Chapter 12
Regulating Phytonutrient Levels in Plants – Toward Modification of Plant Metabolism for Human Health

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Abstract Plants constitute a major component of our diet, providing pigments and additional phytonutrients that are thought to be essential for maintenance of human health and are therefore also referred to as *functional metabolites*. Several fruit and vegetable species already contain high levels of several of these ingredients, while others do not. Nevertheless, efforts have been devoted to increasing and diversifying the content of phytonutrients, such as carotenoids, flavonoids, and vitamins, even in plants that normally produce high levels of such nutritional components. These efforts rely on transgenic and non-transgenic approaches which have exposed complex regulation mechanisms required for increasing the levels of functional metabolites in plants. The study of these regulatory mechanisms is essential to expedite improvement of levels of these metabolites in fruits, vegetables, cereals, legumes, and starchy roots or tubers. Such improvement is important for the following reasons: (1) to increase the efficiency of the industrial extraction of these compounds that are later being used as natural food supplements or fortifiers and as a source of natural colors to replace the chemical alternatives; (2) to improve and diversify the diet in populations of developing countries, where malnutrition may occur through lack of variety in the diet; (3) to provide fresh agricultural products such as fruits and vegetables highly enriched with certain phytonutrients to possibly substitute the chemically synthesized food supplements and vitamins; and (4) to provide an array of new and attractive colors to our diet.

Three basic approaches to modifying a biosynthetic pathway to increase amounts of desirable phytonutrients are available: (1) manipulation of pathway flux, including increasing, preventing, or redirecting flux into or within the pathway; (2) introduction of novel biosynthetic activities from other organisms via genetic engineering; and (3) manipulation of metabolic sink to efficiently sequester the end-products of particular metabolic pathways. These approaches have been effectively demonstrated in relation to the flavonoid and carotenoid biosynthetic pathways in

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tomato (*Solanum lycopersicum*). This chapter is therefore focused on carotenoids and flavonoids, their importance to human nutrition, and approaches used to induce, regulate, and diversify their content in tomato fruits. In addition, several examples of outstanding approaches employed to modulate carotenoid content in other plant species will also be given.

12.1 Introduction

Plants synthesize and accumulate an excess of 200,000 natural products (Fiehn, 2002). Plants also constitute a major component of our diet, providing fiber (i.e., cellulose, hemicellulose, and starch), carotenoids, flavonoids, vitamins, minerals, and additional pigmented and non-pigmented metabolites thought to promote or at least maintain good health (Willcox et al., 2003; Fraser and Bramley, 2004, Davies, 2007). These metabolites are referred to as *phytonutrients*, *functional metabolites*, *phytochemicals*, and lately also *nutraceuticals* (Davies, 2007), defined as certain organic components of plants that are thought to promote human health (The American National Cancer Institute drug dictionary at http://www.cancer.gov/drugdictionary/). Major examples of phytonutrient-rich plant foods and the principle phytonutrients which they accumulate are listed in Table 12.1.

Phytochemicals have been used, even as drugs, for centuries (Yonekura-Sakakibara and Saito, 2006). For example, Hippocrates (ca. 460–370 BC) used to prescribe willow tree leaves to abate fever. The active ingredient, salicin, with potent anti-inflammatory and pain-relieving properties was later extracted from the White Willow Tree (*Salix alba*) and eventually synthetically produced to become the staple over-the-counter drug called *Aspirin*. Noteworthy, the initial conceptual link between food and human health is also related to Hippocrates, who has been referred to as the "father of modern medicine". He stated, "Let thy food be thy medicine and thy medicine be thy food".

The recent completion of the human genome sequence and the advances made in high-throughput technologies brought about the area of *nutragenomics* that is predicted to uncover more precisely the possible relationship between human genetic makeup and nutrients, including phytonutrients. Meanwhile, efforts have been invested in increasing and diversifying the content of nutrients, such as carotenoids, flavonoids, tocopherols, minerals, fatty acids, phytosterols, and vitamins in both model and agricultural plant species (extensively reviewed with selected examples by Galili et al., 2002; Levin et al., 2006; Davies, 2007). While it is not at all clear whether these efforts would necessarily lead to agricultural products with better functional properties for human health benefits, they have exposed regulation mechanisms important for increasing and maintaining high levels of functional metabolites in plant products. The study of these regulatory mechanisms will have an important role in delivering functional attributes through foods, once better relationships between these ingredients and human health will be unraveled.

Table 12.1 Examples of phytonutrient-rich plant foods and the principle phytonutrients they accumulate

Plant food	Phytonutrients
Soybean	Protease inhibitors, β-sitosterol, saponins, phytic acid, isoflavones
Red apples, grapes, blackberries, blueberries, raspberries, red wine	Anthocyanins
Tomato	Lycopene, β-carotene, vitamin C
Broccoli	Vitamin C, 3,3′-diindolylmethane, sulforaphane, lignans, selenium
Garlic	Thiosulfonates, limonene, quercitin
Flax seeds	Lignans
Citrus fruits	Monoterpenes, coumarin, cryptoxanthin, vitamin C, ferulic acid, oxalic acid, flavanones
Corn, watercress, spinach, parsley, avocado, honeydew melon	Lutein, zeaxanthin
Broccoli, Brussels sprouts, kale	Glucosinolates, indoles
Garlic, onions, leeks, chives	Allyl sulfides
Blueberries	Tannic acid, lignans, anthocyanins
Sweet potatoes, carrots, mangos, apricots, pumpkin, winter squash	α-Carotene, β-carotene
Chilli peppers	Capsaicin
Cantaloupe, peaches, tangerines, papaya, oranges	b-Cryptoxanthin, flavonoids
Celery	Flavones
Tea, apple, cocoa	Flavanols
Beans, peas, lentils	Omega fatty acids, saponins, catechins, quercitin, lutein, lignans

Several plant foods already contain high levels of certain phytonutrients, while others do not (Davies, 2007). Nevertheless, efforts have been invested in increasing and diversifying the content of phytonutrients, such as carotenoids, flavonoids, and vitamins in several plant species, even in those that already contain high levels of one or several of these ingredients. The tomato fruit, for instance, is considered to be a good source of lycopene, vitamin C, β-carotene, folate, and potassium (Davies and Hobson 1981; Willcox et al., 2003). The tomato could also potentially be a good source for flavonoids as well (Jones et al., 2003; Willits et al., 2005; van Tuinen et al., 2006; Sapir et al., 2008). Nevertheless, efforts have been invested in increasing the content and diversifying phytonutrients, such as carotenoids and flavonoids, in the tomato fruit (Verhoeyen et al., 2002; Fraser and Bramley, 2004; Levin et al., 2004).

Increasing the levels of phytonutrients, such as lycopene in the tomato fruit, is highly justified from the perspective of the extraction industry due to cost-effectiveness reasons (Levin et al., 2006). Further enriching phytonutrients in plant species that already contain high levels of such ingredients is also directed to possibly substitute the chemically synthesized food supplements and vitamins in human populations that normally consume such supplements (Sloan, 2000; Levin et al.,

2006). Diversifying phytonutrients, including those that contribute to fruit color, can provide an array of new and attractive colors to our diet and also harness synergistic effects among phytonutrients which are important to human health. Increasing the levels of phytonutrients in plant species that normally do not contain high levels of these ingredients, including cereals, some legumes, and starchy roots or tubers/tuberous roots, is important in order to improve the diet in populations of people in developing countries, where nutrition is not diversified enough to provide all of the essential metabolites, primarily vitamins and minerals needed to maintain proper health (Davies, 2007). Due to these reasons, there is now a growing interest in the development of food crops with enhanced levels of phytonutrients. The tomato is an excellent candidate for the following reasons: (1) it is a major crop; (2) it is already a good source of several phytonutrients such as lycopene and vitamin C; (3) it contains many accessions with modulated levels of essential metabolites; (4) it can be easily modified by both classical genetic and transgenic means; and (5) it has been a subject of many studies aimed at increasing and diversifying the content of fruit phytonutrients, mainly carotenoids and flavonoids. Also, excellent analytical and genomics tools have been developed for tomatoes which can facilitate the molecular analysis of a certain gene modification. This chapter will therefore focus on factors that induce, regulate, and diversify carotenoids and flavonoids in tomato (*Solanum lycopersicum*) and their importance to human nutrition. A few outstanding examples of similar factors in other plant species will be also given.

Strategies to increase and diversify the content of either carotenoids or flavonoids in tomato fruits are reviewed here. These efforts rely on transgenic and non-transgenic approaches (i.e., use of spontaneous or induced mutations and/or quantitative trait loci affecting levels of these phytonutrients). The tomato light-responsive *high-pigment* (hp) mutations are an outstanding example of the latter alternative (Levin et al., 2003; 2004) and will therefore be presented in more detail. Due to their impact on fruit lycopene content, these hp mutations were already introgressed into elite tomato germplasm (Levin et al., 2003; 2006). Introgression of one of these hp mutations, hp-2^{dg}, into elite processing cultivars, characterized by an average fruit lycopene concentration of 80–90 $\mu g \cdot g^{-1}$ FW, resulted in cultivars with an average fruit lycopene concentration of up to 280 $\mu g \cdot g^{-1}$ FW, representing an up to 3.5-fold increase in fruit lycopene content. Most notably, recent studies also reinforce earlier ones suggesting that plants carrying these mutations are also characterized by higher levels of other health-promoting metabolites, such as flavonoids and vitamins (Bino et al., 2005). Further, and more recently, it was shown that cross-hybridizing light-responsive hp mutant plants with plants carrying either the Anthocyanin fruit (Aft) or the *atroviolacium* (atv) mutations, known to cause anthocyanin expression in tomato fruits, displayed a significant more-than-additive effect on the production of fruit anthocyanidins and flavonols (van Tuinen et al., 2006; Sapir et al., 2008). This effect was manifested and quantitatively documented as a remarkable ~5-, 19-, and 33-fold increase of petunidin, malvidin, and delphinidin, respectively, in the hp-1/hp-1 Aft/Aft double mutants compared to the cumulative levels of their parental lines (Sapir et al., 2008). These results underlie the importance of

light-responsive hp mutations in modulating phytonutrient content in plants, either on their own or in combination with other gene mutations.

Up to date, five light-responsive hp mutations have been discovered (Lieberman et al., 2004; Galpaz et al., 2008). These mutations, i.e., hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg}, were initially marked as lesions in structural genes of the carotenoid biosynthetic pathway (Stevens and Rick, 1986). However, more recent studies have demonstrated that they represent mutations in two evolutionary conserved regulatory genes active in light signal transduction, known also as *photomorphogenesis* (Mustilli et al., 1999; Levin et al., 2003; Lieberman et al., 2004). The identification of the genes that encode these hp mutant phenotypes has therefore created a conceptual link between photomorphogenesis and biosynthesis of fruit phytonutrients and suggests that manipulation of light signal transduction machinery may be very effective toward the practical manipulation of an array of fruit phytonutrients (Levin et al., 2003; 2006; Liu et al., 2004). Recent studies focusing on the manipulation of light signaling genes in tomato plants, cited in this chapter, support this approach.

12.2 Carotenoids

Carotenoids are orange, yellow, and red pigments that exert a variety of critical functions in plants. They comprise a class of lipid-soluble compounds within the isoprenoid family, which is one of the largest classes of natural products in the plant kingdom with over 22,000 known constituents (Connolly and Hill, 1992; Britton, 1998).

The isoprenoid family also includes gibberellins, phytosterols, saponins, tocopherols, and phylloquinones. Chlorophylls also contain an isoprenoic component, formed from the same precursor of the carotenoid metabolism, geranylgeranyl diphosphate (GGDP) (Fig. 12.1). In addition to their many functional roles in photosynthetic organisms, carotenoids have many industrial applications as food and feed additives and colorants, in cosmetics and pharmaceuticals, and as nutritional supplements (Galili et al., 2002). Carotenoids are C_{40} hydrocarbons with polyene chains that contain 3–15 conjugated double bonds. These double bonds are responsible for the absorption spectrum, and therefore the color of the carotenoid, and for the photochemical properties of the molecule (Britton, 1995).

The carotenoid backbone is either linear or contains one or more cyclic β-ionone or ε-ionone rings or, less frequently, the unusual cyclopentane ring of capsanthin and capsorubin that impart the distinct red color to peppers. Non-oxygenated carotenoids are referred to as *carotenes*, whereas their oxygenated derivatives are designated as *xanthophylls*. The most commonly occurring carotenes are β-carotene in chloroplasts and lycopene as well as β-carotene in chromoplasts of some flowers and fruits, e.g., tomatoes. The most abundant xanthophylls in photosynthetic plant tissues (lutein, violaxanthin, and neoxanthin) are key components of the light-harvesting complexes.

Carotenoids are synthesized in the membranes of nearly all types of the plant plastids and accumulate to high levels in chromoplasts of many flowers, fruits,

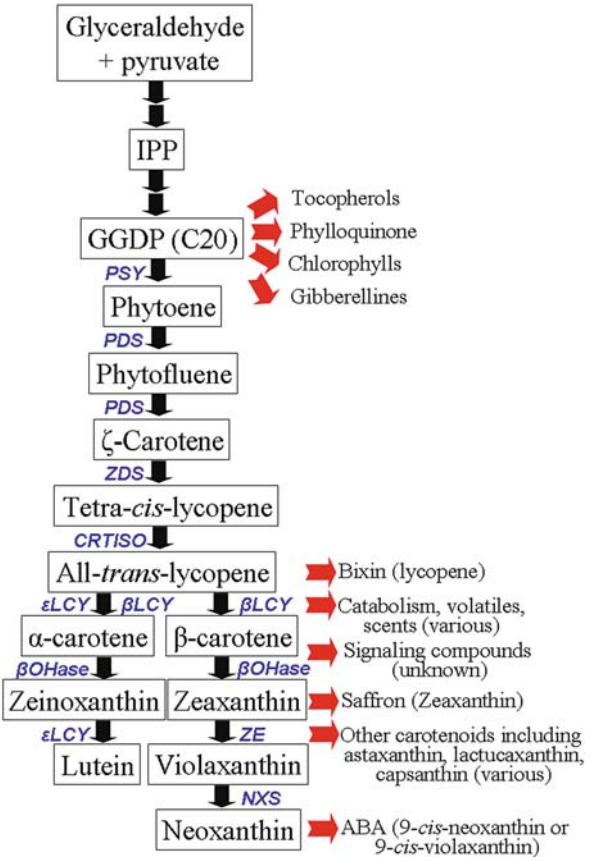

Fig. 12.1 A schematic presentation of the carotenoid biosynthetic pathway and its structural genes. Gene abbreviations: *CRTISO* = carotenoid isomerase, *βLCY* = *β*-lycopene cyclase, *εLCY* = *ε*-lycopene cyclase, *NXS* = neoxanthin synthase, *βOHase* = *β*-carotene hydroxylase, *PDS* = phytoene desaturase, PSY = phytoene synthase, *ZDS* = *ζ*-carotene desaturase, *ZE* = zeaxanthin epoxidase; Metabolite abbreviations: GGDP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate

and roots (Howitt and Pogson, 2006). They are involved in photosystem assembly, light harvesting and photoprotection, photomorphogenesis, non-photochemical quenching, lipid peroxidation, and affect the size and function of the light-harvesting antenna and seed set (Pogson et al., 1998; Havaux and Niyogi, 1999; Niyogi, 1999; Davison et al., 2002; Kulheim et al., 2002; Lokstein et al., 2002; Holt et al., 2004, 2005; Cuttriss and Pogson, 2006; Wang et al., 2008). In chromoplasts, carotenoids serve as pigments that furnish fruits and flowers with distinct colors in order to attract insects and animals for pollination and seed dispersal (Fraser and Bramley, 2004).

Animals as well as humans are unable to synthesize carotenoids de novo and rely upon the diet as a source of these compounds. Over recent years there has been considerable interest in dietary carotenoids with respect to their potential in alleviating age-related diseases in humans, propelling a market with an estimated yield of 100 million tons and a value of about US $935 million per annum (Fraser and Bramley, 2004). Although key carotenoids can be chemically synthesized, there is an increasing demand for the natural alternatives mainly those which are being

extracted or consumed from plants (Sloan, 2000). This attention has been mirrored by significant advances in cloning most of the carotenoid genes and in the genetic manipulation of crop plants with the intention of increasing their levels in the diet.

12.2.1 The Carotenoid Biosynthetic Pathway

During the past decade, a near-complete set of genes required for the synthesis of carotenoids in photosynthetic tissues has been identified, primarily as a result of molecular genetic- and biochemical genomics-based approaches in the model organisms such as *Arabidopsis* (*Arabidopsis thaliana*) and several agricultural crops such as the tomato. Mutant analysis and transgenic studies in these and other systems have provided important insights into the regulation, activities, integration, and evolution of individual enzymes and are already providing a knowledge base for breeding and transgenic approaches to modify the types and levels of these important compounds in agricultural crops (Dellapenna and Pogson, 2006).

In higher plants, carotenoids are synthesized from the plastidic isoprenoid biosynthetic pathway (Lichtenthaler, 1999; Fraser and Bramley, 2004, DellaPenna and Pogson, 2006). They are biosynthetically linked to other isoprenoids such as gibberellins, tocopherols, chlorophylls, and phylloquinones via the five-carbon compound isopetenyl pyrophosphate (IPP). Two distinct pathways exist for IPP production: the *cytosolic mevalonic acid pathway* and the *plastidic mevalonate-independent methylerythritol 4-phosphate (MEP) pathway*. The methylerythritol 4-phosphate pathway combines glyceraldehyde-3-phosphate and pyruvate to form deoxy-D-xylulose 5-phosphate, and a number of steps are then required to form IPP and dimethylallylpyrophosphate (DMAPP) (Lichtenthaler, 1999). IPP is subject to a sequential series of condensation reactions to form geranylgeranyl diphosphate (GGDP), a key intermediate in the synthesis of carotenoids, tocopherols, and many other plastidic isoprenoids (Fig. 12.1).

The initial steps of plant carotenoid synthesis and their chemical properties have been thoroughly discussed in several prior reviews (Cunningham and Gantt 1998; Hirschberg, 2001; Cunningham, 2002; Fraser and Bramley, 2004; Cuttriss and Pogson, 2006). Briefly, the first committed step in plant carotenoid synthesis is the condensation of two molecules of GGDP to produce phytoene (Fig. 12.1) by the enzyme *phytoene synthase* (PSY). Phytoene is produced as a 15-*cis* isomer, which is subsequently converted to all-*trans* isomer derivatives. Two plant desaturases, *phytoene desaturase* (PDS) and *ζ-carotene desaturase* (ZDS), catalyze similar dehydrogenation reactions by introducing four double bonds to form lycopene. Desaturation requires a plastid terminal oxidase and plastoquinone in photosynthetic tissues (Beyer, 1989; Norris et al., 1995; Carol et al., 1999). Bacterial desaturation differs from plants in that a single enzyme, crtI (*phytoene desaturase*), introduces four double bonds into phytoene to yield all-*trans*-lycopene (Cunningham and Gantt, 1998). This bacterial enzyme was therefore used as a target to increase lycopene and other carotenoids content in plant species as will be further outlined.

Until recently, the higher plant desaturases were assumed sufficient for the production of all-*trans*-lycopene. This conclusion was reached despite the accumulation of tetra-*cis*-lycopene in *tangerine* (*t*) tomato and algal mutants (Tomes et al., 1953; Cunningham and Schiff, 1985) and biochemical evidence to the contrary from daffodil (Beyer et al., 1991). Recently, the *carotenoid isomerase* gene, *CRTISO*, was identified in *Arabidopsis* and tomato, which catalyzes *cis–trans* isomerizations and resulting in all-*trans*-lycopene (Isaacson et al., 2002; Park et al., 2002).

In plants, the carotenoid biosynthetic pathway diverges into two main branches after lycopene, distinguished by different cyclic end-groups. Two beta rings lead to the β,β branch (β-carotene and its derivatives: zeaxanthin, violaxanthin, antheraxanthin, and neoxanthin), whereas one beta and one epsilon ring define the β,ε branch (α-carotene and its derivatives). These initial reactions are carried out by two enzymes: *β-lycopene cyclase* (βLCY) and *ε-lycopene cyclase* (εLCY) (Fig. 12.1). βLCY converts lycopene into β-carotene which is later converted to zeaxanthin by *β-carotene hydroxylase* (βOHase). An epoxide group is introduced into both rings of zeaxanthin by *zeaxanthin epoxidase* (ZE) to form violaxanthin. Conversion of violaxanthin to neoxanthin is performed by the enzyme *neoxanthin synthase* (NXS). Both the β- and ε-lycopene cyclase enzymes (βLCY and εLCY, respectively) are initially required to form α-carotene (Cunningham and Gantt, 1998; Pogson et al., 1996), which is being converted to lutein, via zeinoxanthin, by *β-carotene hydroxylase* (βOHase) and *ε-carotene hydroxylase* (εOHase) (Fig. 12.1).

Unlike the flavonoid pathway (see herein below), the regulation of carotenoid biosynthesis at the gene and enzyme level is poorly understood. No regulatory genes involved in carotenoid formation have been isolated thus far. It was reasoned that a heavily branched pathway such as that of carotenoids formation from isoprenoid precursors is unlikely to be controlled by a sole regulatory process (Fig. 12.1). Instead, it was suggested that control points, yet to be identified, are likely to exist at each branch point which probably involve both transcriptional and post-transcriptional regulation events (Fraser and Bramley, 2004). Despite this apparent complexity, several examples exist which resulted in an exceptional up-regulation of the carotenoid biosynthetic pathway by transgenic ("golden" rice) and non-transgenic approaches (the *Or* gene identified in cauliflower and the light-responsive hp mutations identified in tomato). These examples underlie the great potential of current knowledge to modulate levels of these important phytonutrients for the benefit of human health and will, therefore, be separately discussed in a later part of this chapter.

12.3 Flavonoids

Flavonoids comprise a group of plant polyphenols that provide much of the flavor and color to fruits and vegetables (Ross and Kasum, 2002). They are a large family of low-molecular-weight secondary metabolite compounds that are widespread throughout the plant kingdom, ranging from mosses to angiosperms (Koes et al., 1994). Their basic chemical structure, a $C_6-C_3-C_6$ configuration, consists of two

aromatic rings joined by a three-carbon link. This makes the flavonoids good hydrogen and electron donors. Based on their core structure, the aglycone, the flavonoids can be grouped into different classes, such as *flavones* (e.g., apigenin, luteolin), *flavonols* (e.g., quercetin, myricetin), *flavanones* (e.g., naringenin, hesperidin), *catechins* or *flavanols* (e.g., epicatechin, gallocatechin), *anthocyanidins* (e.g., cyanidin, pelargonidin), and *isoflavones* (e.g., genistein, daidzein) (Ross and Kasum, 2002). Within each group, single or combinatorial modifications of the aglycones, such as glycosylation, methylation and acylation, contribute to the formation of individual compounds.

Flavonoids are mainly responsible for the blue to purple, red, and yellowish colors in plants. *Proanthocyanidins* and their monomer units, catechins (Fig. 12.2), are the natural substrates of polyphenol oxidases and are, therefore, involved in the browning phenomenon of fruits.

To date, more than 6,000 flavonoids have been described and the number is still increasing. Notably, most of them are conjugated to sugar molecules and are commonly located in the upper epidermal layers of leaves and fruits as well as in seed coats (Stewart et al., 2000, Willits et al., 2005). In plants, flavonoids are involved in many aspects of growth and development, including pathogen resistance, pigmen-

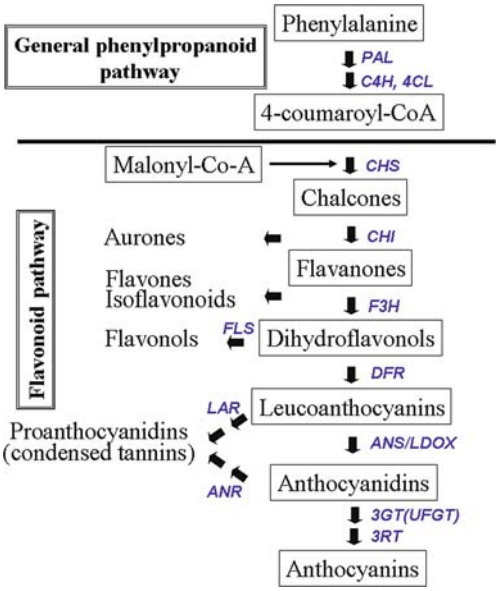

Fig. 12.2 A schematic presentation of the flavonoid biosynthetic pathway and its structural genes. Gene abbreviations: *ANR* = anthocyanidin reductase, *ANS/LDOX* = anthocyanidin synthase, *C4H* = cinnamate 4-hydroxylase, *4CL* = *4*-coumarate-*COA* ligase, *CHS* = chalcone synthase, *CHI* = chalcone isomerase, DFR = dihydroflavonol 4-reductase, *F3H* = flavanone 3-hydroxylase, *FLS* = flavonol synthase, *3GT (UFGT)* = *UDPG*-flavonoid-*3-O*-glucosyltransferase, *LAR* = leucoanthocyanidin reductase, *LDOX* = leucoanthocyanidin dioxygenase, *PAL* = phenylalanine ammonia lyase, *3RT* = anthocyanidin-3-glucoside rhamnosyl transferase

tation, and therefore attraction of pollinating insects, UV light protection, pollen tube growth, plant defense against pathogenic micro-organisms, plant fertility and germination of pollen, seed coat development, and in signaling for the initiation of symbiotic relationships (Harborne, 1986; Dooner et al., 1991; Koes et al., 1994; Dixon and Paiva, 1995; Parr and Bolwell, 2000; Schijlen et al., 2004).

Historically, flavonoids have been an attractive research subject mainly because of the colorful anthocyanins. These eye-catching pigments have been very useful in performing genetic experiments, including Gregor Mendel's study on the inheritance of genes responsible for pea seed coat color and the discovery of transposable elements interrupting maize pigment biosynthetic genes (McClintock, 1967; Lloyd et al., 1992; Koes et al., 1994).

The composition of flavonoids in different fruit species varies greatly (Macheix et al., 1990, Robards and Antolovich, 1997). The main anthocyanins in fruits are glycosides of six anthocyanidins that are widespread and commonly contribute to the pigmentation of fruits. Cyanidin is the most common anthocyanidin, the others being delphinidin, peonidin, pelargonidin, petunidin, and malvidin. Of the flavonols, quercetin, kaempferol, myricetin, and isorhamnetin are common in fruits, quercetin being the predominant flavonol. A third predominant flavonoid group in fruits is proanthocyanidins and their monomer units, catechins (procyanidin) or gallocatechins (prodelphinidins).

Delphinidin-derived anthocyanins are known to be responsible for the bluish colors, whereas cyanidin- and pelargonidin-derived anthocyanins are found in mauve and reddish tissues, respectively. Anthocyanins tend to form complexes with so-called co-pigments that can intensify and modify the initial color given by the pigment. Apparently, almost all polyphenols, as well as other molecules, such as purines, alkaloids, and metallic cations, have the ability to function as co-pigments. The final color of anthocyanins can also be affected by the temperature and pH of the vacuolar solution where they reside (Brouillard and Dangles, 1994; Brouillard et al., 1997; Mol et al., 1998; Cseke et al., 2006).

Because flavonoids impart much of the color and flavor of fruits, vegetables, nuts, and seeds, they form an integral part of the human diet (Parr and Bolwell, 2000). Rich dietary sources of flavonoids include soybean (isoflavones); citrus (flavanones); tea, apple, and cocoa (flavanols); celery (flavones); onion (flavonols); and berries (anthocyanins) (Table 12.1; Rice-Evans et al., 1996; Ross and Kasum, 2002; Le Gall et al., 2003).

12.3.1 The Flavonoid Biosynthetic Pathway

The flavonoid biosynthetic pathway has been almost completely elucidated and comprehensively reviewed (e.g., by Dooner et al., 1991; Koes et al., 1994; Holton and Cornish, 1995; Mol et al., 1998; Weisshaar and Jenkins, 1998; Winkel-Shirley, 2001). Many of the genes controlling this pathway have been cloned from several model plants including maize (*Zea mays*), snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*), gerbera (*Gerbera hybrida*), and more recently, *Arabidopsis* (van

der Krol et al., 1988; Goff et al., 1990; Taylor and Briggs, 1990; Martin et al., 1991; Tonelli et al., 1991; Shirley et al., 1995; Elomaa et al., 1993, Helariutta et al., 1993, 1995; Holton and Cornish, 1995). These genes can be divided into two classes: (1) *structural genes* which encode enzymes that directly participate in the formation of flavonoids and (2) *regulatory genes* that control the expression of the structural genes.

An overview of the flavonoid pathway is presented in Fig. 12.2. Flavonoids are synthesized via the phenylpropanoid pathway, generating organic compounds that are biosynthesized from the amino acid phenylalanine. *Phenylalanine ammonia lyase* (PAL) catalyzes the conversion of phenylalanine to cinnamate. *PAL* also shows activity by converting tyrosine to *p*-coumarate, albeit with a lower efficiency. The *cinnamate 4-hydroxylase* (C4H) catalyzes the synthesis of *p*-hydroxycinnamate from cinnamate, and *4-coumarate:CoA ligase* (4CL) converts *p*-coumarate to its coenzyme-A ester, activating it for reaction with malonyl-CoA. The flavonoid biosynthetic pathway starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, resulting in the yellow-colored naringenin chalcone. This reaction is carried out by the enzyme, *chalcone synthase* (CHS), the key enzyme for flavonoid biosynthesis. In most plants chalcones are not the end-product, as the pathway proceeds with additional enzymatic steps to generate other classes of flavonoids, such as flavanones, dihydroflavonols, and finally, anthocyanins, the major water-soluble pigments in flowers and fruits and root crops like beets. Other flavonoid classes, i.e., isoflavones, aurones, flavones, proanthocyanidins, and flavonols, represent side branches of the flavonoid pathway and are derived from intermediates in anthocyanin formation (Fig. 12.2).

Naringenin chalcone is isomerized to the flavanone naringenin by the enzyme *chalcone isomerase* (CHI). Even in the absence of CHI, naringenin chalcone may spontaneously isomerize to form naringenin (Holton and Cornish, 1995). From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids. *Flavanone 3-hydroxylase* (F3H) catalyzes the stereospecific 3β-hydroxylation of flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, *dihydroflavonol reductase* (DFR) catalyzes the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanins), which are converted to anthocyanidins by *anthocyanidin synthase* (ANS). The formation of glucosides is catalyzed by *UDP glucose-flavonoid 3-O-glucosyl transferase* (UFGT), which stabilizes the anthocyanidins by 3-*O*-glucosylation (Harborne, 1994; Bohm, 1998).

12.4 Health Benefits of Carotenoids and Flavonoids

Diet is believed to play an important role in the development of chronic human diseases (Willcox et al., 2003; Lila, 2007). It is now becoming recognized that certain fruits and vegetables can help prevent or treat chronic human diseases (Heber and Bowerman, 2001; Sloan, 2000; Lila, 2007). However, this recognition is primarily supported by in vitro and epidemiological studies, but by only a limited number of in vivo studies (Willcox et al., 2003). Nonetheless, it is currently believed that not

single components in plant-derived foods but rather complex mixtures of interacting natural chemicals are producing health-protective effects. These phytochemicals accumulate simultaneously in a plant, and they provide a multifaceted defensive strategy for both the plant and the human consumer (Heber and Bowerman, 2001; Lila, 2007).

Many phytochemicals are colorful, providing an easy way to communicate increased diversity of fruits and vegetables to the public (Joseph et al., 2003). These colors have provided various recommended color codes for plant-derived diet, advising consumers to ingest one serving of each color groups daily. For instance, a seven-color code was suggested by Heber and Bowerman (2001) which includes (1) red foods that contain lycopene, the pigment in tomatoes, which becomes localized in the prostate gland and may be involved in maintaining prostate health; (2) yellow-green vegetables, such as corn and leafy greens, that contain lutein and zeaxanthin, which become localized in the retina where age-related macular degeneration occurs; (3) red-purple foods containing anthocyanins, which are powerful antioxidants found in red apples, grapes, berries, and wine; (4) orange foods, including carrots, sweet potatoes, yams, mangos, apricots, pumpkin, and winter squash, which contain β-carotene; (5) orange-yellow foods, including oranges, tangerines, and lemons, which contain citrus flavonoids; (6) green foods, including broccoli, Brussels sprouts, and kale, which contain glucosinolates; and (7) white-green foods in the onion family that contain allyl sulfides. Interestingly five of the above color groups can be assigned to the carotenoid or flavonoid families of phytonutrients, underlying their importance for human nutrition.

Some members of the carotenoid family of compounds, such as β-carotene, are precursors (provitamins) of vitamin A. Following ingestion by humans and animals, β-carotene is being converted into vitamin A. Low dietary intake of fruits, vegetables, and preformed sources of vitamin A consumed from animals, can often lead to vitamin A deficiency that causes acute health disorders. Vitamin A deficiency is an endemic nutrition problem throughout much of the developing world, especially affecting the health and survival of infants, young children, and pregnant and lactating women. One of the earliest manifestations of vitamin A deficiency is impaired vision, particularly in reduced light (night blindness). Other health consequences of vitamin A deficiency include impaired immunity, xerophthalmia, keratomalacia, growth and developmental problems among children, and increased risk of mortality (Mayne, 1996; West, 2003; Wintergerst et al., 2007). Noteworthy, excessive intake of vitamin A, manifested as hypervitaminosis A, can also lead to health disorders such as birth defects, liver problems, and reduced bone mineral density. However, these toxicities are usually related to overconsumption of the preformed sources of vitamin A (i.e., retinyl esters from animal foods, fortified foods, and pharmaceutical supplements). Carotenoid forms, such as β-carotene as found in fruits and vegetables, usually give no such symptoms (Penniston and Tanumihardjo, 2006).

Studies carried out since 1970 displayed a correlation between high intake of carotenoids and health benefits. These studies have suggested that diets high in carotenoids reduce the risk of chronic diseases such as lung, breast, prostate, and colorectal cancers; cataract and macular degeneration; light-induced erythema; and

cardiovascular diseases (recently reviewed by Fraser and Bramley, 2004; Levin et al., 2006).

Recent studies have suggested that the consumption of tomatoes and tomato-based products reduces the risk of chronic diseases such as cancer and cardiovascular diseases. This protective effect has been associated with carotenoids, which are one of the major classes of phytochemicals in this fruit. The most abundant carotenoid in ripe-red tomato is lycopene, followed by phytoene, phytofluene, ζ-carotene, γ-carotene, β-carotene, neurosporene, and lutein (Khachik et al., 2002). Although the proposed health benefits of tomato and tomato-based products are usually related to lycopene, the possibility that other phytochemicals in the tomato fruit also contribute to these protective properties should not be ignored. A recent study, in which the effect of tomato lycopene on low-density lipoprotein (LDL) oxidation in vitro was compared with the effect of oleoresin (a lipid extract of tomato containing 6% lycopene, 0.1% β-carotene, and 1% vitamin E), provides evidence for a concerted and/or synergistic activity of phytochemicals. The tomato oleoresin exhibited higher capacity to inhibit LDL oxidation in comparison to pure lycopene, by up to fivefold. In addition, lycopene was shown to have a synergistic effect on LDL oxidation with vitamin E and, to a lesser extent, with β-carotene (Fuhrman et al., 2000). From a nutritional point of view, these findings reinforce the advantage of consuming tomato oleoresin rather than pure synthetic lycopene as a dietary supplement.

Lycopene, lutein, and zeaxanthin are the major carotenoids found in human blood and tissues and may be protective in degenerative eye diseases because they absorb damaging blue light. These carotenoids may also protect the skin from light-induced damage (Johnson, 2002; Sies and Stahl, 2003).

Carotenoids and flavonoids have been shown to play a role in preventing cardiovascular diseases due to their antioxidative property. These compounds may function individually, or in concert, to protect lipoproteins and vascular cells from oxidation, which is widely hypothesized to be one of the major causes of atherosclerosis. This hypothesis has been supported by studies that associate reduced cardiovascular risk with consumption of antioxidant-rich foods. Other cardioprotective functions provided by plant phytonutrients may include the reduction of LDL, homocysteine, platelet aggregation, and blood pressure (Willcox et al. 2003). Oxidation of the circulating LDL (LDL_{ox}) may play a key role in the pathogenesis of atherosclerosis and coronary heart disease. It is suggested that macrophages inside the arterial wall take up the LDL_{ox} and initiate the process of plaque formation. Dietary antioxidants such as vitamin E and β-carotene have been shown to prevent the formation of LDL_{ox} and their uptake by microphages in vitro (Rao, 2002). Healthy human subjects ingesting lycopene, in the form of tomato juice, tomato sauce, and oleoresin soft gel capsules, for 1 week had significantly lower levels of LDL compared with controls (Rao and Agarwal, 1998). At present, however, the role of lycopene in the prevention of coronary heart disease is strongly suggestive. Although the antioxidant property of lycopene may be one of the principal mechanisms for its effect, other mechanisms may also be involved. Lycopene was shown to inhibit the activity of an essential enzyme involved in

cholesterol synthesis both in vitro and in a small clinical study suggesting a hypocholesterolemic effect. Other possible mechanisms include enhanced LDL degradation, effect on LDL particle size and composition, plaque rupture, and altered endothelial functions (Rao, 2002).

Several studies focusing on dietary assessment suggest that the intake of tomatoes and tomato products may also be associated with a lower risk of prostate cancer. It is possible that lycopene is one of the compounds in raw and processed tomato products that may contribute to the lower risk of that type of cancer. However, this hypothesis remains to be further investigated. A recent study has also found an association between higher plasma lycopene concentrations and lower risk of prostate cancer, among older participants (>65 years of age) without a family history of prostate cancer (Wu et al., 2004). Several carotenoids have also been shown to have an effect on the immune response: β-carotene, lutein, canthaxanthin, lycopene, and astaxanthin are active in enhancing cell-mediated and humoral immune responses in animals and humans (Chew and Park, 2004).

There is an increasing evidence suggesting that flavonoids, in particular those belonging to the class of flavonols (such as kaempferol and quercetin), are potentially health-protecting components in the human diet as a result of their high antioxidant capacity (Rice-Evans et al., 1997; Lean et al., 1999; Sugihara et al., 1999; Dugas et al., 2000; Duthie and Crozier, 2000; Ng et al., 2000; Proteggente et al., 2002) and their ability, in vitro, to induce human protective enzyme systems (Cook and Samman, 1996; Manach et al., 1996; Janssen et al., 1998; Choi et al., 1999; Frankel, 1999; Hollman and Katan, 1999; Shih et al., 2000). Based on these findings, it was postulated that flavonoids may offer protection against major diseases such as coronary heart diseases and cancer (Hertog and Hollman, 1996; Steinmetz and Potter, 1996; Trevisanato and Kim, 2000; Singh and Agarwal, 2006). In addition, several epidemiological studies have suggested a direct relationship between cardioprotection and consumption of flavonols from dietary sources such as onion, apple, and tea (Hertog et al., 1993; Keli et al., 1996). In this respect, anthocyanins have received particular attention because of their very strong antioxidant activity as measured by the *oxygen radical absorbing capacity* (*ORAC*) assay. Antioxidants such as carotenoids and flavonoids are potentially useful agents in the management of human neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and schizophrenia because one of the factors increasing the incidence of those diseases is accumulation of oxidative damage in neurons (Levin et al., 2006).

The antioxidant activity of flavonoids is thought to slow the aging of cells and to protect against lipid peroxidation. In vitro studies have also shown that flavonoids can inhibit, and sometimes induce, enzymatic systems. They are thought to reduce the proliferation of certain types of tumor cells (Zava and Duwe, 1997; Kawaii et al., 1999) and to be involved in the apoptosis of HL-60 leukemia cells (Ogata et al., 2000).

The flavonoid quercetin, also present in the tomato fruit, was shown to have a strong inhibitory action against cholesterol oxidation, a process leading to the formation of oxysterols, a potentially cytotoxic, mutagenic, atherogenic, and possibly carcinogenic compound found in many commonly processed foods. A

supplementation with quercetin was shown to have a blood pressure lowering effect on spontaneously hypertensive rats (Duarte et al., 2001).

As outlined above, flavonoids comprise a group of plant polyphenols. Polyphenols are in general related to human health and were recently related to the *French paradox*. The *French paradox* refers to the observation that the French suffer relatively low incidence of coronary heart disease, despite having a diet relatively rich in saturated fats (Ferrières, 2004). This low incidence of coronary heart diseases was ascribed to consumption of red wine and was initially attributed to resveratrol, a non-flavonoid polyphenol naturally present in red wine. However, a recent study has identified a particular group of flavonoid polyphenols, known as oligomeric proanthocyanidins (condensed tannins) (Fig. 12.2), which are believed to offer the greatest degree of protection to human blood vessel cells and, therefore, to reduced coronary heart diseases (Corder et al., 2006).

In contrast to their suggestive positive effects, potential risks have been associated with excessive intake of carotenoids and flavonoids as supplements. For instance, harmful properties were found for β-carotene (provitamin A) in particular when given to smokers or to individuals exposed to environmental carcinogens. It was hypothesized that under these circumstances β-carotene was acting as a pro-oxidant rather than an antioxidant (Omenn, 1998). Flavonoids, at high doses, may also act as mutagens, pro-oxidants that generate free radicals, and as inhibitors of key enzymes involved in hormone metabolism. For example, although the protective effect of the flavonoid, quercetin, from oxidative stress has been strongly implied, its excessive intake is suggested to have an adverse effect on the body (Formica and Regelson, 1995; Skibola and Smith, 2000; Galati and O'Brien, 2004; Bando et al., 2007). It was further found that catechol-type compounds, including quercetin, are able to act as pro-oxidants by generating *reactive oxygen species* (*ROS*) and semiquinone radicals during the autocatalytic oxidation process (Guohua et al., 1997; Metodiewa et al., 1999; Kawanishi et al., 2005). Thus, the effect of dietary supplement of phytochemicals on human health should be further investigated, taking into account genetic and environmental factors, as well as specific sub-populations such as smokers. Nevertheless, a diet rich in fruits and vegetables as a natural source for those health-promoting phytochemicals is recommended (Heber and Bowerman, 2001; Riboli and Norat, 2003; Key et al., 2004; Srinath and Katan, 2004; Lila, 2007).

12.5 Approaches for Modification of Metabolite Biosynthesis

Strategies to increase and diversify the content of a certain metabolite in plants focused initially on (1) transgenic modulation of structural genes involved in its biosynthesis, (2) transgenic modulation of genes encoding transcription factors or other regulatory genes affecting its metabolic pathway, and (3) mutations (spontaneous or induced) in structural or regulatory genes and/or quantitative trait loci with pronounced effects on such metabolite levels. Recently, manipula-

tions of metabolic sink to efficiently sequester the end-products of the carotenoid biosynthetic pathway were also shown to be very effective in the accumulation of carotenoid compounds in fruits and vegetables (Lu et al., 2006; Diretto et al., 2007; Kolotilin, et al., 2007; Li and Van Eck, 2007; Simkin et al., 2007).

Modifying a biosynthetic pathway to increase the amount of a desirable compound may be further divided into (Davies, 2007) manipulation of its pathway flux within an organism or introduction of its biosynthetic genes from other organisms. The methods for increasing, preventing, or redirecting flux into or within the pathway include increasing levels of a rate-limiting biosynthetic enzyme, inhibition of the activity of a gene that competes for a limited substrate supply, and up- or down-regulation of the pathway using regulatory factors. For reducing production of undesirable compounds, the well-proven approach is to inhibit gene activity for one of the biosynthetic enzymes. *RNA interference* (*RNAi*) is an effective and reliable approach for preventing enzyme production, with examples of better performance than using antisense or sense-suppression constructs (Nakamura et al., 2006).

Successful genetic engineering of biosynthetic pathways requires knowledge of the production and accumulation of the metabolites of interest, the availability of DNA sequences encoding appropriate biosynthetic enzymes or regulatory factors, and gene transfer methods for the target species. Given a sufficient knowledge of the target system, predictive metabolic engineering approaches may be applied, in which data from metabolomics, transcriptomics, and proteomics are used to identify key targets, such as flux control points or regulatory proteins (Dixon, 2005). However, at present, the required information and tools are available only for a few pathways and crops. For most pathways, there is incomplete knowledge of the genes involved, key flux points, regulatory factors, and the impact of cellular compartmentalization or metabolic channeling. Thus, in many cases, a reiterative "trial and error" approach has usually been used to achieve a successful genetic engineering of biosynthetic pathways (Davies, 2007). A detailed checklist of tools and prior considerations needed to obtain a successful metabolic engineering of plant secondary metabolism has been lately presented which properly illustrates the complexity of this issue (Dixon 2005). This checklist includes understanding the target pathway, taking into account knowledge of pathway intermediates and the enzymes/genes associated with it, availability of precursors for an introduced pathway, the choice of the right gene to engineer in the case of multigene families, understanding of related competing pathways, prediction of spillover pathways, understanding the tissue or cell specificity of the pathway, availability of tissue-specific promoters, knowledge of the inter- and intra-cellular transport mechanisms for intermediates and end-products of the pathway, and knowledge of transcriptional regulators of the pathway and their targets.

Mutations (spontaneous or induced) in structural or regulatory genes of biosynthetic pathways as well as quantitative trait loci with pronounced effects on such phytonutrient levels have proven to be an excellent tool for both pathway engineering and gene identification (Table 12.2). Of particular interest are the

tomato light-responsive *high-pigment* (hp) mutations: hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg}. The identification of genes that cause these mutations has created a conceptual link between genes-related light signaling and overproduction of an array of fruit phytonutrients (Mustilli et al., 1999; Levin et al., 2003; 2004; 2006; Lieberman et al., 2004; Sapir et al., 2008). Due to their importance, these mutations and the transgenic modulation of light signaling genes to increase the functional properties of the tomato fruit will be separately discussed in this chapter.

Table 12.2 Gene identification and map location for selected mutants that increase or modulate carotenoid content in ripe tomato fruits

Mutation	Description	Gene	Map location	Reference
R	Yellow color of ripe fruit flesh	*PSY1*	Chromosome 3	Fray and Grierson, 1993
B	Orange color of fruits, fruit β-carotene highly increased	*βLCY*	Chromosome 6	Ronen et al., 2000
og, og^{c}	Corolla tawny orange, fruit β-carotene highly reduced, ∼15–20% increase in fruit lycopene content	*βLCY*	Chromosome 6	Ronen et al., 2000
DEL	Fruit color orange due to the accumulation of δ-carotene at the expense of lycopene	*εLCY*	Chromosome 12	Ronen et al., 1999
T	Fruit flesh and stamens orange colored. Fruits accumulate prolycopene (7Z,9Z,7′Z,9′Z-tetra-*cis*-lycopene) instead of the all-*trans*-lycopene	*CARTISO*	Chromosome 10	Isaacson et al., 2002
hp-1, hp-1^{w}	Fruit carotenoids, including lycopene, highly increased	*DDB1*	Chromosome 2	Lieberman et al., 2004; Liu et al., 2004
hp-2, hp-2^{j}, hp-2^{dg}	Fruit carotenoids, including lycopene, highly increased	*DET1*	Chromosome 1	Mustilli et al., 1999; Levin et al., 2003
hp-3	Fruits accumulate 30% more carotenoids	*ZE*	Chromosome 2	Galpaz et al., 2007
gh	Fruits milky white, fruit phytoene increased	*PTOX*	Chromosome 11	Josse et al., 2000; Mackinney et al., 956

12.5.1 Non-transgenic Approaches of Modulating the Carotenoid Biosynthetic Pathway in the Tomato Fruit

Fruit quality has been a major focus of most classical tomato breeding programs during the past century (recently reviewed by Foolad, 2007). Color and nutritional quality are among the major tomato fruit quality characteristics of interest. The attention to tomato fruit color has recently increased as the health benefits of lycopene, the major carotenoid in tomato that is responsible for the red fruit color, have become more obvious (Di Mascio et al., 1989; Levy et al., 1995; Stahl and Sies, 1996; Gerster, 1997; Kohlmeier et al., 1997). Several major genes with significant contribution to high contents of fruit lycopene (e.g., the genes encoding the hp and og^c mutant phenotypes) and other carotenoids (e.g., beta-carotene, B) were previously phenotypically identified and mapped onto the classical linkage map of tomato (Wann et al., 1985; Stevens and Rick, 1986). In addition, during the past two decades, numerous *QTLs* (*quantitative trait loci*) and candidate genes with significant effects on fruit color and/or lycopene content were identified in tomato wild accessions such as *S. pimpinellifolium*, *S. peruvianum*, *S. habrochaites*, *S. chmielewskii*, and *S. pennellii* and mapped onto tomato chromosomes along with the previously identified genes (Foolad, 2007). While some of the identified QTLs mapped to the chromosomal locations of many of the known genes of the carotenoid biosynthesis pathway, many mapped to other locations (Liu et al., 2003). It was therefore suggested that there might be more genes affecting fruit color in tomato than those known to affect the carotenoid biosynthesis pathway (Liu et al., 2003).

Tomato mutant accessions with divergent color phenotypes in their fruits were the subject of molecular genetic studies, leading to the identification of genes responsible for such phenotypes. A selection of such mutants, their gene identification, and map location are presented in Table 12.2, while their characteristic color is shown in Fig. 12.3. The sequence of these genes can now serve as recombination-free DNA markers to expedite breeding toward altering pigmentation and enhancing nutritional value of plant foods. Of particular interest are the light-responsive hp mutations that will be dealt with herein below. Another mutant that is becoming of special interest is *t* (*tangerine*), which produces orange-colored fruits accumulating prolycopene (7Z,9Z,7'Z,9'Z-tetra-*cis*-lycopene) instead of the all-*trans*-lycopene that accumulates in regular red-fruited tomatoes (Fig. 12.3; Isaacson et al., 2002). *cis* isomers of lycopene, thought to be powerful antioxidants, have been shown to be more bioavailable than the *trans* isomer, indicating that they are more efficiently absorbed and, therefore, deliver lycopene into the plasma more effectively. This might be interpreted to mean that *cis* isomers of lycopene are more beneficial and, therefore, more valuable to human health than the *trans* isomer (Ishida et al., 2007). Results recently published support the hypothesis that lycopene *cis* isomers are highly bioavailable and suggest that special tomato varieties can be utilized to increase both the intake and the bioavailability of health-beneficial carotenoids (Unlu et al., 2007). Because light-responsive hp mutants are characterized by higher total fruit carotenoids, hp-1/hp-1 *t/t* double mutant fruits share almost double the content of *cis* isomers of lycopene, in comparison to non-hp, +/+ *t/t*, mutant fruits,

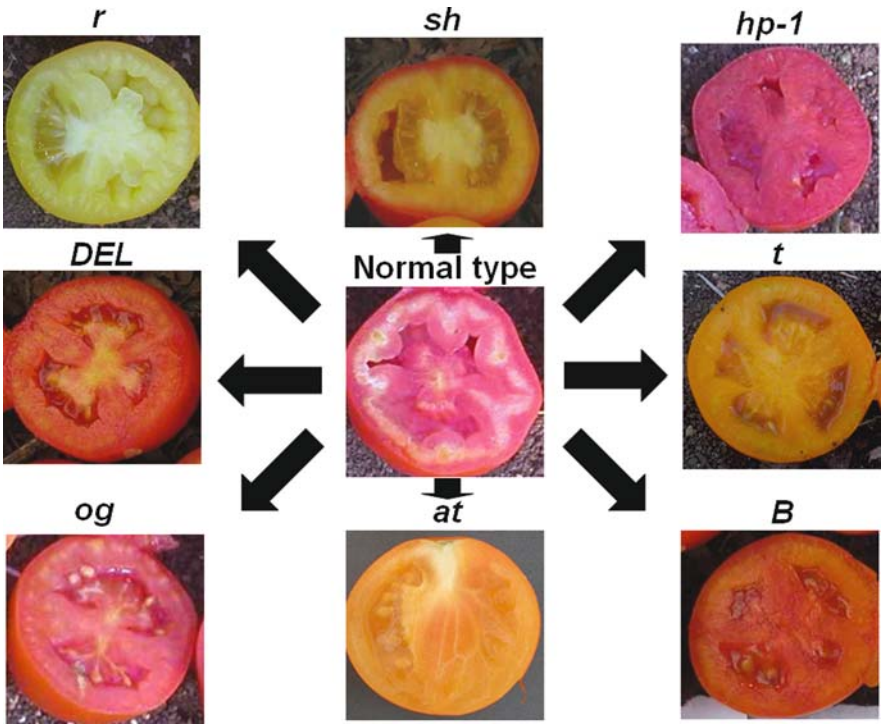

Fig. 12.3 Tomato fruit color mutants related to carotenoids biosynthesis. Abbreviations are as follows: *at* = *apricot*, *yellow-pink* color of fruit flesh; *B* = beta-carotene, high β-carotene, low lycopene in ripe fruit; *DEL* = delta, *Reddish-orange* mature fruit color, due to inhibition of lycopene, and increase of delta-carotene; *hp-1* = *high pigmen-1*, chlorophyll, carotenoids, ascorbic acid content of fruit intensified; *og* = *old gold*, increased fruit lycopene content; *r* = *yellow flesh*, yellow color of ripe fruit flesh; *sh* = *sherry*, fruit flesh yellow with reddish tinge; *t* = *tangerine*; fruit flesh and stamens orange colored

demonstrating the power of classical breeding to both modulate the profile and increase the content of selected carotenoids in the tomato fruit (Levin I, personal communication).

12.5.2 Non-transgenic Approaches of Modulating the Flavonoid Biosynthetic Pathway in the Tomato Fruit

Despite the relative success obtained in increasing flavonoid content in tomato fruits by transgenic modifications, there is an ongoing interest in breeding a high flavonoid tomato without genetic engineering (Willits et al. 2005). This interest is motivated by customers' reluctance to consume transgenic fruits and vegetables.

As recently summarized (Jones et al. 2003; Sapir et al., 2008), fruits of several tomato accessions, as well as species which are closely related to the cultivated

tomato, contain significantly higher amounts of anthocyanins (Giorgiev 1972; Rick 1964; Rick et al. 1994; Fig. 12.4). The Anthocyanin fruit (Aft, formerly Af) from *S. chilense*, Aubergine (ABG) from *S. lycopersicoides*, and the recessive *atroviolacium* (*atv*) mutation from *Lycopersicon cheesmaniae* cause anthocyanin expression in tomato fruit. We have also managed to introgress the trait of fruit anthocyanin expression from *S. peruvianum* accessions (Fig. 12.4), and recently, the wild species *S. pennellii* var. *puberulum* was shown to be a source for enriching tomato fruits with functional flavonoids (Willits et al., 2005).

Another approach to increase fruit flavonoids is through the introgression of the *high-pigment* (hp) mutations hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg}. These mutations are best known for their positive effect on carotenoid levels in ripe-red fruits (Levin et al., 2003; Mochizuki and Kamimura, 1984; van Tuinen et al., 2006; Wann et al., 1985). In addition, mature fruits of plants carrying the hp-1 mutation were also found to exhibit a 13-fold increase of the flavonol quercetin in tomato fruit pericarp relative to their isogenic counterparts (Yen et al., 1997). We have also shown similar increases in quercetin levels in fruits of the hp-2^{dg} mutant and in fruit skin of hp-2 and hp-2^{j} mutants (Bino et al., 2005; Levin et al., 2006).

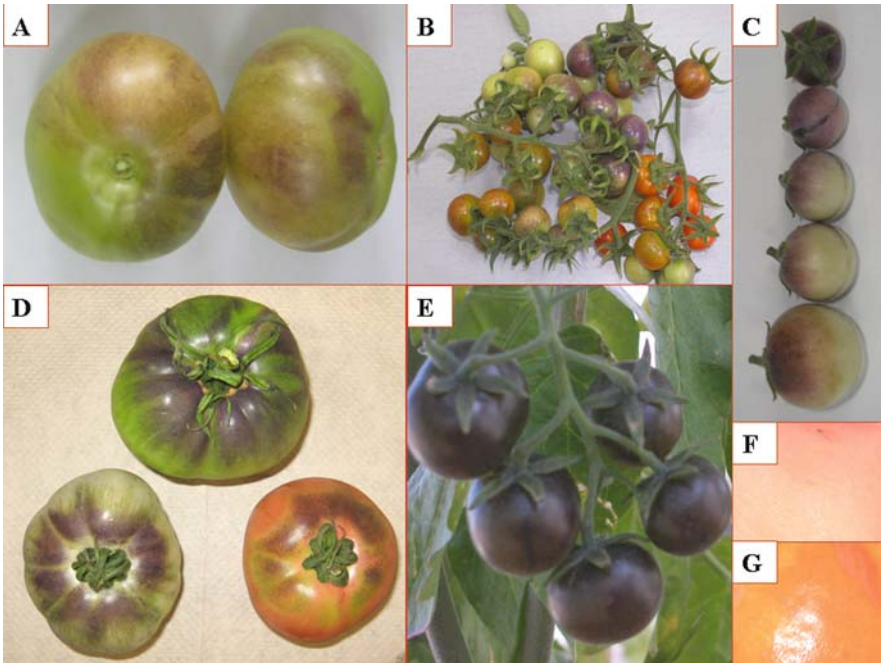

Fig. 12.4 Tomato fruit color phenotypes related to flavonoid biosynthesis. (**A**) Anthocyanin fruit (Aft) from *S. chilense*, (**B**) Aubergine (ABG) from *S. lycopersicoides*, (**C**) *S. peruvianum* (PI 128650), (**D**) Purple Smudge introgressed from *S. peruvianum*, (**E**) fruits of a double homozygous *AFT/AFT hp-1/hp-1* plant, (**F**) fruit skin from a tomato *y* mutant, and (**G**) fruit skin from regular tomato

Results recently presented in a textbook manuscript (van Tuinen et al., 2006), indicated that several phenolic compounds with high antioxidant capacity are new or increased in fruits of double mutant Aft/Aft hp-1^w/hp-1^w, as compared to fruits of single mutant parents. One of these compounds was identified as the flavonoid, rutin (van Tuinen et al., 2006). The hp-1^w mutation is as an extreme mutation (Lieberman et al., 2004), yielding plants with poor horticultural performances in comparison to its allelic hp-1 mutation. Thus, it has been of practical importance to also analyze the interaction between Aft and hp-1 mutants. We have recently shown that (Sapir et al., 2008) (1) Aft fruits are also characterized by significantly higher levels of the flavonols, quercetin and kaempferol, thus enhancing their functional value; (2) the tomato *ANT1* gene, encoding a MYB transcription factor, displayed nucleotide and amino acid polymorphisms between the Aft genotype, originating from *S. chilense*, and cultivated genotypes; (3) a DNA marker based on *ANT1* showed that the Aft trait is encoded by a single locus on chromosome 10 fully associated with *ANT1*; and (4) double homozygotes Aft/Aft hp-1/hp-1 plants displayed a more-than-additive (synergistic) effect on the production of fruit anthocyanidins and flavonols. This effect was manifested by ~5-, 19-, and 33-fold increases of petunidin, malvidin, and delphinidin, respectively, in the double mutants compared to the cumulative levels of their parental lines (demonstrated visually in Fig. 12.4).

Another important mutant related to the flavonoid biosynthetic pathway is the *y* mutant (Fig. 12.4). Fruits of this mutant are typified by colorless fruit epidermis, resulting in pinkish fruits that are preferred by consumers in most Asian countries. It is highly likely that the phenotype of the *y* mutant is attributed to major changes in the flavonoid pathway leading to the formation of naringenin chalcone, the yellow pigment accumulating in tomato fruit cuticle.

12.5.3 Metabolic Engineering of the Carotenoid Biosynthetic Pathway in Tomato

The economic value and health-promoting properties related to the tomato fruit make it an important target for increasing nutritional content either by traditional breeding or genetic manipulation. In view of the health-promoting properties of carotenoids and flavonoids, many attempts have been made to genetically modify the tomato fruit into overproduction of these phytochemicals. In most cases, this was achieved by modulating the expression of structural genes encoding biosynthetic enzymes of the dedicated pathway. In the carotenoid biosynthesis pathway, *phytoene synthase* (PSY), the enzyme that catalyzes the first committed step (Fig. 12.1), has been a preferred target for gene manipulation of the carotenoid biosynthetic pathway (Fraser and Bramley, 2004). The choice in PSY as such a target gene was also due to the fact that it exhibits the highest flux control coefficient among enzymes of the pathway, suggesting that it possess the greatest control over flux through the pathway (Fraser et al., 2002). Constitutive expression of

PSY in tomato plants resulted in earlier production of lycopene in the fruits of these transgenic plants, but the final concentration of lycopene was lower in these plants compared to their azygous controls. In addition, that manipulation has led to dwarfism, which is presumably caused by redirecting geranylgeranyl diphosphate (GGDP) from the gibberellin pathway into carotenoid synthesis (Fray et al., 1995; Fig. 12.1). In a later study, transformation of tomato plants with an additional PSY from *Erwinia uredovora* in a fruit-specific manner has led to a two to fourfold increase in total fruit carotenoids, whereas phytoene, lycopene, and β-carotene levels were increased 2.4-, 1.8-, and 2.2-fold, respectively. The transgene had no effect on the levels of related isoprenoids (tocopherols, plastoquinone, and ubiquinone), and the activities of other enzymes in the pathway were not significantly altered (Fraser et al., 2002).

Interestingly, increase in lycopene levels was also achieved by manipulation of the polyamines biosynthesis pathway. A two to threefold increase in lycopene was observed in tomato fruits expressing the yeast *S*-adenosylmethionine decarboxylase gene fused to a ripening-inducible *E8* promoter (Mehta et al., 2002).

Increase of β-carotene in tomato fruits has been achieved by various genetic manipulations. Constitutive expression of the bacterial phytoene desaturase (*PDS*) gene, which converts phytoene into lycopene, doubled the concentration of β-carotene in the fruit but halved the total carotenoid content. Interestingly, several endogenous carotenoid genes were up-regulated, except for PSY, which was repressed. These findings, coupled with the decrease observed in total carotenoids and the increase in β-carotene levels, suggest feedback inhibition within the pathway (Romer et al., 2000). Transgenic overexpression of the native lycopene β-cyclase gene in tomato fruit resulted in a 3.8-fold increase in the concentration of β-carotene, while the total carotene concentration was unchanged or slightly elevated. Transformation of an antisense construct of this same gene inhibited the enzyme expression by 50% and resulted in a slight increase in lycopene content (Rosati et al., 2000). Transgenic overexpression of the alternative lycopene β-cyclase has led to a greater increase in β-carotene concentration. That increase was accompanied by lower lycopene content in a manner that resembles the situation in the *B* mutant of tomato (Ronen et al., 2000). Recently, transgenic tomato lines containing a bacterial 1-deoxy-D-xylulose-5-phosphate synthase gene targeted to the plastid with the tomato DXS transit sequence resulted in increased carotenoid content (1.6-fold). Phytoene and β-carotene exhibited the greatest increases (2.4- and 2.2-fold, respectively). Extra-plastidic isoprenoids were unaffected in these lines (Enfissi et al., 2005).

Xanthophylls are an important class of target compounds, because of their antioxidant properties, their chemical stability, and the difficulties associated with their chemical synthesis. Metabolic engineering of xanthophyll content in tomato fruit was successfully achieved by overexpressing the *Arabidopsis* lycopene β-cyclase and the pepper β-carotene hydroxylase genes in a fruit-specific manner. This manipulation resulted in about tenfold increase in β-carotene level and accumulation of β-cryptoxanthin and zeaxanthin, two xanthophylls that were not detectable in the Moneymaker parental line (Dharmapuri et al., 2002).

12.5.4 Metabolic Engineering of the Flavonoid Biosynthetic Pathway in Tomato

There is a growing interest in producing food plants with increased amounts of flavonoids because of their potential health benefits. With several exceptions (Fig. 12.4), many tomato accessions contain only small amounts of flavonoids, which are usually produced in the peel of the fruit (Willits et al., 2005). Transformation of tomato plants with the chalcone isomerase (*CHI*) gene from *P. hybrida*, under the control of cauliflower mosaic virus (CaMV) 35S promoter, resulted in a dramatic increase in peel flavonoid levels. An up to 78-fold increase in flavonoids content was observed in transformed ripe-red fruits compared with the control, mainly due to an accumulation of rutin (Muir et al., 2001). In a different study, a significant accumulation of isoquercitrin, the immediate precursor to rutin, was achieved by ectopic expression of *CHI* (Olthof et al., 2000). Isoquercitrin is thought to be more bioavailable than rutin, the quercetin glycoside found in wild-type tomato peel and, thus, is considered to have a higher nutritional value. These studies show that ectopic expression of one gene, i.e., *P. hybrida CHI*, is sufficient to increase flavonol accumulation in tomato peel. However, no increase in flavonol levels were observed in leaves and in green, breaker, and turning tomato flesh from high flavonol transgenic plants, although relatively high levels of *CHI* transcripts were detected in these tissues. This indicates that, in tomato, flavonoid biosynthesis is subject to tissue-specific regulation and that in order to achieve a significant increase in flavonol accumulation in tomato flesh, a different approach is required (Willits et al., 2005). Indeed, flavonoid accumulation in tomato flesh, and hence an overall increase in flavonoid levels in tomato fruit, was achieved by simultaneous overexpression of the maize genes encoding the transcription factors LC and C1. LC and C1 are members of MYC- and MYB-type transcription factors families, respectively, that control the expression of several structural genes in the pathway leading to anthocyanins in maize (Dooner et al., 1991). Fruit-specific expression of both *LC* and *C1* genes had caused an up to 60-fold increase in kaempferol glycosides in tomato flesh tissue and an overall increase in total fruit flavonols of up to 20-fold (Bovy et al., 2002). In an alternative approach, genes encoding four key biosynthetic enzymes from *P. hybrida* leading to flavonols, chalcone synthase (CHS), CHI, flavanone-3-hydroxylase (F3H), and flavonol synthase (FLS), were ectopically and simultaneously expressed in tomato plants. About 75% of the primary transformants containing all four transgenes accumulated very high levels of quercetin glycosides in the peel and more modest but significantly increased levels of kaempferol- and naringenin-glycosides in columella tissue (Verhoeyen et al., 2002). That study has also shown that *CHS* and *FLS* appear to be the key genes leading to flavonol biosynthesis in tomato flesh (pericarp and columella) tissue. While ectopic expression of *CHS* alone resulted in increased levels of naringenin-glycosides, but no increase in flavonols, and the *FLS* transgene showed no significant effect by itself, expression of both *CHS* and *FLS* had a *synergistic effect* resulting in a significant accumulation of both naringenin glycosides and kaempferol glycosides (flavonols) in tomato flesh. Apparently, CHI is the key enzyme for flavonol accumulation in tomato peel,

while CHS and FLS enzymes are required for the production of flavonols in flesh tissue. Therefore, it was reasoned that, in order to achieve increased flavonol accumulation throughout the tomato fruit, ectopic expression of three genes encoding the biosynthetic enzymes CHS, CHI, and FLS would be needed. Indeed, cross harboring these three genes accumulates increased levels of quercetin glycosides in peel and kaempferol glycosides in flesh (Colliver, unpublished results). It is also noteworthy that a similar phenotype can be achieved by crossing tomatoes containing *LC* and *C1* transgenes with tomatoes containing the *CHI* transgene – the only structural gene for the production of kaempferol-type flavonols and pelargonidin-type anthocyanins that was not strongly induced by the LC/C1 transcription factors (Muir, unpublished results).

Further, T-DNA activation-tagging experiments in tomato identified a *MYB* transcriptional regulator of anthocyanin biosynthesis, termed *ANT1*, which shares high homology with *Petunia AN2* (Mathews et al., 2003). These ant1 mutant tomato

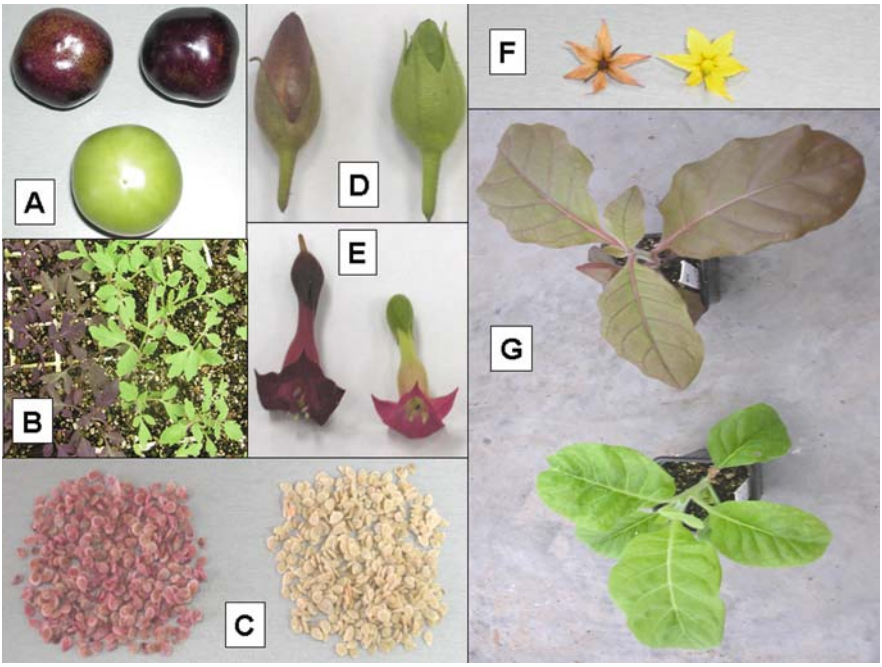

Fig. 12.5 Phenotypes obtained by overexpression of the *ANT1*gene in tomato and tobacco. (**A**) Transgenic tomato fruits (*upper two fruits*) in comparison to a non-transgenic fruit (*lower fruit*), (**B**) transgenic tomato seedlings (*left*) in comparison to their non-transgenic counterparts (*right*), (**C**) transgenic tomato seeds (*left*) in comparison to their non-transgenic counterparts (*right*), (**D**) a transgenic tobacco fruit (*left*) in comparison to its non-transgenic counterpart (*right*), (**E**) a transgenic tobacco flower (*left*) in comparison to its non-transgenic counterpart (*right*), (**F**) a transgenic tomato flower (*left*) in comparison to its non-transgenic counterpart (*right*), and (**G**) a transgenic tobacco seedling (*upper*) in comparison to its non-transgenic counterpart (*lower*)

plants yielded fruits with purple spotting on the fruit epidermis and anthocyanin-pigmented leaves and flowers. Overexpression of *ANT1*, controlled by the cassava vein mosaic promoter, generated similar phenotypes in Micro-Tom tomato plants and anthocyanin-pigmented leaves in tobacco plants. We have recently transformed the *ANT1* gene under the control of 35S promoter and obtained much stronger phenotypes in transformed Moneymaker tomato plants and similar phenotypes in tobacco plants. These results, which are presented in Fig. 12.5, visually demonstrate the immense potential of up-regulating the flavonoid biosynthetic pathway by transcription factors.

Recently, transgenic tomato plants accumulating new flavonoid compounds in their fruit peel were engineered using structural flavonoid genes from different plant sources (Schijlen et al., 2006). In this study, structural flavonoid genes (encoding *stilbene synthase*, *chalcone synthase*, *chalcone reductase*, *chalcone isomerase*, and *flavone synthase*) from different plant sources were used to produce transgenic tomatoes accumulating new phytochemicals. Biochemical analysis showed that the fruit peel contained high levels of stilbenes (resveratrol and piceid), deoxychalcones (butein and isoliquiritigenin), flavones (luteolin-7-glucoside and luteolin aglycon), and flavonols (quercetin glycosides and kaempferol glycosides). Using an online high-performance liquid chromatography (HPLC) antioxidant detection system, it was possible to demonstrate that, due to the presence of the novel flavonoids, the transgenic tomato fruits displayed altered antioxidant profiles. In addition, total antioxidant capacity of tomato fruit peel with high levels of flavones and flavonols increased more than threefold.

12.6 The Tomato Photomorphogenic Light-Responsive hp Mutants

Plants respond to light intensity, direction, duration, and spectral quality by modulating their developmental processes in an array of interactions that are referred to as *photomorphogenesis*. Photomorphogenic mutants have proven to be an excellent tool in the study of the complex interactions between light and plant development, and some of them have also been harnessed in several breeding programs of agricultural crops. Photomorphogenic mutants have been reported in a number of species, including *Arabidopsis*, *Sorghum*, *Brassica*, tobacco, tomato, and pea. In general, these mutants may be classified either as defective in photoreceptors or altered in some element of the light signal transduction pathway (Chory, 1993).

Several photomorphogenic mutants have been described in tomato (van Tuinen et al., 1997). Among these, mutants carrying the monogenic recessive *high-pigment* (hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg}) mutations are characterized by their exaggerated light responsiveness. These mutants display higher anthocyanin levels, shorter hypocotyls, darker foliage, and higher fruit pigmentation than their isogenic normal counterparts (Mochizuki and Kamimura, 1984; Wann et al., 1985, Peters et al., 1989, Mustilli et al., 1999; Levin et al., 2003). The high pigmentation of fruits of these mutants is due to significantly elevated levels of chlorophylls at most of their

pre-mature developmental stages. The mature ripe-red fruits of these mutants are characterized by an intense red color which is mainly due to increased levels of carotenoids, primarily lycopene. Because of their effect on fruit color, attributed to enhanced lycopene content, hp mutations have been introgressed into several commercial processing and fresh-market tomato cultivars that are currently marketed as lycopene-rich tomatoes (LRT) (Wann, 1997; Levin et al., 2006). The processing tomato varieties are primarily cultivated for the purpose of lycopene extraction, which is further used as food additive, food supplement, and food colorant in many processed products (http://www.lycored.com/). Current processing tomato cultivars harboring such mutations can reach a remarkable up to 3.5-fold increase in fruit lycopene content (from 80 to 280 $\mu g \cdot g^{-1}$ FW). Interestingly, this increase is higher than that reported thus far using the genetically modified alternatives discussed herein above.

The origins of hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg} mutations have been lately extensively summarized (Lieberman et al., 2004; Levin et al., 2006). Further, the hp-2, hp-2^{j}, and hp-2^{dg} mutations were mapped to the gene encoding the nuclear protein DEETIOLATED1 (DET1), a central negative regulator of photomorphogenesis (Mustilli et al., 1999, Levin et al., 2003). The gene encoding the hp-1 and hp-1^{w} mutant phenotypes has also been recently identified (Lieberman et al., 2004) and later independently confirmed by an additional laboratory (Liu et al., 2004). Results show that hp-1 and hp-1^{w} are alternative alleles at the tomato gene encoding UV DAMAGED DNA BINDING protein 1 (DDB1), recently shown to interact both biochemically and genetically with the DET1 protein (Schroeder et al., 2002, Liu et al., 2004). DDB1 is a protein evolutionally conserved from fission yeast to humans. It was initially identified, together with DDB2, as a subunit of a heterodimeric protein complex that recognizes the UV-induced DNA lesions in the nucleotide excision repair pathway. Mounting evidence has now established a major role of DDB1 as a substrate-recruiting subunit of the Cullin 4(CUL4)-based E3 ubiquitin ligase complexes that also contain RBX1 (also named ROC1), DET1 and, in the case of *Arabidopsis*, COP10 as well (Wertz et al., 2004; Hu et al., 2004; Bernhardt et al., 2006; Chen et al., 2006).

Tomato hp mutations (hp-1, hp-1^{w}, hp-2, hp-2^{j}, and hp-2^{dg}) are best known for their positive effect on carotenoid (lycopene and carotenes) levels in ripe-red fruits (Mochizuki and Kamimura, 1984; Wann et al., 1985, Levin et al., 2003). Interestingly, however, mature fruits of plants carrying the hp-1 mutation were also found to exhibit a 13-fold increase of the flavonoid, quercetin, in tomato fruit pericarp (Yen et al., 1997) and also some increase in ascorbic acid (vitamin C) (Mochizuki and Kamimura, 1984). In a study carried out during a summer season, similar increases were identified in quercetin levels in the fruit peel of the tomato mutants hp-2 and hp-2^{j} compared to their isogenic normal counterparts (Levin et al., 2006). These results suggest that other metabolites may be increased in tomato hp mutants. To validate this hypothesis, the overall metabolic modifications between hp-2^{dg} tomato mutant fruits and their isogenic non-mutant counterparts were compared (Bino et al., 2005). Targeted metabolite analyses, as well as large-scale non-targeted mass spectrometry (MS)-based metabolite profiling, were used to phenotype the differences

in fruit metabolite composition. Targeted high-performance liquid chromatography with photodiode array detection (HPLC–PDA) metabolite analyses showed higher levels of isoprenoids and phenolic compounds, as well as vitamin C, in hp-2^{dg} fruits. A selected list of such metabolites including their average levels in ripe-red fruits and their fold increase in hp-2^{dg} were presented in Levin et al. (2006). Non-targeted GC–MS profiling of red fruits produced 25 volatile compounds that showed a 1.5-fold difference between the genotypes (Bino et al., 2005). Analyses of red fruits using HPLC coupled to high-resolution quadruple time-of-flight mass spectrometry (LC–QTOF–MS) in both ESI-positive and ESI-negative modes generated, respectively, 6168 and 5401 mass signals, of which 142 and 303 showed a twofold difference between the genotypes. Of this total of 443 mass signals, 383 (∼86%) were up-regulated in the hp-2^{dg} genotype, while only 62 (∼14%) were found down-regulated in that mutant (Bino et al., 2005). Overall, these results show that the hp-2^{dg} fruits are more active metabolically and are characterized by overproduction of many metabolites, several of which are known for their antioxidant or photo-protective activities. It was hypothesized that these metabolites may serve as resources recruited by plants to respond to and manage light stress. Because hp-2^{dg} is highly iso-phenotypic to other tomato hp mutants, similar metabolic responses are also expected in these other mutants.

A transcriptional profiling study was also carried out on fruits harvested from hp-2^{dg} mutant plants in comparison to their isogenic counterparts using microarray technology (Kolotilin et al., 2007). Results show that a large portion of the genes that are affected by hp-2^{dg} mutation display a tendency for up- rather than down-regulation, indicating that this genotype is more active transcriptionally as well. Ontology assignment of these differentially regulated transcripts revealed a consistent up-regulation of transcripts related to chloroplast/chromoplast biogenesis and photosynthesis in hp-2^{dg} mutants throughout fruit ripening. A tendency of up-regulation was also observed in structural genes involved in phytonutrient biosynthesis. However, this up-regulation was not as consistent, positioning plastid biogenesis as a more important determinant of phytonutrient overproduction in hp-2^{dg} and possibly other hp mutant fruits. These results were linked to microscopic observations that revealed a highly significant increase in chloroplast/chromoplast size and number in pericarp cells of mature-green hp-2^{dg}/hp-2^{dg} and hp-2^{j}/hp-2^{j} fruits in comparison to their normal counterparts.

As noted herein, the identification of genes responsible for the light-responsive hp mutant phenotypes has created a conceptual link between light cues and overproduction of fruit phytonutrients, primarily those that accumulate in the plastids. It was further shown that in these mutants plastid biogenesis is the major determinant of the drive that increases these phytonutrients (Kolotilin et al., 2007). Interestingly, these concepts were also lately documented in the characterization of tomato plants mutated at the zeaxanthin epoxidase (*ZE*) gene (Galpaz et al., 2007) and its overexpression in an additional study (Wang et al., 2008). Fruits harvested from the mutant, termed hp-3, displayed 30% more carotenoids in the mature fruit compared to their isogenic normal counterparts. This increase in fruit carotenoids content was accompanied by at least a twofold increase in plastid compartment size (Galpaz et al.,

2007). In addition, constitutive overexpression of *ZE* in tomato plants characterized in a later study was found to display enhanced sensitivity of the tomato plants to photo-inhibition caused by high light stress (Wang et al., 2008).

12.6.1 Light Signal Transduction as a Target for Nutritional Enhancement

As indicated above, tomato hp mutants plants are characterized by overproduction of many metabolites, some of which possess antioxidant or photo-protective activities. The genes responsible for these mutations have been cloned and represent tomato homologs of light signal transduction regulatory genes, previously described in *Arabidopsis*. Therefore, targeting the light signaling pathway might be an effective approach to engineer fruit nutritional quality. Although carotenoid accumulation in edible plant tissues has been manipulated by altering corresponding biosynthetic enzymes (e.g., "golden" rice, Beyer et al., 2002), the outcome of such approaches has at times fallen short of expectations, as summarized above. This is probably because of a lack of understanding regarding endogenous mechanisms of regulation and accumulation of carotenoids and/or undesirable side effects on non-target metabolites derived from the altered pathway (Fray et al., 1995; Beyer et al., 2002; Liu et al., 2004). Engineering of an existing signal transduction network already capable of regulating flux through the carotenoid synthesis pathway in a biologically viable manner might represent an alternative to optimizing the carotenoid-associated nutritional benefit in plant tissues such as fruit (Liu et al., 2004). Indeed, recently it has been shown that manipulating tomato light signal transduction genes homologous to HY5 and COP1 from *Arabidopsis* can result in modified fruit carotenoid accumulation in tomatoes (Liu et al., 2004). Down-regulated LeHY5 plants exhibit defects in light responses, including inhibited seedling photomorphogenesis, loss of thylakoid organization, and reduced carotenoid accumulation. In contrast, repression of LeCOP1like expression results in plants with exaggerated photomorphogenesis, dark green leaves, and elevated fruit carotenoid levels. Manipulation of DET1 expression in tomato resulted in photomorphogenic phenotypes caused by post-transcriptional gene silencing and fruits with increased carotenoids (Davuluri et al., 2004). These results were later supplemented by fruit-specific RNAi-mediated suppression of DET1, resulting in increased fruit flavonoid content in addition to carotenoids (Davuluri et al., 2005).

Antisense tomato plants carrying the C-terminal portion of the tomato cryptochrome 1 (*TCRY1*) gene have also been characterized (Ninu et al., 1999). Synthesis of anthocyanins under blue light was reduced in antisense seedlings. In contrast, carotenoid and chlorophyll levels were essentially unaltered. Tomato cryptochrome 2 overexpression, on the other hand, resulted in a high-pigment phenotype, with overproduction of anthocyanins and chlorophyll in leaves and of flavonoids and lycopene in fruits. The accumulation of lycopene in fruits was accompanied by the decreased expression of lycopene β-cyclase genes (Giliberto et al., 2005). These results finally confirm the hypothesis that genes encoding

components of the light signal transduction machinery also influence fruit pigmentation and thus represent powerful tools for the manipulation of tomato fruit nutritional quality. Because light signaling genes are evolutionarily highly conserved, it seems reasonable that they may have an impact on the nutritional quality in plant species other than the tomato, including species that are distantly related to the tomato.

12.7 Outstanding Examples of Engineering Metabolic Pathways in Other Plant Species

Metabolic engineering of the carotenoid, flavonoid, and other metabolic pathways in the tomato and other species has been recently extensively reviewed (Galili et al., 2002; Fraser and Bramley, 2004; DellaPenna and Pogson, 2006; Yonekura-Sakakibara and Saito, 2006; Davies, 2007; Li and Van Eck, 2007). In addition to the tomato, efforts to up-regulate synthesis of carotenoids were also invested in agricultural species such as the potato (*Solanum tuberosum*) and rice (*Oryza sativa*), while synthesis of flavonoids was successfully up-regulated in potato and corn (*Z. mays*). In addition, major metabolic engineering efforts were carried out to modulate levels of other phytonutrients such as tocopherols, vitamin C, iron, selenium, and zinc (Davies, 2007; DellaPenna and Pogson, 2006; Li and Van Eck, 2007). Outstanding in this regard are the "golden" rice (Fig. 12.6), achieved by a transgenic approach, and the *Orange* (*Or*) gene mutation identified in cauliflower (*Brassica oleracea*, Fig. 12.6). In both cases, accumulation of high levels of β-carotene was conferred in tissues that are normally devoid or contain very low levels of carotenoids.

The apparent lack of high levels of carotenoid accumulation in low-pigmented tissues of crops such as rice endosperm and cauliflower curds could be due to (1) low metabolic flux into the carotenoid biosynthetic pathway, (2) high metabolic flux out of the carotenoid biosynthetic pathway into branching points and/or toward non-carotenoid end-products, (3) inactivation and absence of key genes in the biosynthetic pathway, and (4) lack of a deposition sink to efficiently sequester the end-products of the carotenoid biosynthetic pathway. While modulating metabolic flux by structural or regulatory genes of metabolic pathways was demonstrated above, and recently elsewhere (Davies, 2007; DellaPenna and Pogson, 2006), the "golden" rice and the *Or* gene mutation exemplify, respectively, the latter two possibilities.

The "golden" rice was named for its bright yellow endosperm due to the production and accumulation of β-carotene, a precursor of vitamin A, which is normally not produced in regular rice (Fig. 12.6). Engineering "golden" rice was designed to combat vitamin A deficiency in third-world Southeast Asian countries in which rice is a major nutritional commodity. The "golden" rice was first engineered with the insertion of the *PSY* gene from daffodil (*Narcissus pseudonarcissus*) and the bacterial *phytoene desaturase* (*CrtI*) gene from *E. uredovora*, which can catalyze three enzymatic steps from phytoene to all-*trans*-lycopene (Ye et al., 2000). The *PSY* gene was inserted under the control of an endosperm-specific glutelin

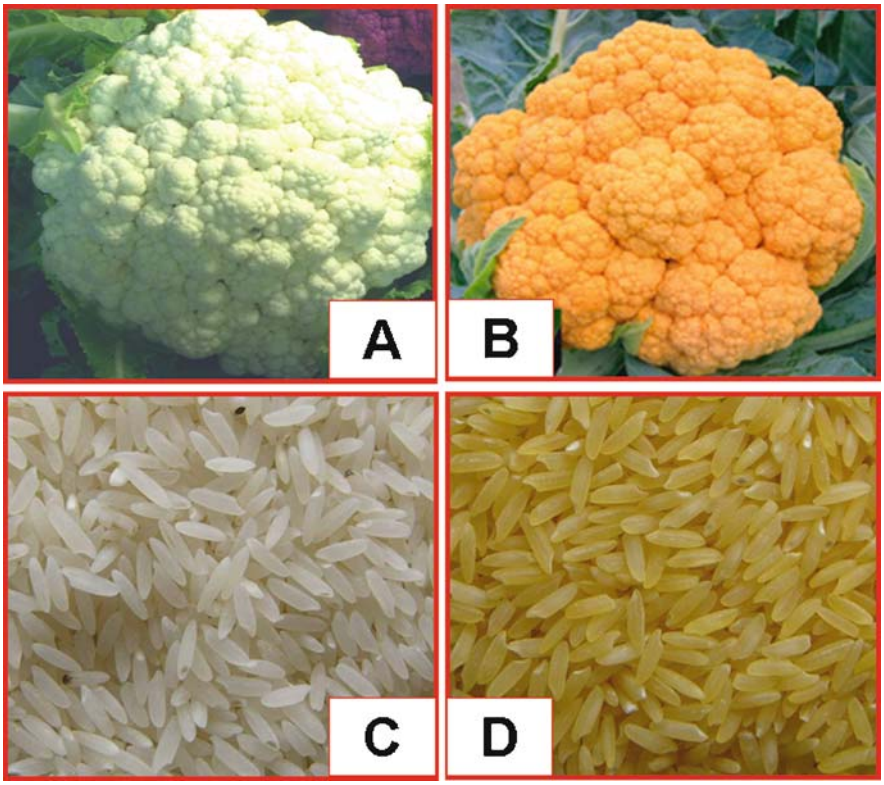

Fig. 12.6 Phenotypes of the *Or*mutant and the "golden" rice. (**A**) Regular cauliflower, (**B**) *Or* mutant cauliflower, (**C**) regular rice, and (D) "golden" rice

promoter, and in order to localize the gene product to the plastids (site of carotenoid biosynthesis), *CrtI* was designed as a fusion with the transit peptide of RUBISCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) small subunit under the control of 35S promoter. An alternative construct was made by co-transformation with constructs carrying the *PSY/CrtI* gene, as described above, and the *LCY* gene under the control of a glutelin promoter. By the latter approach, the carotenoid content of edible rice endosperm was about 1.6 $\mu g \cdot g^{-1}$ dry weight (Ye et al., 2000).

In 2005, "golden" rice 2 was developed and the β-carotene content was increased up to 23-fold (about 37 $\mu g \cdot g^{-1}$ dry weight) compared to the original "golden" rice, a level adequate to provide the recommended dietary allowance of provitamin A for children in an average daily consumption of rice. The higher β-carotene content was achieved by choosing the maize *PSY* gene rather than the *PSY* genes from *Arabidopsis*, daffodil or the carotenoid-accumulating vegetables such as tomato, bell pepper, and carrot (Paine et al., 2005).

Recently, a similar approach has been employed to successfully produce "golden" potato tubers (Diretto et al., 2007). Earlier, seed-specific overexpression of a bacterial phytoene synthase gene (*crtB*) in a seed-specific manner produced

"golden" canola (*Brassica napus*) seeds containing up to 50-fold higher total carotenoids (Shewmaker et al., 1999).

Another novel alternative approach to increasing metabolites in plant tissues emerged from the recent work on isolation and functional characterization of the carotenoid gene mutation, denoted *Or,* in cauliflower (Fig.12.6; Lu et al., 2006). *Or* is a spontaneous semi-dominant mutation that confers the accumulation of high levels of β-carotene in various tissues normally devoid of carotenoids (Li and Van Eck, 2007).

The *Or* gene was found to encode a DnaJ cysteine-rich domain-containing protein. Rather than directly regulating carotenoid biosynthesis, the *Or* gene appears to mediate the differentiation of proplastids and/or non-colored plastids (leucoplasts) in apical shoot and inflorescence meristematic tissues of the curds into chromoplasts for the associated carotenoid accumulation (Lu et al., 2006; Li and Van Eck, 2007). Transformation of the *Or* gene into wild-type cauliflower converts the white color of curd tissue into distinct orange color with increased levels of β-carotene (Fig. 12.6). Examination of the cytological effects of the *Or* transgene revealed that expression of the *Or* transgene leads to the formation of large membranous chromoplasts in the cauliflower curd cells of the *Or* transformants (Lu et al., 2006). Interestingly, when the *Or* gene, under the control of a potato granule-bound starch synthase promoter, was introduced into potato, it resulted in the production of tubers with orange-yellow flesh (parenchymatous tissue). The total carotenoid levels in the *Or* transgenic potato lines were up to sixfold higher than in the non-transformed controls. Further examination of the cellular contents of these transgenic tubers by light microscopy showed that while the tubers in the controls contain exclusively various sizes of starch grains in amyloplasts, the *Or* transgenic tubers have additional orange bodies. These orange bodies include intact chromoplasts and a large number of more sharply outlined orange structures of helical sheets and fragments released from chromoplasts. These results and those of others have led to the conclusion that *Or* gene-associated carotenoid accumulation in these transgenic tubers is most likely due to the formation of carotenoid sequestering structures in chromoplasts, which provide a metabolic sink to facilitate accumulation of carotenoids. It was thus demonstrated that successful metabolic engineering of carotenoid accumulation can be also achieved by creating a metabolic sink (Li and Van Eck, 2007).

This conceptual approach was also recently tested in tomatoes following overexpression of fibrillin (Simkin et al., 2007). Fibrillin is involved in the formation of lipoprotein structures, such as plastoglobules and fibrils in certain chromoplast types, which have been implicated in the overproduction of pigments due to a sink effect. In order to examine its effect in differentiating chromoplasts of a non-fibrillar type, the pepper fibrillin gene was expressed in tomato fruits. Both the transcript and protein were found to accumulate during tomato fruit ripening from an early mature-green stage. However, formation of carotenoid deposition structures in tomato chromoplasts, such as fibrils, was not observed. Nevertheless, a twofold increase in carotenoid content and associated carotenoid-derived flavor volatiles (6-methyl-5-hepten-2-one, geranylacetone, β-ionone, and β-cyclocitral) was observed. The transgenic fruit displayed delayed loss of thylakoids in differentiating chromoplasts,

leading to the transient formation of plastids exhibiting a typical chromoplastic zone adjacent to a protected chloroplastic zone with preserved thylakoids. These results therefore suggest that fibrillin may protect plastids against degradation, thus extending their carotenoid production life span and leading to greater carotenoid accumulation. In this respect, the recent transcriptional profiling carried out on hp-2^{dg}fruits has underlined plastid number as the main contributor to plastid-accumulating phytonutrients (Kolotilin et al., 2007). This study has further shown that in mature-green fruits harvested from hp-2^{dg} mutant plants, the plastid compartment size is 8.4-fold higher as compared to its normal counterpart, suggesting a similar potential to increase fruit carotenoid content. However, upon ripening, a sharp decrease was observed in plastid compartment size in fruits of hp-2^{dg}, primarily attributed to a sharp decrease in plastid number, which was much more attenuated in their normal counterparts. Ripe-red fruits of the hp-2^{dg} mutant were characterized by only ~2.8-fold increase in chromoplast compartment compared to their normal counterpart. This increase corresponds to the 2.3-fold increase usually observed in total carotenoids between these genotypes at this ripening stage. These results cumulatively suggest that prevention of the enhanced plastid degradation observed upon ripening in hp-2^{dg} mutant fruits could potentially be a target to increase carotenoid accumulation in these mutant fruits. Such prevention of plastid degradation could be possibly achieved via overexpression of fibrillin in hp-2^{dg} mutant plants. An alternative approach to achieve higher carotenoid accumulation in hp-2^{dg} mutant plants could be via overexpression of DnaJ to create an alternative metabolic sink.

12.8 Concluding Remarks and Perspectives

It is now becoming recognized that consumption of fruits and vegetables can prevent or even be used to treat chronic human diseases. However, this recognition is mainly supported by in vitro and by epidemiological studies that seem to vary between sub-populations. There is therefore a need for more clinical in vivo trials to substantiate these effects on a whole organism basis and in different human sub-populations. There is also a need to formulate appropriate directives for recommended daily allowance for each metabolite in each sub-population.

It is predicated that the recent completion of the human genome sequence, the advances made in high-throughput technologies, and the emerging area of nutragenomics will uncover more precisely the possible relationship between human genetic makeup and the type and quantity of phytonutrients needed to maintain proper health. This may position phytonutrient consumption behavior in humans more at the level of pharma- rather than nutraceuticals with recommendations for a critical dosage rather than a daily allowance. In other words, food may become medicine and vice versa, in accordance with Hippocrates statement, “Let thy food be thy medicine and thy medicine be thy food”.

Meanwhile, transgenic genetic modifications (GMO) have already been exploited and found to be useful in enriching and diversifying the content of phytonutrient metabolites in a variety of plant species. As outlined in this chapter, these

modifications can be justified, but it is not entirely clear whether consumption of plant foods highly enriched with a certain phytonuterients will indeed contribute to maintenance of proper health and/or to treat chronic human diseases.

Despite the relative success obtained in increasing the phytonutrient content of plant foods by GMO modifications, consumers, in particular those that share higher health awareness, are reluctant to consume transgenic plant foods. Luckily, several genetic resources such as the tomato light-responsive hp mutants and the *Or* gene mutation identified in cauliflower show that there are efficient non-GMO alternatives to increase phytonutrient content in plant foods. Of particular interest are the tomato hp mutants characterized by higher levels of both carotenoids and flavonoids in the fruits. Moreover, ripe-red fruits, harvested from these mutants, also display increased levels of several other metabolites, including vitamins C and E. Thus, consumption of fruits of this type may maximize positive synergistic health effects that were already documented among several of these phytonutrients.

The genes that cause hp mutant phenotypes were cloned and identified as two evolutionarily conserved genes active in light signal transduction, known also as photomorphogenesis. The identification of the genes that encode the hp mutant phenotypes has therefore created a conceptual link between photomorphogenesis and biosynthesis of fruit phytonutrients and thus point to modulation of light signal transduction machinery as an effective approach toward practical manipulation of the kinds and amounts of fruit phytonutrients. The high-evolutionary conservation of these genes also suggests that similar effects may be obtained by manipulating these genes in plant species other than the tomato either by transgenic or non-transgenic methodologies.

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