that S11-RNase entered pollen tubes of both compatible and incompatible S haplotypes [73], the inhibitor model and its modification, the general inhibitor model, have been generally accepted [74]. Upon real identification, the pollen S encodes an F-box protein that could be involved in proteolysis; the cytotoxic effect of the S-RNase has been considered to be evaded due to S-RNase degradation rather than inhibition

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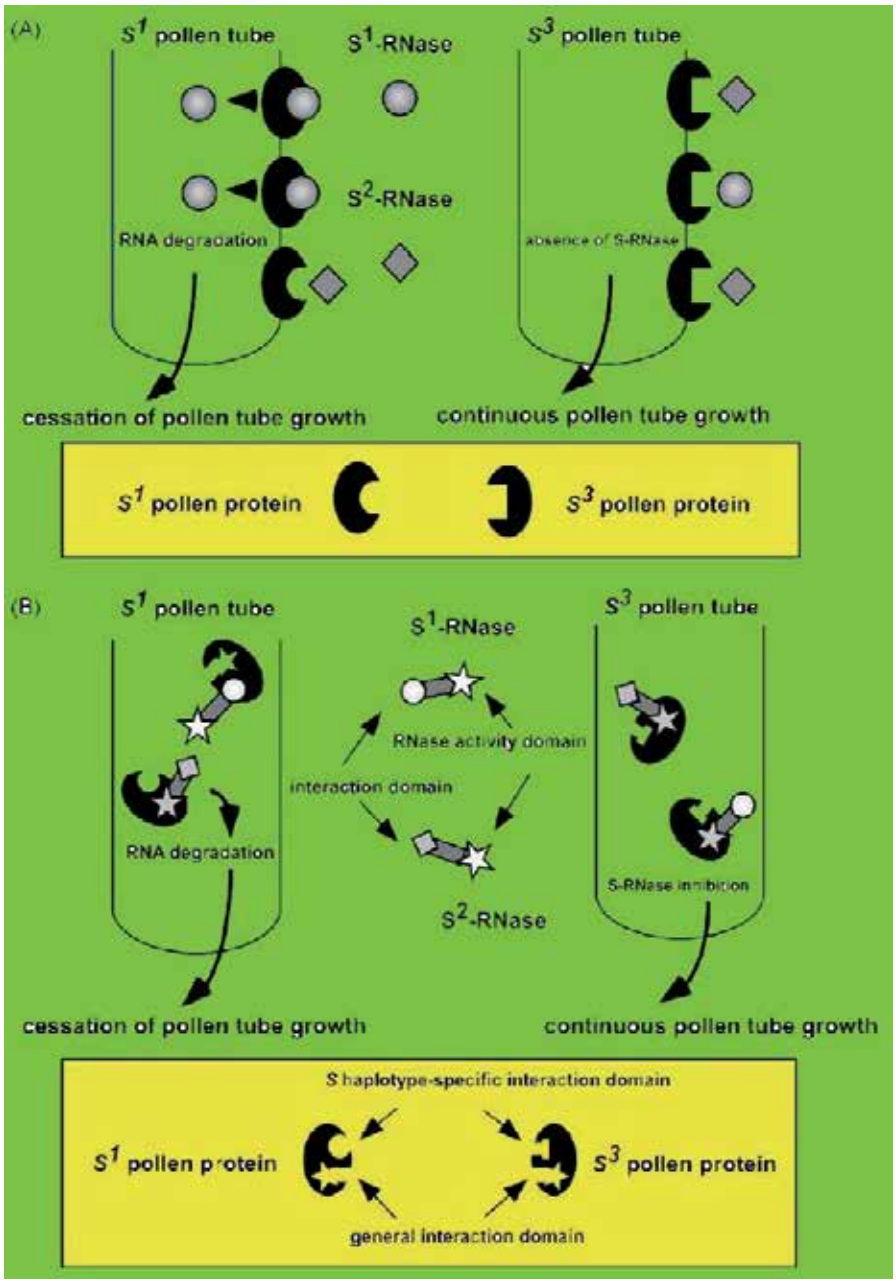

Figure 3. Gatekeeper and inhibitor models for the self-incompatibility reaction. The growth of S1 and S3 pollen tube in the S1S2 pistil is schematically described for the gatekeeper (A) and inhibitor (B) models [With permission of 118].

of S-RNase activity [39, 127-129, 143] (Fig. 5). Even though it remains to be seen whether or not both self- and non-self-S-RNases enter the pollen tube of Rosaceae, a degradation model has been offered for pollen-pistil acknowledgement. Although as mentioned previously, S-

RNases are highly polymorphic, sequence comparison in the 12 S-RNase sequences in 1991/1992 recognized five regions of conservation, known as C1 to C5 [123]. Of these, two (C2 and C3) share a high degree of sequence similarity while using corresponding regions of fungal RNases, RNase T2 [58], and RNase RH [38]. This similarity that led to the discovery that S-proteins are themselves RNases [83].

Discovery of a self-compatible S-allele in *Lycopersicon peruvianum*, in which one of the two catalytically essential histidine residues was mutated, provided a strong inference that RNase activity was necessary for SI function [104], but in the absence of same specificity, or identity of the pollen component of the interaction, this was not conclusive. Using a transgenic approach, where a mutant S-RNase gene with the codon for one of the two catalytically essential histidines replaced with an asparagine codon was introduced into plants, Huang et al. (1994) affirmed that the creation with this mutant SRNase was not ready to present an increment of S-capacity (dissimilar to the genuine wild-sort protein) so as a result of this, characteristic RNase activity is a bit of the reason behind S-RNases.

S-RNases are glycoproteins with one or more N-connected glycan structures, increasing the likelihood that allelic specificity may be encoded with the sugar moieties inside glycan structures. This question was addressed by engineering an S-RNase gene in which the asparagine codon of the only N-glycosylation site of the protein was replaced with an aspartic codon; however, N-glycosylation site in the protein has been supplanted with the aspartic codon. Investigation of transgenic vegetation communicating this sort of mutant S-RNase indicated that non-glycosylated S-RNase has the capacity to act similarly and effectively as wild-type S-RNase by releasing dust containing exactly the same S-allele [57]. Henceforth, the advancement of S-specificity is not found inside glycan feature chains in the protein spine of S-RNases.

A set of hypervariable regions, termed HVa and HVb, was additionally uncovered by Ioerger et al. (1991). These are the numerous hydrophilic locales of the S-RNase, realizing the speculation that HVa and HVb are the prime candidates for the determinant of S-RNAse specificity [49, 124]. The crystal structure of SF11-RNase has recently been determined by X-ray diffraction [44], confirming that both HVa and HVb regions are where they might play a role in determining allelic specificity. Ishimizu et al. (1998) found four regions of rosaceous S-RNases that demonstrate a crucial abundance of non-synonymous substitution around synonymous substitution and appear to be under positive selection, of which two overlap with HVa and HVb. In an investigation of Scrophulariaceae, extremely variable HVa and HVb ranges were discovered; however, but did not find evidence of diversifying selection [131]. It can be fascinating to see that a large proportion of these proteins are communicated at abnormal states in an exceedingly short period of time in the genuine procurement of SI. To date no codon usage studies have been carried out for S-RNases from any family but is an area of research that may provide interesting results.

There are several reports of experiments employing transgenic methods to identify regions and amino acid residues involved in the encoding of allelic specificity. Chimeric S-RNase genes have been created and presented in transgenic plant life intended of the S-specificity displayed

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by hybrid S-RNases. Further, no gain of the new S-specificity of the donor allele was found, despite the fact that all hybrid S-RNases exhibited normal levels of RNase activity [55, 146].

A good but apparently contradictory result has been gained via a research employing a few very tightly related S-RNases (S11 and S13) of *Solanum chacoense* [80]. Both these S-RNases differ by a total of only 10 proteins, three that are within HVa and another of which in HVb. Substitution in the HVa and HVb regions of S11-RNase together with S13-RNase produced S-RNase found to exhibit S13- specificity; however, there was no S11 specificity in transgenic plant life.

These results seem to claim that HVa and HVb collectively are sufficient for S-haplotype specificity. However, any domain swapping experiment can only address the role of those amino acids which differ between the two proteins under study. If the outcomes of the specific three trials are obtained together, it would seem that proteins outside HVa and HVb (conserved S11-RNase and S13-RNase) are suitable to be engaged inside the allelic specificity of S-RNases [130]. Nevertheless, it is clear that the HVa and HVb areas play a vital role in encoding allelic specificity in S-RNases.

4. Pistil S determinant

4.1. Identification of S-RNase

The physiology and mechanisms of GSI are actually most substantially studied in solanaceous vegetable species. The development of cDNA glycoproteins co-segregated together with S alleles was first cloned via *Nicotiana alata* [2-3]. The deduced amino p sequence clearly implicated stylar RNase involvement in the recognition and rejection reaction inside the style. Along with other studies, it has been shown that in Solanaceae, the S allele product inside pistil is usually a highly simple glycoprotein comprising sequence motif characteristics in the active site in the fungal RNase T2 [58] and RH [38], termed S-RNase [83, 84].

At one point when transgenic analyses with *Petunia inflata* and *Nicotiana* proved that the S-RNase alone is enough for determining the specificity in the GSI pollen rejection response inside Solanaceae [67, 88], Sassa et al. (1996) and Broothaerts et al. (1995) reported that SRNases are associated together with GSI of *Pyrus* and *Malus* in Rosaceae. The finding that these families recruited the same molecule as the GSI pistil determinant has been unexpected because Solanaceae (*Asteridae*) and Rosaceae (*Rosidae*) are phylogenetically remote [12, 45]. Although deduced amino p sequences from cDNAs of S-RNases of *Pyrus* and *Malus* may be similar and support the active site of T2/S-type RNases, differences were clear involving the rosaceous and solanaceous S-RNases [105-108, 126]. Later, Xue et al. (1996) cloned the real S-RNase throughout *Antirrhinum* inside Plantaginaceae, a tightly related family of the Solanaceae.

As *Prunus* is one of the Rosaceae, it had been readily predicted that *Prunus* even offers an S-RNase-based GSI system. However, S-RNases remained unidentified for countless years after the real cloning in the *Pyrus* and *Malus* S-RNases, because polymerase chain reaction (PCR) cloning solutions for *Prunus* S-RNase were hindered by its fairly low DNA string, similar to

Pyrus and *Malus* SRNases as well as the presence of *Prunus* RNase genes which cannot involved with GSI. The first clue for the cloning of *Prunus* S-RNase was obtained when N-terminal sequences of almond (*Prunus dulcis*) SRNase were reported [120]. By the N-terminal amino p sequences, sweet cherry (*Prunus avium*) [120] and almond [125] S-RNases were cloned. Currently, sequences of over 100 *Prunus* S-RNase alleles are actually deposited in GenBank.

4.2. Pollen S determinant

4.2.1. Identification of F-box gene

The pollen S determinant in the S-RNase-based GSI in Rosaceae, Solanaceae, and Plantaginaceae was discovered decades after the real stylar determinant, S-RNase. The subcentromeric location in the solanaceous and plantaginaceous S locus experienced had long prevented chromosome walking [23, 142]. The first clue for the identification in the pollen S was from the S locus in the Plantaginaceae. Sequencing analysis in the *Antirrhinum hispanicum* S locus exposed the presence of the pollen-expressed F-box gene (AhSLF for a. *hispanicum* S locus F-box) located 9 kb downstream of S2-RNase [65].

Even though it was speculated that the F-box protein gene encoded the real pollen S, only one particular allele has been cloned. The S locus of *Prunus*, which is located right at the end of the linkage group 6 [20], was much more compact than those of Solanaceae and Plantaginaceae. Two separate groups in Japan successfully sequenced the real S locus of *Prunus* beginning from the S-RNase [22, 127]. Ushijima et al. (2003) did DNA sequencing and transcriptional analyses for the genomic areas that flank real almond (*P. dulcis*) S-RNase and identified polymorphic and non-polymorphic S locus F-box genes, called SFB of S haplotype-specific F-box gene and SLF for S locus F-box, respectively. At present, SLF continues to be referred to as SLFL1 [79] after the nomenclature of Entani et al. (2003). The options that come with SFB, including the high degree of allelic polymorphism, pollen-specific appearance, and the close physical distance to the S-RNase many supported that SFB may be the male determinant of GSI in almond. The same research group also found SFB in the cherry (*Prunus*) S locus in their attempt to compare the same S-RNase allele on SC and SI kinds of cherries, *R. cerasus* and *P. avium*, respectively [140]. Another study group in Japan reviewed the S locus place of a couple different S haplotypes in Japanese apricot (*Prunus mume*) and found some F-box genes [22]. Among them, SLF of S locus F-box, that includes a different name but can be orthologous to SFB throughout almond and cherries, shows a high level of allelic string diversity and was supposed to be a prospect of pollen S. The other F-box genes found, SLFL1, SLFL2, SLFL3 of SLF including gene 1, 2 and 3, respectively, showed far lower allelic string diversity. The pollen S candidate of Japanese apricot has been independently cloned in a study by Yamane et al. (2003d) and named differently as PmSFB. Since then, the *Prunus* pollen S was initially referred to by a couple of different words or terms such as ''SFB'' and also ''SLF''. We used ''SFB'' in this particular review to show the various features in *Prunus* SFB than the SLF in Solanacae and Plantaginaceae.

Direct evidence that S locus F-box gene adjustments of allele specificity in the pollen was from a transgenic research in *R. inflata* [109]. This kind of experiment employed a well-known

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phenomenon termed ''competitive interaction'', where heteroallelic pollen which has two unique pollen S alleles work in pistils together with one as well as both cognate S haplotypes [18, 26-29].

Although transgenic analyses in *Prunus* are hindered by a long period and lacking a useful transformation system, molecular characterization in the S haplotypes throughout SC mutants supplied indirect but very good supporting facts for SFB staying the pollen S [125]. Later, multiple F-box genes, called SFB (SFBBs), were found in candidate pollen S genes in *Malus* and *Pyrus* [105-107]. Even though it has also been shown that the SFBB-gamma gene is not likely involved with the specificity determination of GSI reaction [131], it is still possible that just as with the other families and genera, one or a number of the SFBBs could be the pollen S.

4.3. Pollen S structure

SFB has just a single intron inside 50 untranslated places, where zero intron was within solanaceous and plantaginaceous SLFs. Although intron dimension varies together with different alleles, the difference inside the intron dimension is too small to be detected the intron length polymorphism for for S genotyping. Hence, fluorescent primers and an automatic sequencer were used to detect the real intron period polymorphism of S genotyping depending on SFB alleles [128]. Nonetheless, because fairly large-scale recombinational research using 1022 meioses confirmed no recombination involving the Prunus S-RNase and SFB [46] and S-RNase genotyping is often much easier, SFB genotyping can be used as a supplementary research for S haplotype determination. SFB research, on the contrary, is usually a powerful tool as well as the sole way to detect SC pollen-part mutant (PPM) S haplotypes, where only the real pollen S continues to be mutated [47-48, 126].

Another essential auxiliary examination of SFB uncovered the event of a few variables (V1 and V2) and two hypervariable (HVa and HVb) territories [47]. These hypervariable regions appeared to be hydrophilic or at least not strongly hydrophobic, which suggests that these regions may be exposed on the surface and function in the allele specificity of the recognition response. The fact that positively selected sites appear to concentrate in the variable and hypervariable regions further supports the possibility that these regions could play an important role in the SC/SI recognition.

Phylogenetic analyses with F-box genes inside *Arabidopsis thaliana* genome indicated that *Prunus* SFB, Petunia SLF, and Antirrhinum SLF might have a monophyletic beginning [134], as continues to be reported for that pistil S-RNase [45, 116]. Vieira et al. (2009) also concluded that there's no effective evidence to declare that the pollen S was independently recruited many times while in evolution. Nonetheless, phylogenetic reconstructions in the pollen S across all three families highlight numerous differences. As opposed to the phylogenetic tree of the S-RNase and S-like RNases of plants, the pollen-determinant F-box genes of a given family or genus are more closely related to its own S locus F-box genes that have no role in SI than to the pollen-determinants of the other families or genera [105, 126, 136, 79].

Another difference is available in the allelic string diversity in SFB of *Prunus* and SLF in the Solanaceae and also Plantaginaceae [92]. Deduced amino p sequences via SLF alleles prove as

extremely excessive high pairwise identities of over 90%. When it comes to plantaginaceous alleles, the actual pair wise identities about SLF alleles are 97% and 99% [144], while those of the respective S-RNase alleles are 30% and 60% [139]. On the contrary, the pair wise allelic string identities on Prunus SFB alleles is lower and a lot like those of S-RNase alleles; the degrees of divergence is comparable around 60–90% [45, 92]. Nonetheless, the phylogenetic associations among SFB are generally incongruent together with one of several S-RNases for the same S haplotype, regardless of the expectation of co-evolution in the pollen and pistil determinants [46, 92]. In Solanaceae and Plantaginaceae, it usually is plausible to consider that more allelic string identities can be achieved within SLF compared with SFB of *Prunus* and can show faster evolutionary heritage. Interestingly, the S-RNase of Solanaceae and also Plantaginaceae show higher degrees of allelic string diversity when compared with that of *Prunus*. Therefore, it is clear the *Prunus* S locus and also solanaceous and plantaginaceous S loci evolved in a variety of ways.

4.4. Pollen S-gene and function

The point that an acknowledgement event occurs involving the pollen and pistil inside operation of gametophytic self-incompatibility (GSI) dictates that recognition molecules have to be present in both tissues. For the real pistil and pollen components for being encoded by different body's genes raises many interesting conceptual issues both inside generation of new allelic specificities and inside maintenance in the genes to be a genetically associated unit. Therefore, many early types of the mechanism of gametophytic SI were relying on an individual gene, with inhibition occurring by using a dimerization event from the pollen tube or due to differential processing of a single gene as well as operon to create pistil and S-gene products [69]. There is a lot of evidence that pollen and pistil S-components are, in reality, separate body genes.

1. The expression of S-RNases throughout transgenic plants while using endogenous supporter causes a big difference in the pistil, but not pollen S-specificity [67].
2. The expression of S-RNases in transgenic plants employing a pollen particular promoter will not alter the real SI behavior in the pollen [21].
3. Through a phenomenon known as ''competitive connection,'' SI in time breaks down in pollen grains that carry a couple of different alleles. Plants holding duplication in the S-locus are actually generated by X-ray mutagenesis and these duplications are brought on the pollen to shed S specificity, presumably through the possession of two pollen S-specificities and in many of these self-compatible mutants, the real S-RNase is not present around the duplicated fragment [27-29].

A self-compatible mutant of *Pyrus serotina* (Rosaceae) has been identified in which the S-RNase has been deleted; this deletion affects the pistil, but not pollen S-function [104].

Major effort continues to be directed to the identification of the pollen S-gene and product. It must have a number of characteristics, such as ancestral linkage to S-RNase, allele particular polymorphism, and gene solution interaction in some way with S-RNases—either together with self-S-RNase, cross- S-RNases or both (but in many ways). Pollen meats that connect to

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S-RNases are actually identified such as calcium-dependent protein kinases, which phosphorylate S-RNases at the very least in vitro, however, not in an allele specific manner [63], and more recently, any protein comprising a RING-HC domain, which potentially may be mixed up in ubiquitin ligase-mediated protein degradation pathway, but again will not interact within an S-allele in a particular way [112].

Several studies have focused on mapping the position of the pollen S gene. Golz et al. (2001) determined the order of these marker genes for the S3-haplotype of *N. alata*, and placed the pollen S-gene between a marker (48A) and the S-RNase gene. Ushijima et al. (2001), in their study of a self-compatible cultivar of *Prunus dulcis* (Rosaceae), used a different approach to map the position of the pollen S-gene.

A study on this region via genomic the particular the southern part of subject of blotting prompted that 70 kb in the region quickly flanking the particular real S-RNase generally may seem to comprise sequences that demonstrate S-haplotype certain diversity. It will infer the particular plant pollen S-gene in this haplotype can be found by way of this kind of 70 kb place. It is actually established the real S-locus could be sub-contract centromeric interior Solanaceae with the knowledge that polymorphism of alleles generally appears to be improved with more than just one megabase in this relatives (see below). Certainly, polymorphism provides for just 70 kb through the*Prunus*, this resolves the dilemma about whether or not the real chromosomal site within the S-locus could be conserved among the Solanacaeae as well as the Rosaceae. Additional work would be likely essential to handle this kind of issue.

The latest focus of attempts to recognize the pollen S-gene that continues to be connected to the areas flanking S-RNase. Indeed, this repetitive nature of non-coding sequences flanking the real S-RNase gene, has, up to now, dissuaded attempts at chromosome walking in this area and string data via genomic clones (cloned throughout lambda phage) has been available only for a couple of kb upon each side in the S-RNase gene [14].

The latest technological advances, nonetheless, with unique advancement of the Bacterial Artificial Chromosome (BAC) local library, have greatly increased the size limit of genomic clones, increasing the number of sequence information per clone and rendering that more chromosome walking can be done. BAC clones containing S-RNase genes are actually identified throughout *Petunia inflata* [86-87] and *Antirrhinum hispanicum* [65]. BAC replicated from *Antirrhinum* encodes any 63.7 kb region in the S2-locus comprising the S-RNase and it has been sequenced [65]. Six putative body's genes were recognized whose deduced amino p sequences demonstrate homology together with known meats and of the four encode retrotransposons. The most significant finding has been a gene, known as SLF (S-locus F-box), encoding a great F-box comprising protein found about 9 kb downstream from S-RNase gene which can be expressed in the pollen as well as the tapetum [65]. Sequencing contig of 3 BAC clones, which signify a 328 kb region in the *P. inflata*S2-locus comprising the S-RNase, has shown a comparable abundance of retrotransposons and, interestingly, also has a gene a lot like SLF. SLF gene in *P. inflata* exhibits ~90% identity (at the real amino-acid level) concerning three haplotypes in support of ~30% personality to *Antirrhinum* SLF-S2. With both kinds, SLF genes look like the nearest pollen expressed gene for the S-RNase. Therefore, these represent very good candidates for the pollen S-gene. F-box comprising proteins are generally compo-

nents of ubiquitin–ligase processes, which, along with ubiquitin- initiating enzymes and ubiquitin-conjugating enzymes, mediate protein degradation with the 26S proteosome. Experiments are underway, making use of transgenic plants to discover if SLF is definitely the pollen S-gene.

Predicted amino acid sequences frompollen SFB and SLF from all functional SI S haplotypes conserve the F-box motif at the Nterminal, indicating that they function as F-box proteins. The F-box protein is known to be a component of a class of E3 ubiquitin ligases, the SCF complex, which regulates protein degradation in the ubiquitin/proteasome proteolytic pathway [19]. The F-box protein functions as a receptor to incorporate proteins targeted for polyubiquitination into the SCF complex. The polyubiquitinated targeted proteins are degraded by the 26S proteasome. It was, therefore, first proposed that the SCF complex that contains SFB (SCFSFB) and SLF (SCFSLF) might polyubiquitinate all nonself-SRNases for degradation but specifically interact with its cognate SRNase to leave it active, leading to the arrest of self-pollen tubes [56, 126-128].

Biochemical experimental results supporting this hypothesis have been obtained with Solanaceae and Plantaginaceae. Coimmunoprecipitation and yeast two hybrid analyses showed that *Antirrhinum* SLF (AhSLF) physically interacted with S-RNase in a nonallelic fashion and polyubiquitination of S-RNase was observed after incubating pollen proteins with compatible but not with incompatible stylar proteins [100]. Furthermore, AhSLF was shown to interact with ASK1 and CUL1-like proteins, suggesting that AhSLF makes an SCF complex. Later, AhSSK1 (*A. hispanicum* SLF-interacting SKP1-like1) that may work as an adaptor of the putative SCFSLF was cloned [42]. In contrast to AhSLF, *Petunia* SLF preferentially interacts using its nonself-S-RNase rather than its self-S-RNase [42]. This can be unexpected because the interactions of an allelic product in the pollen S using its self-S-RNase were long viewed as thermodynamically favored over the interactions using its nonself- S-RNase [56]. It would appear that *Petunia* SLF includes a domain that functions of S-RNase presenting (FD2) and two fields that control S-RNase-binding (FD1 and FD3) [41]. The FD1 and FD3 fields are shown to weaken the strong interaction involving FD2 and also S-RNase while in self-interactions, bringing about preferential connection and polyubiquitination of nonself-S-RNase.

The existence of different mechanisms of self-recognition in *Prunus* GSI can be suggested by means of molecular analyses of SC PPM S haplotypes as well as the SC/SI behavior in polyploids in *Prunus*. In contrast to the sign that the substrate of SCFSLF can be nonself-S-RNase in Solanaceae and Plantaginaceae, there are many indications that the substrate of SCFSFB might be a different molecule versus SRNase.

5. Genes that modulate the real SI reaction

Although the real S-locus encodes real determinants of S-haplotype specificity, there is evidence for the existence of other unlinked body's genes, termed modifier genes which can be required for SI result (reviewed in [16, 56, 81-84,], which is to be discussed in this article.

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6. The pistil modifier variables

6.1. HT-B and glycoprotein

HT-B protein, a smaller asparagine-rich protein expressed late in style development, was initially identified by differential cDNA hybridization to screen fashion genes which were expressed in self-incompatible kinds such as *N. alata* however, but not in *N. plumbaginifolia*, any closely related self-compatible kinds [84]. Homologs of HT-B have also been recognized in other genera in the Solanaceae, *Lycopersicon* and *Solanum* [61, 94]. Down-regulation of HT-B appearance by anti-sense shift and RNAi was starving the transformants of the chance to reject self-pollen, suggesting that it is essential for SI [85, 94]. Within a comparative research of self-incompatible and self-compatible taxa of *Lycopersicon*, the appearance of HT-B gene had not been detected in all the self-compatible taxa [61]. Given that no direct interaction of HT-B and S-RNases has been detected, the complete role of HT-B of SI result was not yet determined until a newly released immunolocalization research [25].

This sort of revealed that in self-pollen pipes, HT-B was more likely to help S-RNase move from a great endomembrane compartment for the cytoplasm, where they might exert cytotoxicity, bringing about the arrest of pollen tube growth, when it is in compatible pollen pipes; the HT-B amount was appreciably down-regulated as well as the S-RNases were compartmentalized [25].

The 120 kDa glycoprotein (120K) is an abundant protein inside the stylar ECM and taken up by the real growing pollen pipes [71]. This 120K protein binds to S-RNase in vitro and, like HT-B, reductions of its expression by means of RNAi stopped self-pollen rejection [16, 33]. With recent immunolocalization trials, antibodies for the 120 kDa glycoprotein were found to label the real compartment tissue layer that enters the S-RNases inside the pollen pipes. However, given that S-RNase uptake is usual in 120K protein defective plant life; its specific role in SI continues to evade researchers [25].

7. Pollen modifier variables

7.1. SSK1

F-box proteins often serve as adaptors that bind specific substrate proteins to the SCF (Skp1-Cullin-F-box) E3 ubiquitin ligase complex [99]. This raised the possibility of whether the SLF involved in the SI also participates in an SCF complex, mediating S-RNase ubiquitination. Identification of other components in such a putative complex is obviously necessary to address this question. SSK1 (SLF-interacting SKP1-like1), a homolog of SKP1, was originally isolated in *A. hispanicum* through a yeast two-hybrid screening against a pollen cDNA library using AhSLF-S2 as bait [42] (Fig. 4.).

Pull-down assays encouraged that AhSSK1 could be an adaptor that connects SLF to CUL1 protein. Therefore, it can be thought that SLF and SSK1 will tend to be recruited to some anonical complicated SCF, which could be responsible for S-RNase ubiquitination.

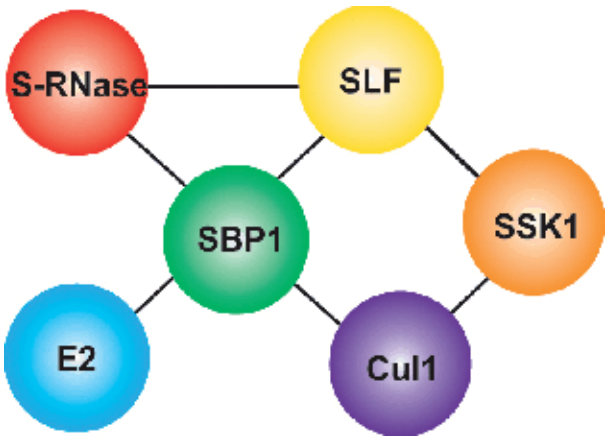

Figure 4. Protein interactions in gametophytic self-incompatibility [110]

7.2. SBP1

In trying to isolate the real pollen S, Sims and Ordanic (2001) screened a yeast twohybrid library from mature pollen of *P. hybrida* making use of *P. hybrida* S1-RNase because bait, and identified any gene known as PhSBP1 (S-RNase Presenting Protein1). Its homolog in *S. chacoense* was obtained depending on a comparable approach [95]. Nonetheless, the SBP1 gene displayed no haplotype polymorphismand was found to be expressed in almost all tissues. Additionally, it has been unlinked for the S-locus and so is unlikely to encode the real pollen S-determinant. Nonetheless, sequence research revealed that SBP1 has a RING-finger website, which can be characteristic of E3 ubiquitin ligases [60], indicating a possible role of SBP1 in S-RNase ubiquitination and degradation [95, 110-113]. Oddly enough, *P. inflata* SBP1 (PiSBP1) has recently been shown to interact together with PiSLFs, Pi CUL1 and an ubiquitin-conjugating enzyme, along with a novel E3 ligase complex continues to be suggested, with the possibility that PiSBP1 has a mixed role in SKP1 and RBX1 [111, 40].

8. S-like RNases in plants

Non-S RNases (syn. S-like RNases) in the T2/S-type are actually distinguished via different vegetable species. Non-S RNases are divided into two kinds, acidic and fundamental [127, 45]. A type of acidic non-SRNases is included in phosphate reuse as a result of phosphate confinement and tissue maturation [6, 121]. Other acidic non-S RNases can be up-controlled as a result of injury and vaccination together with pathogenic organisms. Albeit some essential non-S RNases are actually accounted of, for illustration, RNase Lc1 and Lc2 of *Luffa cylindrica*, RNase of *Momordica charantia* [46-48], and RNase X2 of *Petunia inflata* [68], their physiological capacities are not yet distinct.

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Members of the real of category to which S-RNases fit in, exemplified with the fungal RNase T2, are actually identified creatures as varied as worms, bacteria, fungus, slime molds, Drosophila, and oysters [32]. In addition, plants are actually found to obtain T2 category RNases that are not involved with SI. Completion in the Arabidopsis genome string has revealed five T2 group RNases in this particular self-compatible kinds (GenBank Accession Nos.: NP_178399; NP_030524; NP_178399; NP_563940; NM_101288). With plants, the similarity of the T2 RNases to S-RNases has generated those S-like RNases. Although S-like RNases are closely related to S-RNases, there are important differences in their design, expression, and function [120] and they do not take part in the control of SI.

S-like RNases are actually found in all the plants examined and constitute an essential family of RNA-degrading meats in plant life. In distinction to S-RNases, their expression is not restricted for the pistil— they are expressed in a number of plant parts and caused by several unique stimuli. There is experimental evidence that S-like RNases are involved in phosphate starvation, senescence, wounding, programmed cell death, defense against pathogens, and light signaling (for review, see [6].

A particular class of S-like RNase has recently been given and called relic S-RNases. These are generally S-like RNases which can be expressed throughout pistils but are not S-linked and so are presumed not to be involved with SI. Relic SRNases are actually identified throughout both SI *Petunia* [68] and *Antirrhinum* and also SC *Nicotiana* [26, 64]. It has already been proposed that these relic SRNases include arisen by using a duplication process that takes place where a fragment in the S-locus, containing the real S-RNase that continues to be integrated elsewhere inside the genome and that has evolved independently [26]. This is founded on the fact that sequences of relic S-RNases are extremely closely linked to the S-RNases from the genus where they may have been found, unlike S-like S-RNases. Therefore, it is significant to make a distinction between relic S-RNases and S-like RNases when contemplating the evolution of this group because it seems likely that they may have very unique evolutionary histories. Whether relic SRNases have a new purpose or signify a non-functional similar gene is not yet determined. Nonetheless, it is clear that a number of processes that S-like RNases are actually involved in, in unique defense and senescence, are of significant importance in pistil tissue.

9. Evolutionary elements

S-RNases are actually involved in gametophytic SI in 3 distinct groups of eudicots, the real Solanaceae [2]; Rosaceae [105]; and also Scrophulariaceae [139]. This addresses the issue of whether or not primarily S-RNase-based SI includes a single beginning or whether they have arisen independently on multiple occasions. The Solanaceae and Scrophulariaceae are part of the subclass *Asteridae* in contrast to the Rosaceae that is part of the subclass *Rosidae*. A single origin in the S-RNases in these 3 families would suggest that primarily S-RNase-based SI was within the popular ancestor of its subclasses, which collectively form ~75% off dicot individuals. In improvement, it would suggest that there was extensive lack of primarily S-RNase- based SI and some gains of other forms of SI in higher dicots [45]. Even

though informative, estimating evolutionary relationship among S-RNases can be challenging for many reasons: the genes are fairly short long (~220AA residues), time since divergence can be long ~110 Mbps involving the *Asteridae* and *Rosidae*) along with a strongly adverse frequency-dependent assortment is likely to have generated extensive string divergence after the system comes [45].

A number of studies have attempted to discuss evolutionary associations among S-RNases and related S-like RNases [45, 101, 104-107, 116, 125, 139]. Preliminary studies found that solanaceous, rosaceous and scrophulariaceous S-RNases just about every formed monophyletic clade [105, 139], pointing to the idea that SRNases share a typical ancestor. Later studies, nonetheless, found simply very vulnerable bootstrap support for nodes uniting S-RNases [101, 125-128], but simply included a finite number about S-like RNases (7 and 14, respectively), limiting the chance to distinguish the single and also multiple beginnings of SRNase mediated SI. The two most up-to-date studies have taken advantage of the significant amount of completely new sequence information now available together with different phylogenetic approaches than those used previously to check phylogenetic associations among S-RNase sequences.

Igic and Kohn (2001) carried out a maximum likelihood analysis using 67 S-RNase and S-RNase-like DNA sequences, Steinbachs and Holsinger (2002) executed a Bayesian research of 72 DNA sequences. Together with maximum chance analysis, Igic and Kohn also analyzed intron presence/absence and position in 29 S-RNase and also S-RNase including genes, and found a high degree of congruence regarding relationships deduced by intron/exon structure as well as the tree extracted by utmost likelihood research. While not really unequivocal, the finest interpretation in the trees generated in similar studies is that S-RNases indeed carry out a kind ofmonophyletic clade (with the actual addition of one or two S-like RNases). Thus, primarily S-RNase-based GSI generally seems to have arisen from a popular ancestor, which covers three-quarters of dicot individuals, indicating that primarily S-RNase-based GSI has been the ancestors stated in nearly all dicots [45, 115].

10. Completely new perspectives

To address a number of unresolved difficulties surrounding the actual evolution of S-RNase-based SI systems, we analyzed the actual S-RNase information in two ways: (1) we explored the actual variation throughout S-RNase gene DNA and also amino p sequences, particularly in relation to structural and functional motifs described previously. (2) We also S-RNase string data of potential patterns of assortment and/or recombination. Previous works [44, 123] include suggested specific types of the structure in the S-RNase genes that we tested employing a broader phylogenetic test than previously used, as well as exploring the variation in gene sequence in a phylogenetic context. Some scientific tests have encouraged that recombination may play a role in the real diversification of alleles in S-RNase gene family, at very least within populations [134]. We explored an opportunity of assortment and/or recombination happening across many major S-RNases and S-like RNases. The gene structure of S-RNases has been

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previously described as including five conserved domains (C1–C5), two hypervariable regions (Hva and HVb), and several highly conserved amino acid residues including five cysteine residues, one leucine residue, and two tryptophan residues outside of the conserved domains and two histidine residues (one in C2 and one in C3) that are considered to be catalytic residues for RNase function [44, 123]. We first explored the real variation of proteins and nucleotides in S-RNase sequences employing a previously released dataset [116] by means of counting the amount of inferred changes on the phylogenetic tree using MacClade 4.03 [75]. During these evaluations, it has been noted the published alignment failed to align every one of the conserved fields previously identified [123]. The patterns seen together with nucleotide and amino p sequences suggested the same patterns; consequently, amino p variability was also evaluated.

Roalson et al. (2003) in this respect analyzed amino variety in SRNase position for 3 clades: (1) the whole S- and S-like RNases clade, (2) the real clade of Solanaceae and Scrophulariaceae S-RNases, and (3) the genuine clade of S-like II RNases. Comparative examples were essentially in different clades on the grounds that are alluded to here of these 3 clades. The examples of deviation for a large portion of these 3 clades are than the speculated monitored fields, hypervariable territories, and moderated amino p deposits.Amino p variety in the Solanaceae/Scrophulariaceae clade is for the most part than the recommended basic components proposed by Ida et al. (2001). Altogether, there was substantially more amino p substitution surmised, even at hypothetically saved locales, than keeps on being beforehand recognized. At the point when numerous S- and S-like RNases are considered, in any event various amino acids in every one of the saved spaces may be variable, with upwards of 19 substitutions found at a few locales. In change, amino p positions outside the "hypervariable" zones show comparative degrees of amino p change inside a phylogenetic wording as is found inside HVa and HVb. Preserved amino p deposits (cysteine, leucine, tryptophan, and histidine) are several slightest variable amino p buildups, in spite of the fact that other amino p deposits were discovered which can be correspondingly saved. For example, the glycine deposit in C2 near the preserved histidine buildup, a tryptophan deposit in the middle of HVb and C3, the genuine lysine and glycine buildups all through C3 that encompass the genuine saved histidine deposit, and any tyrosine/phenylalanine set about halfway about C3 and C4. A couple of different positions appear to have minor variety, yet this is a result that does not have (crevice all through amino p arrangement) the amino p positions in numerous groupings. At the point when just probably utilitarian S-RNases are thought, for example, the S-RNases through Solanaceae and Scrophulariaceae, a comparable example of amino p variety is found, despite the fact that the aggregate number of changes at locales is lower. As a comparative example of variety is found in utilitarian S-RNases as is discovered when numerous S- furthermore S-like RNases are thought, it is not clear in the occasion the lower level of variety at positions is generally a capacity in the higher protection inside useful duplicates and additionally lower testing (26 versus 72 amino p successions). Since the discovery when numerous groupings were viewed, there is a huge level of variety inside all the moderated fields and there are heaps of amino acids outside the hypervariable fields that show comparative degrees of variety as is found in HVa also in HVb. As has been discovered, extreme S- and S-like RNases and the Solanaceae/Scrophulariaceae S-RNases, huge degrees of a variety were discovered over the whole quality

if S-like RNase clade II alone is viewed as (Fig. 5). Just 16 groupings in this specific clade can be discovered, which can be reflected inside aggregate quantities of derived adjust at every amino p deposit, however, the genuine example of amino p substitutions over the quality takes after that inside alternate examinations. Also, most in the saved amino p buildups are correspondingly rationed inside S-like RNase II clade as theS-RNase clades (Fig. 5).

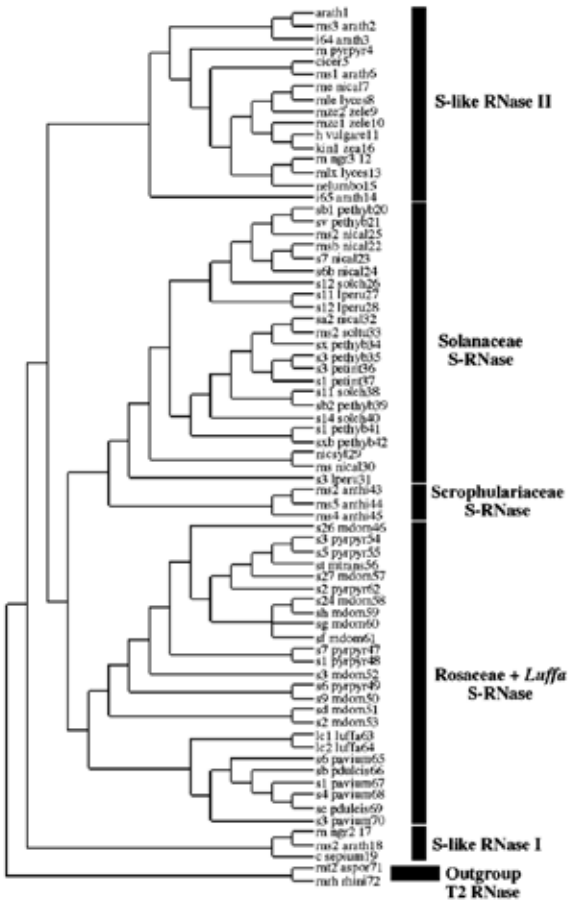

Figure 5. Phylogenetic hypothesis of relationships among S- and S-like RNase gene sequences with major clades labeled according to the plant family of origin and their inferred function (S- or S-like). Modified from Steinbachs and Holsinger (2002); Bayesian analysis of DNA sequence data [with permission from ref. 102].

In the analysis of S-RNase sequences via *P. inflata*, Wang et al. (2001) supplied evidence pertaining to recombination from the S-RNase gene. It has been determined that at the very least, now and again, homology between S-RNases varied from one end of the gene to the other, as an example, the 50 stop of S19-RNase has been closely linked to S2-RNase and 30 stop was closely linked to S8-RNase. Hence, while youwill find conceptual issues in accepting that recombination cannot only happen from the S-locus; however, within S-RNases themselves,

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it is undoubtedly a probability that uncommon recombination functions have played a role in the actual evolution of the genes.

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