Chapter 3

Functional Fruits Through Metabolic Engineering

Luis Quiroz-Iturra, Carolina Rosas-Saavedra and Claudia Stange Klein

Additional information is available at the end of the chapter

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Abstract

Metabolic engineering is a main focus of many plant biotechnology programs that look for the production of novel plant varieties with improved human health benefits. Among the most interesting goals are those that are focused in the production of functional fruits. A fruit can be considered as functional if it produces additional benefits to human health and well-being, beyond nutrition. Fruits that present higher levels of beneficial compounds such as essential vitamins, antioxidants, and phytochemicals can be considered as functional as those compounds have long-term benefits in reducing the occurrence of certain diseases. Through the expression, silencing, or mutagenesis strategies, many functional fruit crops have been produced during the last 40 years. Novel plants produce higher amount of carotenoids, antocyanins, and folic acid in their fruits, as well as higher color, sweetness, flavor, and aroma. The improvement of postharvest and resistance to biotic and abiotic stress in commercial plants has been also enhanced as it can led to a better fruit production. Taken together, this chapter will present a revision of the main fruits that have been improved by means of metabolic engineering within the framework of functional foods and super foods.

Keywords: biotechnology, functional food, fruits, metabolic engineering

1. Introduction

Today, the quality of life is becoming one of the pivotal reasons for people to be concerned about its health. Also, the steady increase in life expectancy accompanied by the growing cost of health care and the increasing rate of metabolic disorders (heart disease, obesity, diabetes, and arthritis) are the factors to consider in terms of life quality. On the other hand, vast scientific evidence determines a pivotal link between diet and human health, showing the crucial

role of nutrition in the prevention of chronic diseases, such as coronary heart diseases, cancer, neurodegenerative, and respiratory disease, along with aging. Therefore, this scenario had led to the development of functional foods as a recognized category of foods to be part of an international strategy to overcome diseases related to human diet and life style.

The first conceptual approach developed by the European Commission Concerted Action on Functional Food Science in Europe (FUFOSE), coordinated by the International Life Sciences Institute (ILSI) Europe established that "A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules but part of a normal food pattern" [1, 2]. Today, an universal accepted definition for the term functional food is not accorded yet [1, 3]. In fact, this concept has been defined several times [4], although in most countries there is not a legislative definition [3].

From a practical point of view, a whole fruit that contains sufficient quantities of beneficial components represents the simplest example of functional food and therefore may be considered as functional fruit. The main characteristic of functional fruits is their high content of bioactive compounds, which promote a state of health and well-being and/or reduce the risk of some diseases and may even be used to cure some illnesses. However, there is a slight difference between conventional and functional fruits, even for experts such as nutritionists, because many if not most fruits contain natural components that provide benefits beyond basic nutrition, such as lycopene in tomatoes [5] or anthocyanins in pomegranate [6]. Moreover, not all fruits can be considered as functional because the health benefits strongly depend on the absorption and transformation of nutrients (bioaccesibility) during gastrointestinal digestion [7]. The bioaccesibility of these compounds permits to have a clear idea of their potential bioavailability, term that involves the biological activity of these compounds.

To meet future demand for functional foods, the food industry must address critical challenges such as developing strategies to increase the yield of healthy compounds in fruits or to add nutritional value with new components to improve the quality standards aiming to maintain the well-being. The beneficial components of functional fruits can be enhanced through special growing conditions [8–10], through breeding techniques [11] or through metabolic engineering for delivering truly unique health benefits [12]. In this context, metabolic engineering plays a key role in developing functional fruits, in terms of being defined as "the direct improvement of production, formation, or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology" [13, 14]. In plants, metabolic engineering can be used to develop functional foods and super foods by handling the flow of primary and secondary metabolic pathways, allowing the redirection of carbon flow toward products of interest or the synthesis of new products.

Nowadays, metabolic engineering has been used to produce new plant varieties with higher levels of valuable compounds such as pro-vitamin A, antocyanins, folic acid, antioxidants, as well as higher color, sweetness, flavor, and aroma. Many novel functional fruit crops have been

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generated during the last 40 years by using molecular strategies such as the over-expression, silencing, or mutagenesis of specific genes. The improvement of postharvest and resistance to biotic and abiotic stress in commercial plants has been also enhanced as it can lead to a better fruit production.

Taken together, in the following sections, we will review the progress in biotechnological approaches in developing functional fruits by describing strategies employed in metabolic engineering, and the characteristics that have been improved in several agronomic traits to insert novel functional fruits into the market.

2. Metabolic engineering strategies

In plants, metabolic engineering has been developed through different approaches. Within them, the most widely used are the gain-of-function by transgenesis (including cisgenia and intragenia) and the loss-of-function carried out through gene silencing and mutagenesis. These strategies are achieved by *Agrobacterium tumefaciens* or biobalistics transformation systems.

2.1. Production of transgenic plants that express a functional gene

Transgenesis is the process by which one (or more) exogenous gene (called transgene) is inserted into a living organism, giving them a new feature that has to be stable over next generations. Cisgenia and intragenia terms are used when the exogenous genes belong to the same or related plants, respectively [15]. The functionality of the transgene in the new plant is accomplished due to the fact that the genetic code of all living organisms is exactly the same. This means that a specific DNA sequence may encode the same protein in any living organism. Transgenesis itself is a process that occurs in nature without human intervention. The better example is taken from plants that are infected by the bacteria *A. tumefaciens*. This bacteria produces the disease known as "grown call" in which *Agrobacterium* induces the formation of a tumor in the stem of more than 140 species of Eudicotyledoneae. The symptoms are caused by inserting a DNA segment in a semirandom manner in the plant genome. This DNA segment (known as T-DNA, 'transfer DNA') codifies for plant hormones that induce the generation of tumors when produced together in high levels in plant cells [16]. This feature has allowed scientists to use a modified strain of *A. tumefaciens* for inserting genes of interest instead of the phytohormone-inducing-tumor genes [17]. For instances, several commercial transgenic plant varieties produced through Agrobacterium transformation are cultivated and consumed in around 25 countries in the world. Most of them are transgenic varieties of maize, soybean, cotton, and canola that are tolerant to herbicides and resistant to insects, among others. Actually, fruits have also been improved through this technique and will be described later.

2.2. Gene silencing of specific genes in plants

In the case of plant gene silencing, also termed RNA interference (RNAi) or post-transcriptional gene silencing (PTGS), a small fragment of 100–400 pb of the gene in antisense orientation is

introduced into the plant, causing the degradation of the target RNA, diminishing thus the amount of mRNA and of the protein from 40 to 99%. The process for gene silencing has been described extensively and is stated through the degradation of a double-stranded RNA molecule (dsRNA) in the cell coming from the hybridization of the antisense RNA and the endogenous sense RNA of the target gene, which triggers the RNAi pathway [18, 19]. The fragments generated include small interfering RNAs (siRNA) of about 21–23 nucleotides in length that through the host RNA-induced silencing complex (RISC) allows the systemic degradation of the target mRNA. It is believed that siRNA system has evolved as a cellular defense mechanism against RNA viruses or to combat the proliferation of transposons within the cell [19]. Currently, siRNAs are now widely used to suppress the expression of specific genes and to assess gene function. Gene silencing is considered a mechanism of gene knockdown, where the expression of a gene is reduced at least 99% but not completely [20].

2.3. Plant mutagenesis

Several standardized procedures that induce mutations have been used for the production of new crops varieties of commercial interest [21]. This technique uses physical or chemical mutagenic agents. The chemical procedure, by using EMS (ethyl-methane sulfonate), is simpler to achieve and has demonstrated to be one of the most reliable inducers of mutagenesis [21]. EMS tends to generate random changes in nucleotides, generating single-nucleotide polymorphisms (SNPs) or deletions (indel) that affect the functionality of some gene(s) in the genome [22]. This is translated in the modification of phenotypes and/or physiological characters. Mutations are inherit events in any alive species and naturally and randomly happen around 50,000 times per year which genetically change from normal cells to mutated cells every day [23]. Many of those alterations are repaired, but when not, a mutation persists being a key piece in the evolution of the species. Therefore, chemical mutagenesis has been used for more than 60 years in breeding programs in the world. Plants generated by mutagenesis do not require long evaluation processes as transgenic or silenced plants and are accepted and introduced to the market more efficiently. Approximately 2965 induced mutagenesis cultivars such as crops that include wheat, barley, and rice, and among others have been generated and released during the last 40 years. Novel varieties of fruits, which include Kiwifruits, produced through EMS mutagenesis, have also been approved for commercialization [21–24].

One of the most innovative system, which has gained great impact few years ago in the field of metabolic engineering, is the genome editing system CRISPR/Cas. This is a versatile and effective tool for editing genomes in a site-specific manner [25, 26]. The CRISPR/Cas system is a natural defense mechanism in eubacteria and Aequeas against plasmids and viruses [27, 28]. For metabolic engineering, a chimeric guide RNA (gRNA) that contains 20 nucleotides must specifically bind to their target sequence in the DNA. The target sequence must also contain the protospacer adjacent motif (PAM) sequence that is recognized by Cas9, cutting 3–4 nucleotides upstream of the PAM sequence [25]. The most common editing events are small Indels (insertion or deletion) of 1–10 nt [29]. The CRISPR/Cas9 system has been effectively used as a tool for editing the genome of numerous plants including *Arabidopsis thaliana, Nicotiana tabacum, Oryza sativa, Zea mays, Glycine max, Triticum aestivum,* and citrus [29].

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3. Metabolic engineering for fruit-trees improvement

There are several strategies for metabolic engineering of plants. Nevertheless, the nutritive fruits are normally produced from trees or from "recalcitrant" plants that are difficult to regenerate *in vitro* and to transform [30]. Usually, the success of genetic transformation depends on the success of the regeneration process for each plant species. This is influenced by several factors, such as the genotype of the variety, the source of explant, and the degree of tissue differentiation. Therefore, the tissue culture conditions must be optimized for each range of each crop independently [31]. Generally, two methods of tissue culture have been used for regeneration of transgenic plants: organogenesis and somatic embryogenesis. Organogenesis is the process in which the regeneration of a new seedling occurs directly from the explants while in somatic embryogenesis, the formation of embryos from somatic cells that are present in the explant tissue is produced [32]. Somatic embryogenesis has many advantages over organogenesis, including its potentially high rates of multiplication, the genetic uniformity among embryo clones, the potential for expansion in bioreactors, and cryopreserved through synthetic seeds [33]. Despite the above, somatic embryogenesis has not been standardized for most fruit species, and therefore, somatic organogenesis is normally carried out for transgenic plant regeneration.

3.1. Disease control in plants to improve fruit crops

Owing to the economic importance that represents fruit production in various countries, there have been many efforts to generate plants with increased resistance against diseases and pathogens, whether fungi, bacteria, or viruses. Today, only virus resistance is used commercially, which only represents a small proportion of all transgenic plants grown in the world. Most of the plant-infecting viruses are of single-stranded RNA+ type, which codify for a capsid protein, movement protein, and replicases, among others. Within the first cases of success in the generation of virus-resistant plants through metabolic engineering, there are those using viral capsid protein (CP) as the transgene [34]. In this case, plants expressing the CP gene confer viral resistance mediated by RNAi gene silencing when plants are infected by the specific virus.

In 2004, the first example of a genetically engineered horticultural crop that has made it to market was produced in Hawaii. The genetically engineered Rainbow and SunUp Papaya, which are resistant to the papaya ringspot virus (PRSV) were successfully commercialized and adopted by farmers in the Puna area of Hawaii [35]. In addition, after 20 years of testing and risk assessment in the laboratory, in greenhouse and field, plum 'HoneySweet' is now used as a GM crop resistant to Sharka disease caused by the plum pox virus (PPV), which has been validated for US cultivation [36]. PPV protection is based on RNAi, and resistance has been shown to be highly effective, stable, durable, and heritable as a dominant trait. Extensive testing has also demonstrated on the safety and the ability of the RNAi technology for fruit production [36]. Chandrasekaran et al. [37] developed a virus-resistant variety of cucumber (*Cucumis sativus L.*) by CRISPR/Cas9 technology. The Ipomovirus infects cucumber and produces a vein yellowing of the leaves. During the infection, the virus requires the plant eIF4E

gene (eukaryotic initiation factor of translation 4E) to carry out the recognition and transcription of their genes [38]. With the CRISPR/Cas9 tool, mutations in the eIF4E gene were introduced in cucumber, and the transgenic plants present small deletions or single-nucleotide polymorphisms (SNPs) in the mutated region of eIF4E. By the culture of the plant to next generations, homozygous mutant progeny was selected. The obtained one showed immunity to the Ipomovirus Cucumber vein yellowing infection and increased resistance to Zucchini yellow mosaic virus and papaya ring spot mosaic virus-W. A system for cucumber virus resistance was generated for the first time, without the need to produce a transgenic organism [37].

Regarding the generation of plants resistant to fungal and bacterial diseases, the main strategies are the expression of resistant genes coding for resistance receptors (R) and those involved in the defense mechanisms such as pathogenic-related proteins (PR proteins), peptides, and antimicrobial metabolites, and genes involved in detoxification mechanisms [39]. The plant defense response is triggered by the recognition of pathogen avirulence factors (avr) by the resistance receptors (R) equipped in the host plant [40]. The avr and R interaction activates one or more signal transduction pathways and eventually triggers a local response termed hypersensitive response (HR) and a systemic acquired resistance (SAR). Both of them induced by the signal-molecule salicylic acid (SA) which permits the accumulation of PR proteins through the activation of a signal transduction machinery [41]. Arabidopsis NPR1 gene (PR gene nonexpresser) is well recognized as a key regulator of signal transduction by SA leading to SAR. The NPR1 overexpression in citrus showed a positive response to the increased citrus canker resistance, caused by the bacterial pathogen *Xanthomonas citri* subsp. *Citri* [42]. In apple (*Malus domestica*), transgenic plants overexpressing the MdNPR1 gene exhibited increased resistance to two important fungal pathogens of apple, *Venturia inaequalis* and *Gymnosporangium juniperi-virginianae* [43]. The expression of the R gene was achieved in apples to also overcome the infection by the fungus *V. inaequalis*, which causes Scabies disease which is one of the most serious diseases that hinder the apple crop production. The gene that confers resistance to this disease is termed Rvi6/Vf scab and is present originally in the *Malus floribunda* 821 wild species. This gene has been incorporated in different commercial apple cultivars by classical breeding. However, as *M. floribunda* 821 has not an edible and attractive fruit, the new breeded species that have the resistance gene Rvi6/Vf produce low-quality fruits that do not reach the market [44]. In order to obtain high-resistant species, the Rvi6 gene (formerly HcrVf2) was inserted in susceptible apple cultivar 'Gala', thereby obtaining a commercially attractive variety that is resistant to Scab disease [45]. Unlike other crops, this variety is considered a cisgenic line, as the Rvi6 gene was taken from another variety of apple.

The hydrolytic enzymes chitinase and glucanase, the best characterized class of PR proteins, are able to degrade the cell wall of pathogenic fungi invaders. PR proteins are important components of the response of plant defense against fungal and bacterial pathogens [46, 47]. Transgenic plants expressing genes encoding for chitinase and glucanase showed increased resistance to fungal diseases in many fruit plants [34]. Furthermore, the use of antimicrobial peptides expressed constitutively in plant tissues has been recommended for genetic engineering of plants to increase disease resistance against fungal and bacterial pathogens [39].

Defensins, one of the classic examples of small antimicrobial peptides, play an important role in the response of plant defense against fungi. Defensins produce the permeabilization of the membrane inhibiting the growth of the fungi through the interaction with membrane components of the fungus [48]. Two defensins genes derived from petunia (PhDef1 and PhDef2) were expressed in banana. *In vitro* and *ex vivo* assays clearly suggested that transgenic banana plants were resistant against the pathogenic fungus *Fusarium oxysporum* sp. cubense [49]. Moreover, antimicrobial proteins from other organisms, such as insects or animals, have been used to increase the resistance to pathogens. Some of these nonplant antimicrobial proteins, such as attacin or cecropin from *Hyalophora cecropia* and magainin from *Xenopus laevis*, showed antimicrobial activity and increased resistance to pathogens in transgenic fruit, such as apple, papaya, pear, potato, sugarcane, and grape [39, 50]. Another alternative is to enhance the production of phytoalexins, antimicrobial metabolites that contribute significantly to the resistance against pathogens [51]. Even so, the production of antimicrobial metabolites generally requires coordinated action of a number of biosynthetic enzymes, which means that many genes are required. This feature makes very difficult to increment antimicrobial metabolites to generate plant resistance varieties [39].

Usually, necrotrophic pathogens produce toxins and enzymes that degrade the cell wall to invade the plant cell for a successful infection. Detoxification and degradation of these phytotoxins by the generation of transgenic plants could provide an opportunity to improve resistance to several diseases [52]. However, the strategy to develop disease-resistant plants is not accessible because some phytotoxins are harmful to mammals, and the product of a detoxification reaction could remain toxic in the plant [39].

3.2. Abiotic stress tolerance in fruit crops

Resistance to abiotic stresses is a challenging goal to develop biotechnological fruit cultivars and varieties because many plants face rough conditions of drought, salinity, cold, and heat, among others. These environmental factors are significant plant stressors, and their effects on plant development and productivity are reflected in serious agricultural yield losses [11].

Survival of plants under adverse environmental conditions is realized by structural and metabolic changes into endogenous developmental programs. Therefore, methods for agronomic processes and crop improvement are required to enhance these adaptive responses. Efforts have been made to introduce traits with improved drought tolerance, but in many cases, the strategies involved the insertion of a wide range of genes into plants [53].

Drought, salinity, extreme temperatures, and oxidative stress are interconnected environmental stresses [54] that often activate similar cell signaling pathways [55, 56] and cellular responses, such as the accumulation of compatible solutes, production of stress proteins, and the up-regulation of anti-oxidants [57, 58]. Therefore, plant modification for abiotic-enhanced tolerance is mostly based on the manipulation of one or several genes that are either involved in signaling and regulatory pathways [59, 60] or that encode enzymes present in pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants [61, 62] or that encode stress-tolerance-conferring proteins [63]. For example, the

overexpression of SK3-type DHN gene (ShDHN) in transgenic tomato, which codes for a type of dehydrin (DHN), increased tolerance to drought and cold stresses and improved seedling growth under salt and osmotic stresses [63]. DHN is also known as Group 2 LEA (late embryogenesis abundant) proteins [64], and the overexpression of ShDHN in tomato accumulated more proline, maintained higher enzymatic activities of superoxide dismutase and catalase, and suffered less membrane damage under cold and drought stresses.

Another interesting example in abiotic tolerance is the transformation effect of an important rootstock for lemon, *Citrus macrophylla* W. that constitutively expresses the CBF3/DREB1A transcription factor from *Arabidopsis*. CBF3/DREB1A is a member of transcription factors induced on abiotic stress conditions [65]. Transgenic lemon lines showed normal development and, under salt stress, showed greater growth, better stomatal conductance, and similar accumulation of chloride and sodium in the leaves, in comparison with wild-type plants [66].

The adaptation to stress often affects metabolic and energy requirements that sometimes result in deleterious collateral effects such as yield penalty, which mask and limit its benefit to agriculture. In consequence, some authors have succeeded to enhance the abiotic stress tolerance of agricultural species by combining traditional and molecular breeding [67, 68] with the transformation of specific genes [54] such as those reported in this section. Therefore, these strategies have been applied to other species such as soybean, corn, cotton, and canola, which are currently on the market, although additional research is still necessary to evaluate stress resistance of fruit trees varieties in field trials under real stress conditions [68]. Even more, the problems of high costs on the development and releasing processes and safety requirements in regulatory demands must be solved in this kind of fruit crops.

4. Metabolic engineering for functional fruits development

4.1. Nutritional improvement in fruits

Fruits and their processed derivatives are important nutritional sources, not only for carbohydrates, but also for a wide range of secondary metabolites. That are beneficial to human health. Most important metabolites are carotenoids, flavonoids, and anthocyanins, which are widely known to be powerful antioxidants and anticancer agents. Therefore, since many years ago, a great effort has been done to increase the content of these metabolites in plants to improve the nutritional value of fruits.

4.2. Functional fruits with improved carotenoid content

Carotenoids are the second most abundant pigments found in nature, with more than 750 structurally different compounds responsible for yellow, orange, and red colors [69]. Carotenoids are metabolites synthesized in plants, algae, fungi, and yeasts, and some bacteria where they have photosynthetic, antioxidants, and/or photoprotectant functions [70]. In vertebrates, carotenoids are precursors of vitamin A, and they are also involved in the

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formation and maintenance of bones and retina. Mammals such as human are not able to synthesize vitamin A, and therefore, they have to include carotenoids in the diet [71]. From a pharmaceutical point of view, these pigments are used as nutritional supplements and antioxidants, highlighting by their protection against UV damage, anticarcinogenic properties, prevention of cardiovascular diseases, cataracts, and macular degeneration [70–73]. Owing to all these important features for human health, the improvement of enhancing carotenoids content in plants and fruits has been carried out since many years, and the production of new varieties of fruits with enhanced amount of carotenoids has been succeeded a few years ago.

The most recognized fruit produced by metabolic engineering is the "super banana," which is part of an Australian project [74]. The aim of this project is to increase the levels of pro-vitamin A in the pulp of commercial banana using metabolic engineering. The overexpression of Psy gene (Apsy2a) from wild banana Asupina, which accumulates carotenoid in the pulp, showed the highest increase in β-carotene levels in the transgenic super banana variety (**Figure 1A**) [75]. Psy gene encodes for the phytoene synthase (PSY), which is the first and the key step in the biosynthetic pathway of carotenoids. Currently, the super banana is in the human-feeding trial stage in the United States. The aim is to transfer the technology to East Africa, where bananas are one of the major basic foods and the levels of vitamin A deficiency are high [76]. Another fruit, which has been modified to improve carotenoid concentration by metabolic engineering, is the kiwifruit (*Actinidia deliciosa*). Transgenic kiwi lines with improved carotenoid content were generated by overexpression of geranylgeranyl diphosphate synthase gene (GGPS), which is part of the metabolic precursors route of carotenoids and Psy of *Citrus unshiu* [77]. Pons et al. showed that it is possible to increase the content of β-carotene in orange fruit (*Citrus sinensis*) through gene silencing of β-carotene hydroxylase gene (CsB-CHX) [78]. This gene is involved in the conversion of β-carotene into xanthophylls. Transgenic fruits showed a dark yellow (golden) phenotype (**Figure 1C**). The levels of β-carotene in transgenic fruit pulp were 36 times higher. Besides, *in vivo* studies performed in *Caenorhabditis elegans* suggested that antioxidant effect in golden fruit was 20% higher than that in conventional fruits [78]. Overexpression of key genes for enhancement of β-carotene synthesis has been extended to other fruits such as tomato (*Solanum lycopersicum*) [79, 80] or the Hong Kong kumquat (*Fortunella hindsii*) [81].

4.3. Functional fruits with improved antocyanin content

Anthocyanins are one of the most important water-soluble plant pigments. They are synthesized by the flavonoid branch in the phenylpropanoid pathway. In plants, anthocyanins are secondary metabolites involved in multiple processes, such as attracting pollinators, protection against damage from UV light, seed dispersal, and pathogen attack [84]. For humans, anthocyanins have taken great importance due to its antioxidant and anti-inflammatory effects [85]. Tomato, which is a red fruit, owes its color to the accumulation of carotenoids in the pulp and peel (mainly lycopene) [86]. However, the content of anthocyanins in tomato is very low. Metabolic engineering has been used to enrich the anthocyanin content in tomatoes. For instance, the expression of Delila (DEL) and Rosea1 (ROS1) transcription factors, from

Antirrhinum majus, in transgenic tomato allowed the increase of anthocyanin content in the fruit [83]. It was observed that DEL and ROS1 transcription factors were able to activate multiple genes related to anthocyanin biosynthesis, such as phenylalanine ammonia lyase (PAL) and flavonoid 3′5′ hydroxylase (F3′5′H). Thus, transgenic tomatoes exhibit intense purple coloration in the pulp and fruit peel as shown in **Figure 1E**, because they contain high concentration of anthocyanin [86]. Several studies on the regulation of the biosynthetic pathway of anthocyanins showed that the family of MYB transcription factors regulates the expression of genes involved in this pathway [87]. Espley et al. reported that greater accumulation of anthocyanins in apple fruits (**Figure 1B**) was obtained by transforming with the transcription factor *MdMYB10* [82]. Moreover, the expression of *FaMYB10* in strawberry generated an increase in anthocyanins in the root, leaf, and strawberry fruit [88].

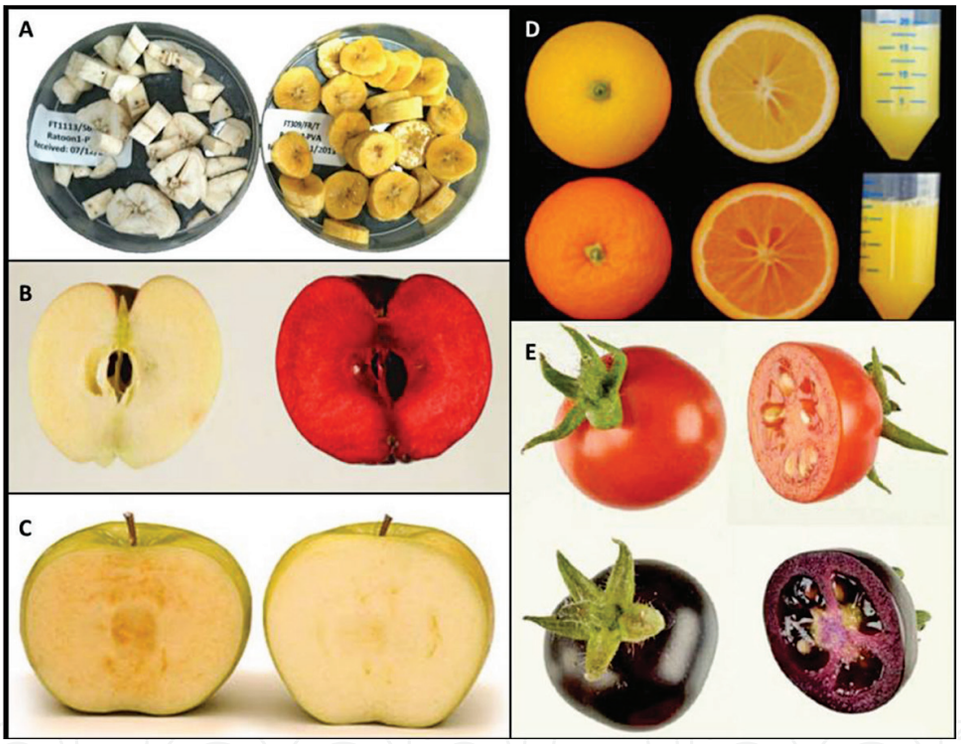

Figure 1. Examples of transgenic fruits. (A) Fruits of wild banana (*left*) and transgenic golden banana fruits with higher levels of β-carotene (*right*) [75]. (B) Wild apple fruit (*left*) and transgenic fruit that accumulate higher levels of anthocyanins in the pulp (*right*) [82]. (C) Wild apple fruit which presents pulp oxidation (*left*) and arctic apple which has a higher resistance to oxidation of the pulp (*right*). (D) Wild orange fruit (*bottom*) and golden orange fruits with higher levels of β-carotene [78]. (E) Wild tomato fruits (*top*) and transgenic tomato fruits that accumulate higher levels of anthocyanins in the pulp [83].

4.4. Functional fruits with improved folic acid content

Folic acid, also known as vitamin B9, is part of the water-soluble vitamin B complex, which is necessary for the formation of structural proteins and hemoglobin [89]. Folate deficiency is considered as a worldwide problem because it is mainly caused by poor nutrition in poor

countries. Folate deficiency during pregnancy causes premature births and babies with low weight and possible defects in neural tube development. In adults, the clearest sign of folate deficiency is anemia while children can also slow growth [89]. Folates are synthesized from pteridine, p-aminobenzoate (PABA) and glutamate precursor [90]. Diaz de la Garza et al. [90] developed transgenic tomatoes, which overexpressed the genes involved in the first steps in the biosynthesis of pteridine and PABA proteins: GTP cyclohydrolase I and amynodeoxychorismate synthase, respectively, in a fruit-specific manner [90]. The amount of folic acid contained in ripe tomato fruits was 25 times higher than in nontransgenic one, and the amount responds to the daily requirement for adult consumers with less than a standard serving.

5. Functional fruits as oral vaccines

Metabolic engineering has been used not only to increase its content of healthy secondary metabolites but also for generating edible oral vaccines. The strategy of oral vaccines offers several advantages over the classical system of injection, as oral vaccine is a cheaper strategy to produce immunization and is a more practical system to be implemented in universal vaccination programs [91]. An example is the strategy to immunize against the enterovirus 71 (EV71), which is known to cause seasonal epidemics of hand, foot, and mouth and can reach fatal neurological complications in young children. Tomato plants expressing the VP1 epitope and the coat protein of the enterovirus 71 (EV71) were produced to induce immunization when eaten [92]. Tomato fruit–expressing VP1 protein was firstly tested in mice, which presented an increment of specific IgA and IgG immunoglobulins against VP1. Besides, the serum from mice fed with transgenic tomato was able to neutralize EV71 infection in rhabdomyosarcoma cell culture and the proliferation of spleen cells in orally immunized mice was also enhanced by VP1 tomatoes, which activated both humoral and cellular immunity. The results of this study not only demonstrated the feasibility of using transgenic tomato as an oral vaccine to generate protective immunity against EV71 in mice but also the likelihood of vaccine development against other kinds of enterovirus [92].

Another example of oral vaccine aims to immunize against the enterobacteria *Yersinia pestis*, which is a gram-negative bacilli, anaerobic facultative and pathogenic to humans that causes pneumonic and bubonic pest. This pathogen affects mainly people in Africa, Asia, and Latin America. Owing to the increasing reports of the emergence of antibiotic-resistant *Y. pestis* strains, the need for a safer and cheaper vaccination system increases. Among all *Y. pestis* antigens, only the F1 and V antigens have generated immunogenicity in conventional vaccines. Alvarez et al. reported the expression of a fusion protein F1-V in tomato plants. The immunogenicity of transgenic tomatoes F1-V was tested in mice, and the immune response of mice vaccinated with antigens of bacterial origin (conventional system) and oral transgenic vaccine was compared. The results showed a similar level of immunization with both strategies [93].

6. Enrichment of organoleptic properties in fruits

Despite the best efforts in metabolic engineering of fruits which have been focused on increasing the nutritional value and defense against biotic and abiotic stress, this technology is also applied in handling the organoleptic properties of fruits such as color, texture, flavor, and aroma to get new varieties more attractive and pleasant to the consumer.

6.1. Color improvement in fruits

The color is a key feature of the quality of fruits and flowers and is often associated with carotenoids, flavonoids, and anthocyanins. As we described in the previous section (nutritional improvement), many fruits have been modified in their metabolism to increase these beneficial molecules for health, but most of them are also responsible for giving color to several organs of the plant. Taken the last example of the "super banana" and tomato with increased content in β-carotene, it is important to note that both fruits have an orange color in the pulp. On the other hand, fruits of tomato, apple, and strawberry modified for increasing anthocyanins accumulation have a more bluish (tomato) [83, 86] and red (apple and strawberry) [82, 88] pulp and peel (**Figure 1**). Thus, the organoleptic property of color can be modified by creating more striking and novel varieties.

6.2. Sweetness increment in fruits

Sweetness is one of the major determinants of the quality of fruits and generally depends on two factors, the composition and content of sugars. In plants, sugar also works as substrates in carbon metabolism and energy [94]. ADP-glucose pyrophosphorylase (AGPase), a key enzyme in the metabolic pathway from sucrose to starch, catalyzes the rate-limiting step of the biosynthesis of starch by generating the ADP glucose and inorganic pyrophosphate from glucose 1-phosphate and ATP. Transgenic plants of strawberry were developed by gene silencing using an antisense sequence for *FaGPS* gene, which codes for AGPase. A decrease in starch content and an increase in total soluble sugar content of 16–37% were obtained in transgenic fruits [95].

Currently, there are many alternative sweeteners that have been approved by the European Union regulators. Some of these are aspartame, saccharin, cyclamate, neohesperidin DC, acesulfame-K, and thaumatin. The first five compounds are low-molecular-weight molecules and are obtained by technology of traditional organic synthesis, although it should be noted that aspartame is an unnatural peptide. In contrast, thaumatin, is a natural protein that is produced normally in the *Thaumatococcus benth* plant, which is native to West Africa. In addition to thaumatin, there are several other sweet proteins in nature. Some of them have been isolated, purified, and characterized. Their genes have been cloned, and in some cases, recombinant versions of the natural protein have been obtained. Consequently, many of these sweet proteins can be used for the development of transgenic plants to enhance sweetness and fruit quality [96]. The tridimensional structure of thaumatin was determined at a high

resolution and shows a marked homology with the PR-5 proteins (pathogenesis-related protein-5), which are involved in biotic stress defense [97, 98]. Despite their structural similarity, it has not been reported that any PR-5 proteins produce a sweet taste [99]. Thaumatin is about 100,000 times sweeter than sucrose on a molar basis (about 1600 times on a weight basis). Thus, the threshold sweetness value of thaumatin is about 50 nM [100]. The thaumatin II gene was expressed in cucumber plants. The transgenic fruits accumulating thaumatin II showed a sweet phenotype and a positive correlation between the levels of accumulation of thaumatin and the intensity of sweet taste [101]. In addition, the concentration of E,Z-2,6-nonadienal, which is the main molecular odorant in cucumber, was enhanced in the transgenic cucumber fruits. Thus, transgenic expression of the thaumatin II gene resulted not only in a sweeter taste of fruits compared to control but also in a greater aroma intensity [102]. Tomato, pear, and strawberry were also transformed with the thaumatin II gene, and they also showed a direct correlation between amount of protein expression and the increase in sweetness [103–105]. As a particular case, the strawberry expressing thaumatin II showed a significantly higher resistance to gray mold caused by *Botrytis cinerea* [105].

6.3. Aroma as an important feature in fruits

Aroma has an important influence at the moment of choosing foods. The threshold for human perception of a volatile molecule can be low as 0.007 μg/L in water [106]. Thus, both the unique combination of volatile compound and the specific proportions of each of the volatile components, determine the properties of flavor in fruits and other foods. Additionally, plant volatiles greatly influence pollination and fruit defense responses and are therefore critical for breeders. Thus, the aroma is presented as a complex mixture of a large number of volatile compounds, whose composition is species specific and often for the particular variety of a fruit [107]. Although different fruits share many aromatic characteristics, each fruit has a characteristic aroma, which depends on the combination of volatile compounds, concentration, and perception of volatiles. The most important aromatic compounds are derived from amino acids, lipids, phenols, and mono and sesquiterpenes [107]. Lewinsohn et al. showed that transgenic tomatoes that express the gene for S-linalool synthase (LIS), under the control of the fruit-specific promoter E8, are able to synthesize and accumulate linalool S-terpenoid and 8-hydroxylinalool compounds. No other phenotypic alterations were observed, including levels of other terpenoids such as γ- and α-tocopherols, lycopene, β-carotene, and lutein, and the results show that it is possible to improve levels of monoterpenes in fruit ripening by metabolic engineering [108]. Transgenic tomato with higher levels of other terpenoids was developed by overexpression of geraniol synthase (*GES*) gene from *Ocimum basilicum*, which catalyzed the synthesis of geraniol from geranyl diphosphate, under the direction of the polygalacturonase gene (PG) fruit-specific promoter, it was possible to increase the content of geraniol in tomato fruits, although pigments were decreased. This would indicate that geraniol accumulation occurs at the expense of the accumulation of lycopene probably because geranyl diphosphate is a common precursor for the synthesis of both metabolites. The aroma of transgenic tomatoes was stronger compared to nontransgenic ones. Aroma test was performed to clarify the matter by several panelists, showing that they preferred the aroma of transgenic tomatoes [109].

7. Improvement of the characteristics in post-harvest

A critical decision for fruit growers is the time to harvest a crop. The time depends on factors such as the time required to reach the market and the management in route. Therefore, harvest is carried out when it has reached "harvest maturity" in any type of fresh fruits.

All fruits continue their metabolic processes after harvest. Therefore, the maintenance of the postharvest life is important to avoid that the product become inedible. In other words, many efforts are focused on preserve certain fruit traits, including nutritional value, processing qualities, flavor, and shelf life until the development of the ideal condition for consumption. Otherwise, the time lag of the postharvest life has the risk to expose serious losses in an ever-growing market. Indeed, the postharvest losses of fruits and vegetables, including roots and tubers, reach almost 50% of the production in developing countries, and the ratio of wastage is highest among food products [110, 111]. Also more than 40% of the food products losses occur at postharvest and processing levels in developing countries [112].

Within the postharvest life, fruit ripening occurs through physiological and biochemical reactions that alter visual appearance, flavor, aroma, texture, and fruit firmness [113, 114]. Therefore, many breeders and researchers have studied the complexity of fruit ripening and the development of engineered plants with high quality levels of fruit production in terms of flavor, color, and aroma.

7.1. Inhibition of ripening

The inhibition of fruit ripening has been achieved by reducing ethylene production [115]. Inhibition of ethylene production can be carried out by downregulating genes encoding key enzymes in the biosynthetic pathway of ethylene [116, 117] or by diverging the metabolic flux away from ethylene synthesis through the overexpression of enzymes degrading its immediate precursor, the 1-aminocyclopropane-1-carboxylic acid (ACC) [118, 119]. Even though most tomatoes have successfully lower levels of ethylene and an extended shelf life, most of them also compromised fruit-quality traits. An interesting work employed RNAi technology in which three homologs of 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) gene were silenced during the course of ripening. Engineered fruits exhibited delayed ripening, prolonged shelf life for ~45 days, and improved juice quality. Indeed, total soluble solids (TSS) recorded in RNAi-ACS tomatoes increased up to ~40–45% compared to control [120]. In melon and papaya (*Carica papaya*), inhibition of fruit ripening and shelf life extension have also been achieved by silencing of genes coding for the enzymes or regulators of the ethylene biosynthesis pathway [121, 122].

Through Targeting Induced Local Lesions in Genomes (TILLING) approach [123–126], a melon mutant was isolated, which showed delayed fruit ripening and rind yellowing and an increase of fruit firmness and shelf life. The missense mutation G194D occurred in a highly conserved amino acid position of the ethylene biosynthetic enzyme, ACC oxidase 1 (CmACO1), and was predicted to affect the enzymatic activity of CmACO1.

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By using RNAseq analysis, it was recently reported that the Polycomb-group (PcG) protein multicopy suppressor of IRA1 (MSI1) negatively regulates a large set of fruit-ripening genes along with the MADS-box protein RIN (ripening inhibitor) and its regulons. In fact, the genetic manipulation of SlMSI1 and RIN transcription factor successfully prolonged the fruit shelf life in tomato [127]. This may be an optimal approach to improve the post-harvest life of functional fruits by employing high-throughput techniques and addressing multiple metabolic pathways such as light-signaling pathways, which have modulatory components to adjust pigmentation during ripening and could be a selective advantage for primeval fleshy fruited plants [128].

7.2. Reduction of softening in ripe fruits

Reduction in fruit firmness due to softening that accompanies ripening plays a major role in determining the cost factor because exacerbates damage during handling and shipping processes having also a pivotal effect on shelf life, palatability, consumer acceptance, and postharvest resistance to pathogens [129–131]. The excessive softening causes losses around 35–40% of fruits and vegetables produced by India known as the second largest producer of these crops in the world [110]. It is suggested that the cell wall modifications are the major determinant of fruit softening induced as a consequence of the increased levels of cell wall–degrading enzymes [132]. Some strategies to control fruit softening include the manipulation of genes coding cell wall–degrading enzymes such as polygalacturonase or β-galactosidase [133–136]. This is the case of the first genetically modified tomato with reduced levels of expression of polygalacturonase gene through PTGS that was marketed as Flavr Savr™ to remark the potentially positive effect on the flavor [137, 138]. Although this strategy was developed to improve the flavor traits, delayed ripening with an expanded shelf life also occurred in the transgenic tomatoes by delaying cell wall softening.

In nonclimateric fruits such as strawberry and capsicum, efforts to control fruit ripening based on slowing down the rate of fruit softening has been successfully achieved by targeting genes involved in cell wall modification. In strawberry (*Fragaria* × *ananassa* Duch.), the down expression of pectate lyase or the fruit-specific polygalacturonase (FaPG1) genes by antisense technology resulted in extended fruit firmness and postharvest shelf life [139–143].

However, the other studies on the suppression of the expression of cell wall–degrading enzymes have not enough impact in prevent softening of genetically engineer fruits [133, 144]. This may be due to the redundant functionality of components taking part of a complex metabolic process [114, 145]. Therefore, the improvement of fruit shelf life constitutes a strong challenge for the identification of new targets to achieve this goal.

7.3. Browning reduction in fruits

The postharvest storage and quality of fresh fruits are also affected by the enzymatic browning having negative effects on color, flavor, taste, and nutritional value. This reaction may be responsible for up to 50% of total losses of fruits and vegetables production [146]. Browning

is triggered by the oxidation of phenolic compounds to quinones catalyzed by the polyphenol oxidase (PPO) enzyme [147, 148]. The subsequent nonenzymatic polymerization of the quinones results in the brown pigments formation that induces the postharvest deterioration [149]. This is particularly easy to appear in apples, which are highly susceptible to enzymatic browning and contain high levels of polyphenols [150, 151]. In order to reduce postharvest browning, the silencing of PPO gene in an apple was accomplished. The apples produced less PPO activity, and 50% of browning was inhibited compared to wild type control in golden delicious (GD) and granny Smith (GS) [152, 153]. Transgenic apples can keep the original color of the apple flesh when they are subjected to mechanical damage, such as bruising or slicing. This "nonbrowning" phenotype minimizes shrinkage caused by harvest and postharvest damage and also decreases the need for antibrowning compounds on cut fruit (**Figure 1C**). Similar approaches have been performed to reduce grape berry darkening [149], blackheart in pineapple [154], and the browning process in fruits of Yali pear [61].

8. Conclusion

Metabolic engineering generally involves the redirection of cellular metabolism by modifying the expression of genes and enzymes affecting to the regulatory functions within the cell. For a successful metabolic engineering, rate-limiting step is the target for the increase of specific molecules or newly introduced molecules.

Traditional strategies for modifying gene expression such as using *A. tumefaciens* for overexpressing and silencing genes will be continue to be used but the strategies to manipulate the gene expression have been gradually refined. For instance, the selection markers have been removed, and only the transgene or mutagenic effect remains in the plant. In case of using CRISPR/Cas vectors, this methodology induces site-specific mutations and can produce thereby specific knock-out plants with the absence of external DNAs requirements. Since the modified plants do not contain a transgene, they are not included in the category of GMO.

Even more sophisticated metabolomic tools based on biochemical, genetic, environmental, and developmental parameters will offer the possibility to study the production of metabolites through the improvement of primary and secondary metabolic pathways in fruits. The increasing number of plant genomes sequenced, and the availability of many molecular markers can be used to track candidate genes that are associated with the desired trait and feature. However, traditional plant breeding together with genetic engineering provides greater opportunities to develop fruit crops with the desired amount and/or composition of specific metabolites. The most relevant characteristics for genetic improvement of plants that bear economic interest were discussed in this chapter. These characteristics included higher resistance to biotic and abiotic stress and improvement of pre-harvest and post-harvest features to face those problems that affect the vast cultivars and cause a high degree of economic losses. On the other hand, nutritional and organoleptic improvement of functional fruits are in direct benefit for end consumers.

Despite the benefits of metabolic engineering, the development and marketing of genetically modified fruit plants are hampered by many regulatory and social barriers. From the biosafety and consumers point of view, the presence of selectable marker genes, which are essential for the initial selection of transgenic plants, is undesirable. Therefore, the production of transgenic fruit plants without markers is now an essential requirement for commercial exploitation. The techniques such as RNAi in rootstocks for virus silencing, cisgenesis, or intragenesis show great potential and greater acceptance when generating genetically modified organisms. Additionally, selection marker free plants may improve the confidence and bring the benefits of genetically modified products to consumers.

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Author details

Luis Quiroz-Iturra, Carolina Rosas-Saavedra and Claudia Stange Klein*

*Address all correspondence to: cstange@uchile.cl

Department of Biology, Faculty of Science, Centre of Plant Molecular Biology, University of Chile, Santiago, Chile

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