Chunxian Chen *Editor*

Pigments in Fruits and Vegetables

Genomics and Dietetics

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Preface

Colors are ubiquitous in nature, particularly in living organisms ranging from bacteria and fungi to plants and animals. Many organisms have developed their own characteristic colors that vary by parts and developmental stage. These colors are not just visually decorative and attractive, but biologically essential in reproduction, coevolution, and ecosystem sustenance. Colors in plants, flowers, and fruits attract animals for pollination to produce seeds and for consumption to disperse seeds, which both help in species reproduction and diversification. Coloration-based camouflage in ecosystems to enhance survival is a good example of coevolution. The importance of colors in living organisms cannot be overstated. An old saying is apt: Colors can please the eye, gladden the heart, and nurture the mind. Biological pigments, the chemical components able to generate a full spectrum of visual colors in nature, are in fact much more important and valuable; they are biosynthesized behind the scene in living organisms and ultimately ingested in our daily diet.

Pigments produced in plants include four major classes: chlorophylls, carotenoids, flavonoids, and betalains. Chlorophylls are the primary green pigments for photosynthesis. The latter three are complementary nongreen pigments with diverse functions. Extensive research on the genetic mechanisms of their biosynthesis has yielded many exciting and insightful results over the last decades. On the other hand, many pigment-rich fruits and vegetables are consumed daily by human and animals. Potential nutritional and medicinal benefits from these pigments in fruits and vegetables have attracted nutritionists and clinical functional food researchers to study their health effects and encourage people to increase the daily consumption of these pigment-abundant foods.

Colorful fruits and vegetables attract visitors and eaters. Eating freshly harvested colorful vegetables while helping in my parents' vegetable garden remains among the most memorable moments in my childhood. Green cucumber and pea, red tomato and radish, and orange carrot and sweet potato, to name a few, are my favorites. My horticultural career might have started when I helped and wondered in the garden. Not only did the vegetables constantly attract me with their vibrant colors, but also ultimately nourished a future garden lover by their abundant tastes and nutrients. A time in the garden remained a joyful routine during every hometown visit. A small garden has been a must in my own family residence. If we believe there is

a connection between an early childhood wonder and a later adulthood career, this book may give a casual explanation on it, and a delayed answer as well to my early curiosities about the distinct colors and tastes of the vegetables I ate in the garden.

This comprehensive treatise provides a systemic and insightful overview of current advances in the biosynthetic genomics/genetics and preventive dietetics of carotenoids, flavonoids, and betalains, from a general perspective, and in specific fruits and vegetables as well. Genomics/genetics focuses on what and how enzymatic and regulatory genes are involved in pigment biosynthesis. Dietetics emphasizes how these pigments contribute nutritional/medical benefits to health, prevent diseases, and act as potential nutraceuticals in the diet. The goal is to provide research scientists, nutrition specialists, healthy food advocates, students, and rainbow food (fruit and vegetable) lovers with an integrated resource on the biosynthetic and dietetic mechanisms of these pigments.

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Chunxian Chen

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Chapter 1
Overview of Plant Pigments

Chunxian Chen, PhD

Introduction

Pigments make nature colorful and likable. Plant pigments usually refer to four major well-known classes: chlorophylls, carotenoids, flavonoids, and betalains (Table 1.1). Each class may contain various numbers of chemical compounds that can be structurally categorized into distinct subgroups. Most pigments are colored. In general, the visible colors are the emission of certain wavelengths of light by colored pigments after they selectively absorb others specific to their molecules. The color spectra of pigments are illustrated in Fig. 1.1 with some examples of pigment-rich fruits and vegetables. Absorbed light may be captured by a few capable pigments as energy to fuel plant photosynthesis and biochemical reactions. These colored pigments not only visually attract animals for flower pollination and seed dispersal but also function in critical biological processes for plants and play essential coevolutionary roles in ecosystems [1–4]. The biological, ecological, and evolutionary importance of plant pigments and the derived colors cannot be overstated. On the other hand, many pigment-rich fruits and vegetables are critical in the human and animal diet. Some pigments are essential nutrients, and others may serve as nutraceuticals with additional medical benefits, including the prevention and treatment of certain diseases. Chlorophylls are the source of green in all land plants and green algae and function as the primary pigment to capture yellow and blue light for photosynthesis to power plant development and growth. Unlike chlorophylls, the other three are accessory pigments (generally with the absorbance spectrum complementary to chlorophylls) and secondary metabolites that possess much more diverse structures and functions in plants and offer more potential nutritional and

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Table 1.1 Four major classes of plant pigments

Pigment class	Basic structure	Main subgroups	Typical colors	Examples[a]
Carotenoids	40-carbon polyene hydrocarbon chain	Carotenes and xanthophylls	Orange, yellow, pink, red	Citrus, banana, carrot, pepper, leaf veggies
Flavonoids	15-carbon benzo-γ-pyrone skeleton	Anthocyanins; flavonols; 7 others	Purple, blue, red, yellow	Blueberry, blackberry, eggplant, red cabbage
Betalains	Indole-derived glycoside	Betacyanins and betaxanthins	Red, violet, orange, yellow	Dragon fruit, cactus pear, beet, Swiss chard
Chlorophylls	Chlorine ring	a and b	Green	Any green plants

[a] All their scientific names are listed in Fig. 1.1 except cactus pear (*Opuntia ficus-indica*)

health benefits in the diet [3, 4]. In this context, attention is only given to carotenoids, flavonoids, and betalains, with emphasis on the basic biological attributes and dietary benefits of each class.

Carotenoids

Carotenoids are a large family of lipid-soluble tetraterpenoids with a basic 40-carbon polyene hydrocarbon chain structure. This family of over 600 members can be generally divided into two subgroups, carotenes ($C_{40}H_{56}$) and xanthophylls ($C_{40}H_{56}O_2$ or $C_{40}H_{56}O$, the oxygenated derivatives of carotenes), which differ in the terminal rings and oxygenation [5, 6]. For example, α-carotene, β-carotene, and lycopene are carotenes; lutein, zeaxanthin, and violaxanthin are xanthophylls. In plants, certain carotenoids function as complementary light-harvesting pigments to precisely absorb wavelengths of light not gathered by chlorophylls, the primary photosynthesis pigment. They also provide photo-protection against excess light damage to the photosynthetic reaction center by quenching excited species such as singlet oxygen and free radicals or by other carotenoid enabled mechanisms [7]. In addition, carotenoids can be potent antioxidants in lipid formations due to the linearly conjugated carbon bonds that provide high reduction potential. Apocarotenoid hormones, including abscisic acid (ABA) and strigolactone, are produced at the extended steps following the carotenoid biosynthesis pathway, and may play a regulatory or signaling role in the pathway [8, 9]. Most genes and enzymes in the plant carotenoid biosynthesis pathway have been well characterized, and the transcriptional and posttranscriptional regulations are being elucidated as well. This information facilitates more efficient selection of carotenoid-rich varieties through conventional breeding and large-scale biotechnological production of carotenoids via metabolic engineering in microorganisms [10].

A large number of fruits and vegetables are colored by carotenoids, such as citrus (*Citrus* spp.), banana (*Musa paradisiaca*), papaya (*Carica papaya*), tomato

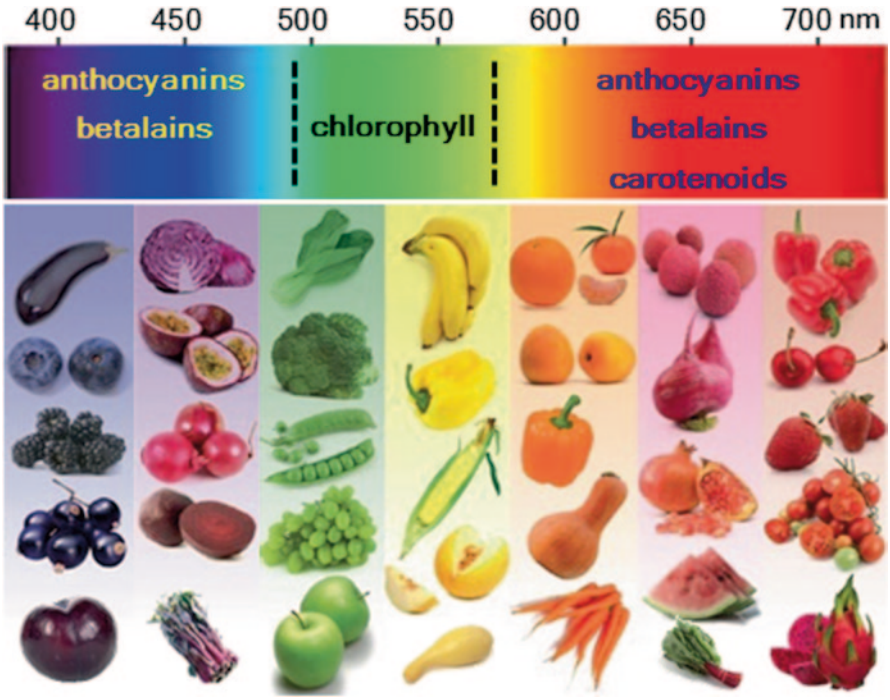

Fig. 1.1 Schematic color spectrum of carotenoids, anthocyanins, betalains and chlorophylls, and examples of pigment-rich fruits and vegetables. Each pigment class was marked in the zones with the approximately corresponding wavelengths (400-700 nm) of visible light and the examples given in the columns below with the symbolic colors. The two black dash lines flanking the green zone was to split and show the approximate color ranges of each pigment class. Some carotenoids are also found in green vegetables and many other colored fruits, however, the colors of these carotenoids at relatively low concentrations are usually masked by those of other predominant pigments. A for anthocyanins, B for betalains, and C for carotenoid were used to mark the primary pigment class in each colored fruit and vegetable, but no abbreviation for chlorophylls was used to mark the class in green fruits and vegetables. From left to right, column **1** from top to bottom are: eggplant (*Solanum melongena*, A), blueberry (*Vaccinium spp*., A), blackberry (*Rubus spp*., A), acai berry (*Euterpe oleracea*, A), plum (*Prunus domestica*, A); **2**: red cabbage (*Brassica oleracea* var. *capitata*, A), passion fruit (*Passiflora edulis*, A), red onion (*Allium cepa*, A), beet (*Beta vulgaris*, B), Zi cai tai ('purple shoot') (*Brassica campestris ssp. chinensis* var. *purpurea*, A); **3**: Shanghai bok choy (*Brassica rapa spp. chinensis*), broccoli (*Brassica oleracea*), pea (*Pisum sativum*), grape (*Vitis vinifera*), 'Granny Smith' apple (*Malus domestica*); **4**: banana (*Musa paradisiaca*, C), yellow bell pepper (*Capsicum annuum*, C), sweet corn (*Zea mays* var. *saccharata*, C), canary melon (*Cucumis melo*, C), squash (*Cucurbita pepo*, C); **5**: citrus (*Citrus spp*., C), persimmon (*Diospyros virginiana*, C), orange bell pepper (C), pumpkin (*Cucurbita maxima*, C), carrot (*Daucus carota*, C); **6**: litchi (*Litchi chinensis*, A), radish (*Raphanus sativus*, A), pomegranate (*Punica granatum*, A), watermelon (*Citrullus lanatus*, C), Swiss chard (*Beta vulgaris* spp. *cicla*, B); **7**: red bell pepper (C), sweet cherry (*Prunus avium*, A), strawberry (*Fragaria ananassa*, A), tomato (*Solanum lycopersicum*, C), dragon fruit (*Hylocereus undatus*, B).

(*Solanum lycopersicum*), carrot (*Daucus carota*), pepper (*Capsicum annuum*), sweet corn (*Zea mays* var. *saccharata*), squash (*Cucurbita pepo*), pumpkin (*Cucurbita maxima*), canary melon (*Cucumis melo*), and watermelon (*Citrullus lana-*

tus) (Fig. 1.1). Some light-colored carotenoids are also found in green vegetables and many other colored fruits, but these carotenoid colors may be masked by green and other predominant colors. Depending on the concentrations and types, carotenoid-rich flowers, fruits, and other plant organs can show a wide spectrum of characteristic yellow, orange, or red colors (Fig. 1.1) [9]. For example, α-carotene, β-cryptoxanthin, and zeaxanthin primarily produce colors in the range of yellow, β-carotene, and lutein in the range of orange, and lycopene in the range of red. These six are also the most common carotenoids in the human diet. In contrast to lycopene, lutein and zeaxanthin that have no β-ionone ring at either terminal of the tetraterpenoid structure, α-carotene, β-carotene, and β-cryptoxanthin have at least one β-ionone ring. As a result, the latter three and the like are provitamin A carotenoids that can be converted to vitamin A in the human and animal body [11]. Therefore, intake of these provitamin A carotenoids can help the acquisition of vitamin A that is essential for human and animal vision, immune system function, normal development, and growth. For example, the immune system can be enhanced by β-carotene by strengthening T and B lymphocyte proliferative responses, stimulating effector T cell functions, and increasing the production of certain interleukins [11]. Lutein and zeaxanthin are the predominant carotenoids within the macula lutea; their physicochemical properties make them suitable candidates considered to act as photoprotectant preventing retinal degeneration. β-carotene and other carotenoids have also demonstrated antioxidant properties and singlet oxygen quenching capacities to prevent chronic disease in vitro and in animal models; the consumption of a diet rich in carotenoids has been epidemiologically correlated with a lower risk for several diseases [12]. A comparison showed cumulative incidence of liver cancer in β-cryptoxanthin-rich orange juice with carotenoids mixture capsules-treated group was statistically ($p = 0.05$) lower than that in the control group [13]. There were also conflicting results from intervention studies on β-carotene to prevent certain cancers and cardiovascular diseases [12]. Data from many population-level intervention studies still suggest a daily intake of fruits and vegetables, which indeed is positively associated with reduced risks of obesity and related chronic diseases and with improved biomarkers of good health [14]. In addition, cooking oil and other food fat may facilitate dissolution of water-insoluble carotenoids and efficient absorption of the nutrient in the diet.

Flavonoids

Flavonoids are a huge family (over 9000 members) of water-soluble polyphenolic compounds with a basic 15-carbon benzo-γ-pyrone (C6–C3–C6) skeleton [15, 16]. Numerous combinations of several substitution groups diversify the chemical structures, properties, and biological functions, and result in at least nine major subgroups: anthocyanins (anthocyanidins), condensed tannins (proanthocyanidins), flavonols, flavones, flavandiols, isoflavonoids, chalcones, aurones, and phlobaphenes [17]. In plants, these colored and colorless flavonoids are synthesized in the

complex flavonoid biosynthesis pathway and play much more diverse biological roles, compared to carotenoids. Only anthocyanins can provide a full spectrum of visible colors with the modification of substitution groups, change of pH values and metal ions [18]. As a result, they probably are the most studied flavonoids in plants [17, 19]. Anthocyanins, synthesized in the cytosol and stored in vacuoles, are also complementary pigments to help chlorophyll collect other available light for photosynthesis and to provide antioxidant protection for plant cells [4]. For example, in cotton plants, red cyanidin-3-O-β-glucoside (an anthocyanin, $C_{21}H_{21}ClO_{11}$) protects living cells from toxic effects of the plants' own phytoalexins by light filtering [20]. In addition to giving flowers and fruits attractive colors and providing the potent protection against oxidation and ultraviolet damage, some flavonoids can function as antimicrobial or defensive agents against biotic and abiotic stresses, and others secretively act as signaling molecules for the plant–microbe interaction in rhizosphere, for example, to establish the symbiosis between plants and nitrogen fixing rhizobia [21].

Flavonoids, particularly anthocyanins, are high in many colored fruits and vegetables, such as blueberry (*Vaccinium* spp.), blackberry (*Rubus* spp.), raspberry (*Rubus idaeus*), strawberry (*Fragaria ananassa*), grape (*Vitis vinifera*), sweet cherry (*Prunus avium*), plum (*Prunus domestica*), peach (*Prunus persica*), pomegranate (*Punica granatum*), red cabbage (*Brassica oleracea* var. *capitata*), and eggplant (*Solanum melongena*) (Fig. 1.1). In addition to the diverse structures and functions of flavonoids in plants, a wide range of biological and antioxidant activities are also observed among flavonoids in the human and animal diet against pathogens, allergens, carcinogens, and other agents causing inflammation. Diverse dietary benefits also include prevention and alleviation of some medical damaging conditions. Some of the activities are accomplished through interaction with host essential enzymes such as cytochromes P450 (CYPs). For example, the risk of some hormone-dependent breast and prostate cancers can be reduced, and menopausal symptoms prevented as well [22]. Accumulated knowledge of flavonoid biosynthesis and the structure–function relationship in plants and humans will facilitate production of targeted flavonoid compounds through metabolic engineering for use as more effective drug and/or chemopreventive agents [23].

Betalains

Betalains are a class of water-soluble indole-derived glycoside pigments that are found only in the order Caryophyllales (e.g., beets, cacti, and amaranths) and never coexist in plants with anthocyanins. In other words, betalains substitute for anthocyanins that are completely absent in the Caryophyllales plants [2, 24, 25]. Betalains differ from anthocyanins in the chemical structures and some properties, but share similarities to anthocyanins in the color spectra, biological functions, and other properties. For example, betalains contain nitrogen but anthocyanins do not. Similarly, betalains are also localized in vacuoles and reportedly offer potent antioxidant

capacity and strong chemoprevention function [25]. Betalains can be structurally divided into betacyanins and betaxanthins that color flowers, fruits, and sometimes vegetative organs primarily into yellow, red, or violet. Betanin, with the molecular formula $C_{24}H_{27}N_2O_{13}$, is an important food colorant produced by beetroot. Compared to those many with carotenoids and flavonoids, betalains-containing fruits and vegetables are rather limited and less well known, but include beet (*Beta vulgaris*), Swiss chard (*Beta vulgaris* spp. *cicla*), dragon fruit (*Hylocereus undatus*), and cactus pear (*Opuntia ficus-indica*; Table 1.1; Fig. 1.1). The betalain biosynthesis pathway has not been elucidated as well as the other two pathways. Some main steps and key enzymes in the pathway were gradually uncovered in recent years, but many other questions remained to be answered [26].

In the end, it is worth noting that the United States Department of Agriculture (USDA) National Agricultural Library maintains a searchable National Nutrient Database (http://ndb.nal.usda.gov/) for over 8000 foods, which includes the main pigment compounds and other phytonutrients for almost all fruits and vegetables. For example, the database for the flavonoid content of selected foods (Release 3.1, December 2013) contains values for 506 food items and for five subgroups of flavonoids: flavonols, flavones, flavanones, flavan-3-ols, and anthocyanidins. These components, and benefits from potential functional foods and nutraceuticals, are also summarized to help people gain relevant knowledge and be encouraged to consume such foods on a regular basis. This book offers a more detailed coverage on the biosynthesis genomics and dietetics of these pigments and representative fruits/vegetables in the following chapters.

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Chapter 2
Carotenoid Biosynthesis Genomics

Amanda Ferreira Da Silva Mendes, Virgínia Lúcia Fontes Soares, and Marcio Gilberto Cardoso Costa

Introduction

Carotenoids are a family of isoprenoid molecules that are widespread in nature. They are responsible for the typical yellow, orange, and red colors of most fruits, flowers, and vegetables, and for the characteristic colors of many birds, insects, fish, and crustaceous intaking carotenoids through the diet. The basic chemical structure of any carotenoid molecule is the long polyene chain, which may extend from 3 to 15 conjugated double bonds, acting as a chromophore that determines the absorption spectrum of the molecule, and hence its color [1]. This basic structure can be further modified in a number of ways, such as cyclization and oxygenation, to yield a family of more than 600 different carotenoids generally divided into two subgroups, carotenes (hydrocarbon carotenoids) and xanthophylls (oxygenated derivatives) [1]. Their oxidative breakdown products are called apocarotenoids.

Carotenoids perform a broad range of metabolic and ecological functions. Carotenoid pigments are essential components of the photosynthetic membranes in all photosynthetic organisms, including plants, algae, and cyanobacteria, protecting them against photooxidative damage [2, 3]. This protective function is crucial in oxygen-evolving photosynthetic organisms, since an impairment to produce cyclic carotenoids is eventually lethal [1]. In higher plants, carotenoids also act as accessory pigments in the light-harvesting antennae of chloroplasts, transferring energy to chlorophylls, and as precursors for biosynthesis of the phytohormones abscisic acid (ABA), which controls abiotic stress signaling pathways, and strigolactone, which controls lateral shoot growth [3–5]. An additional and important role of carotenoids in higher plants is as coloring agents in flowers and fruits to attract pollinators and

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agents of seed dispersal [2]. In these tissues, carotenoids accumulate in nonphotosynthetic chromoplasts, where they are found in association with lipid–protein complexes in plastoglobules and/or in carotenoid-accumulating structures of globular, crystalline, membranous, fibrillar, or tubular forms [6].

Since animals are unable to synthesize carotenoids de novo, they rely upon the diet as the source of these compounds. Dietary carotenoids contribute to animal health and behavior, because they stimulate the immune system and aid in the preferential selection by the sexual partner [7, 8]. In mammalians, including humans, carotenoid species containing a β-ring can be converted into retinal (the main visual pigment), retinol (vitamin A), and retinoic acid (a substance that controls morphogenesis) [9]. Additional beneficial effects of carotenoids in human health are attributed to their antioxidant and anti-inflammatory activities in vivo, which help to prevent certain cancers, cardiovascular diseases, light-induced erythema, and age-related diseases of eye such as cataract and macular degeneration [10, 11].

Here we present the current knowledge about the carotenoid biosynthesis gene families in higher plants from a genomic perspective. This includes information about the basic structure, function, and evolution of the genes and enzymes, as well as the molecular mechanisms regulating carotenoid biosynthesis in different plant tissues.

Carotenoid Biosynthetic Pathway

The biosynthetic pathways involved in carotenoid formation were elucidated during the second half of the twentieth century using both classical biochemical approaches and modern molecular biology techniques [12]. Nevertheless, the major advances in the identification of genes and enzymes of carotenoid biosynthesis occurred in the 1990s. Isolation of carotenoid-defective mutants in plants, and the information resources of the *Arabidopsis thaliana* EST database and the genome sequence of the cyanobacterium *Synechocystis* PCC6803 contributed to such advances [9, 12, 13]. Also important was the dissection of carotenoid biosynthesis pathway in bacterial systems, such as *Rhodobacter capsulatus*, *Erwinia uredovora*, and *Erwinia herbicola*. It allowed engineering strains of *Escherichia coli* accumulating a variety of carotenoid precursors for use as a simple and powerful in vivo system for the assay of enzyme function and substrate specificity [12]. In addition, the different colors exhibited by carotenoid-accumulating *E. coli* strains were exploited to visually screen complementary DNA (cDNA) and genomic libraries, in a procedure referred to as "color complementation," enabling the identification of a number of previously unidentified plant, algal, and cyanobacterial carotenogenic genes based on the visualization of color changes in *E. coli* colonies [14].

Carotenoids make a part of the plethora of chemical compounds that are produced via the general isoprenoid biosynthetic pathway (Fig. 2.1). As all other isoprenoids, carotenoids are built from the five-carbon (C_5) compound isopentenyl diphosphate (IPP) and its allylic isomer, dimethylallyl diphosphate (DMAPP)

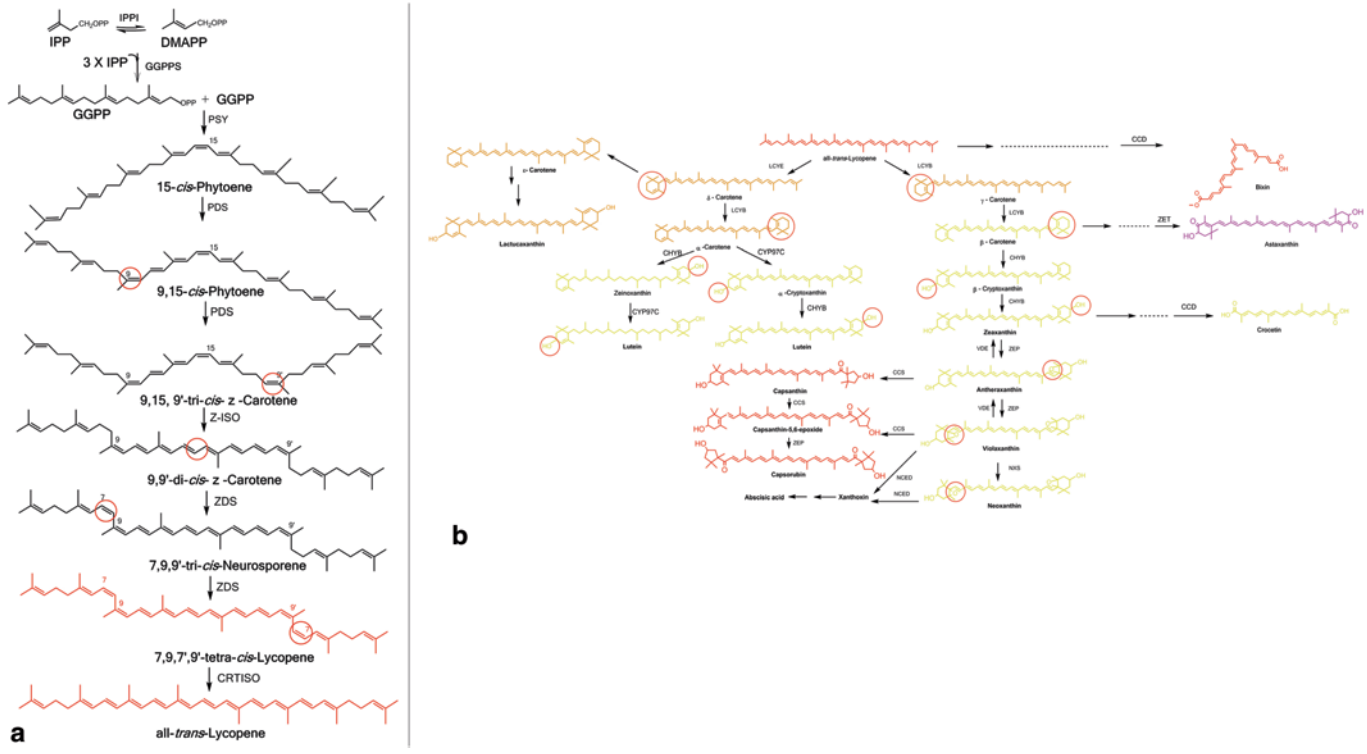

Fig. 2.1 The general isoprenoid carotenoid biosynthetic pathway in plants. **a** Formation of acyclic carotenoids from the precursor GGPP. **b** Formation of cyclic carotenoids from the precursor lycopene. Enzyme abbreviations are as follows: *IPPI* isopentenyl diphosphate isomerase, *GGPPS* geranylgeranyl diphosphate synthase, *PSY* phytoene synthase, *PDS* phytoene desaturase, *Z-ISO* ζ-carotene isomerase, *CrtISO* carotenoid isomerase, *ZDS* ζ-carotene desaturase, *LCYE* lycopene ε-cyclase, *LCYB* lycopene β-cyclase, *CHYB* β-ring hydroxylase, *CYP97C* ε-ring hydroxylase, *CCS* capsanthin/capsorubin synthase, *ZEP* zeaxanthin epoxidase, *VDE* violaxanthin de-epoxidase, *NXS* neoxanthin synthase, *CCD* carotenoid cleavage dioxygenase, *ZET* β-ring hydroxylase, *NCED* 9-cis-epoxycarotenoid dioxygenase, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GGPP* geranylgeranyl diphosphate

[10, 13, 15]. Until recently, it was assumed that IPP was synthesized from acetyl-coenzyme A (CoA) via mevalonic acid (MVA) pathway [16]. However, in the early 1990s retro-biosynthetic studies established the presence of an alternative, MVA-independent pathway for the formation of IPP and DMAPP, termed 1-deoxy-D-xylulose-5-phosphate (DXP) or 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway [17]. Several reports have indicated that eubacteria (*E. coli* and other pathogenic bacteria) and protozoans of apicomplexan phylum (*Plasmodium falciparum*) synthesize isoprenoids only via the MEP pathway, while archaebacteria, fungi, and animals (including humans) contain only the MVA pathway [18, 19]. The formation of isoprenoids in plants, however, can proceed from both MEP and MVA pathways [15, 18]. IPP is synthesized in plastids through the MEP pathway and in the cytosol through the MVA pathway. Thus, MEP pathway is a potential target for the development of new herbicides and anti-malarian and antimicrobial drugs that, besides the large spectrum of action, are not toxic for humans.

The MVA pathway involves a set of six reactions proceeding sequentially from acetyl-CoA to produce IPP and DMAPP [15, 16, 19]. Initially, two acetyl-CoA molecules, obtained through CO_2 fixation, are condensed to yield acetoacetyl-CoA, in a reaction catalyzed by acetyl-CoA *C*-acetyltransferase (AACT, EC 2.3.1.9). Then, a third acetyl-CoA molecule is condensed to acetoacetyl-CoA, forming 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by the action of HMG-CoA synthetase (HMGS, EC 4.1.3.5). The nicotinamide adenine dinucleotide phosphate (NADP)-dependent HMG-CoA reductase converts the CoA-derived in (*R*)-MVA, which is phosphorylated to (*R*)-MVA 5-diphophate by the sequential action of mevalonate kinase (MK, EC 2.7.1.36) and diphosphomevalonate kinase (PMK, EC 2.7.4.2). (*R*)-MVA 5-diphophate is further decarboxylated by the mevalonate diphosphate decarboxylase (MDC, EC 4.1.1.33), producing a pool of IPP. Finally, IPP isomerase catalyzes the reversible conversion of IPP into DMAPP, maintaining the equilibrium between these two compounds [16, 20].

The MEP pathway consists of seven sequential reactions starting from the condensation of pyruvate and glyceraldehyde 3-phosphate (G3P) to yield 1-deoxy-$_D$-xylulose 5-phosphate (DXP) [10, 17, 18]. This transketolase reaction is catalyzed by DXP synthase (DXS, EC 4.1.3.37), an enzyme that requires thiamine pyrophosphate and a divalent cation (Mg^{2+}or Mn^{2+}) as cofactors [18, 21]. DXP is subsequently rearranged and reduced to MEP in a single step, in a reaction catalyzed by DXP reductoisomerase (DXR, EC 1.1.1.267). DXR requires NADPH and Mn^{2+}as cofactors [18, 21]. MEP is converted to IPP and DMAPP via 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME), 2-phospho-4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-MEP), 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MCP), and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-phosphate (HMBPP). The enzymes responsible for these reactions are, respectively, MEP cytidyl transferase (MCT, EC 2.7.7.60), CDP-ME kinase (CMK, EC 2.7.1.148), MCP synthase (MCS, EC 4.6.1.12), and HMBPP synthase (HDS) [18, 21]. IPP/DMAPP synthase (IDS) is responsible for the conversion of HMBPP to a 5:1 mixture of IPP and DMAPP [10, 20, 21].

IPP and DMAPP are subsequently used as blocks in a modular assembly process that produces compounds of 5, 10, 15, 20, or more carbons (in multiples of 5),

allowing the biosynthesis of the basic skeletons for the various isoprenoids, including carotenoids, with a relatively small number of basic reaction steps [10, 15, 19]. For instance, the C_{20} geranylgeranyl diphosphate (GGPP), which serves as the immediate precursor for carotenoids, is formed by the sequential and linear addition of three molecules of IPP to one molecule of DMAPP. The enzyme that catalyzes these reactions, the GGPP synthase (GGPS; EC 2.5.1.29), is encoded by a multigene family of 12 members in the *Arabidopsis* genome, suggesting the involvement of different isozymes in the production of specific groups of isoprenoids [10, 13].

Basic Structure, Function, and Evolution of Carotenoid Biosynthesis Genes and Enzymes

The C_{40} skeleton of all plant carotenoids is assembled from the condensation of two molecules of GGPP, which then suffer a series of enzymatic reactions of desaturation, cyclization, and oxidation [10, 19]. Genetic and molecular evidences indicate that all enzymes of carotenoid biosynthetic pathway in plants are encoded by nuclear genes and post-translationally imported to plastids [12, 22]. Here, the genes and enzymes of carotenoid biosynthesis are discussed sequentially in their order within the pathway, giving specific details for one or more examples of each in *Arabidopsis* and other higher plants whenever possible.

Phytoene Synthase

Phytoene synthase (PSY, EC 2.5.1.32) catalyzes the first committed step in the formation of carotenoids, by the condensation of two GGPP molecules to produce 15-*cis* phytoene (Fig. 2.1a) [10, 12]. Detailed biochemical characterization of tomato and pepper PSYs has demonstrated that they can be either thylacoid membrane associated, but not integral, or stroma-localized proteins [10, 22]. The catalytic site of PSY, at the carboxy terminus, contains a large central cavity, formed by antiparallel alpha-helices, with two aspartate-rich motifs (DELVD and DVGED) that are positioned on opposite walls of the central cavity [19, 23]. The high degree of sequence conservation of these motifs suggests that they are required for the interaction of enzyme with upstream products [23]. PSY also contains an active site (YAKTF) at the amino terminus and a squalene synthase (SQS) domain type located between the catalytic and active sites (Fig. 2.2) [19, 23]. There is a low sequence similarity at the amino terminus among PSYs of different plant species, partially due to the existence of plastid transit peptide sequences that are known to show a low degree of sequence conservation.

Arabidopsis possesses only one *PSY* gene, while tomato and tobacco have two *PSY*s and plants belonging to the Poaceae have three *PSY*s [24–29]. Scenarios of gene duplication and sub-functionalization can be invoked for the evolution of *PSY* genes and enzymes from an ancestral *PSY* gene prototype. The *PSY* paralogs are

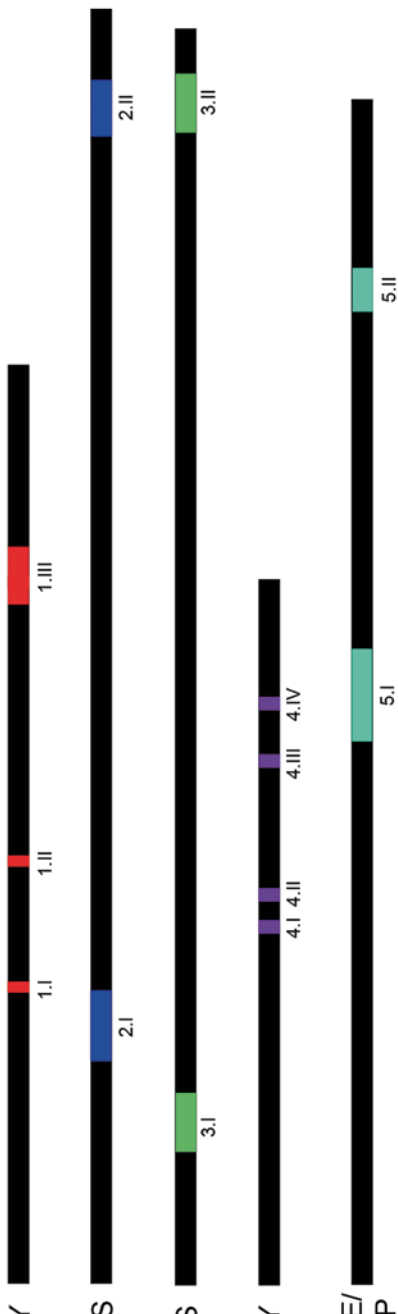

Fig. 2.2 Major functional domains present in some carotenogenic enzymes. 1.I, Active site of PSY; 1.II, squalene synthase domain type; 1.III, catalytic site of PSY. 2.I, Dinucleotide (FAD/NADP)-binding site domain of PDS; 2.II, carotenoid-binding domain of PDS. 3.I, Dinucleotide (FAD/NADP)-binding site domain of ZDS; 3.II, carotenoid-binding domain of ZDS. 4.I, Histidine-binding motif HXXXXXXH type of β-CHY; 4.II, histidine-binding motif HXXHH type of β-CHY; 4.III, histidine-binding motif HXXXXXXH type of β-CHY; 4.IV, histidine-binding motif HXXHH type of β-CHY. 5.I, Cystein-rich region of VDE and ZEP; 5.II, glutamate-rich region of VDE and ZEP. See text for further details. *PSY* phytoene synthase, *FAD/NADP* flavin adenine dinucleotide/nicotinamide adenine dinucleotide phosphate, *PDS* phytoene desaturase, *ZDS* ζ-carotene desaturase, *VDE* violaxanthin de-epoxidase, *ZEP* zeaxanthin epoxidase

involved with the carotenoid synthesis in different plant tissues. For instance, *PSY1* encodes a fruit- and flower-specific isoform in tomato, whereas *PSY2* encodes an isoform that predominates in photosynthetic tissues [25, 27]. In maize, *PSY1* and *PSY2* are required for endosperm carotenoid accumulation and photomorphogenesis in photosynthetic tissues, while *PSY3* is associated with root carotenogenesis and necessary for drought and salt stress-induced production of ABA [28, 30].

A comparison of the gene structures of *PSY*s among different plant species shows that the *Arabidopsis PSY* contains seven exons, as well as *Vitis vinifera*, while rice and maize *PSY*s show a loss of exon 1 (Fig. 2.3a). The gene structures at the

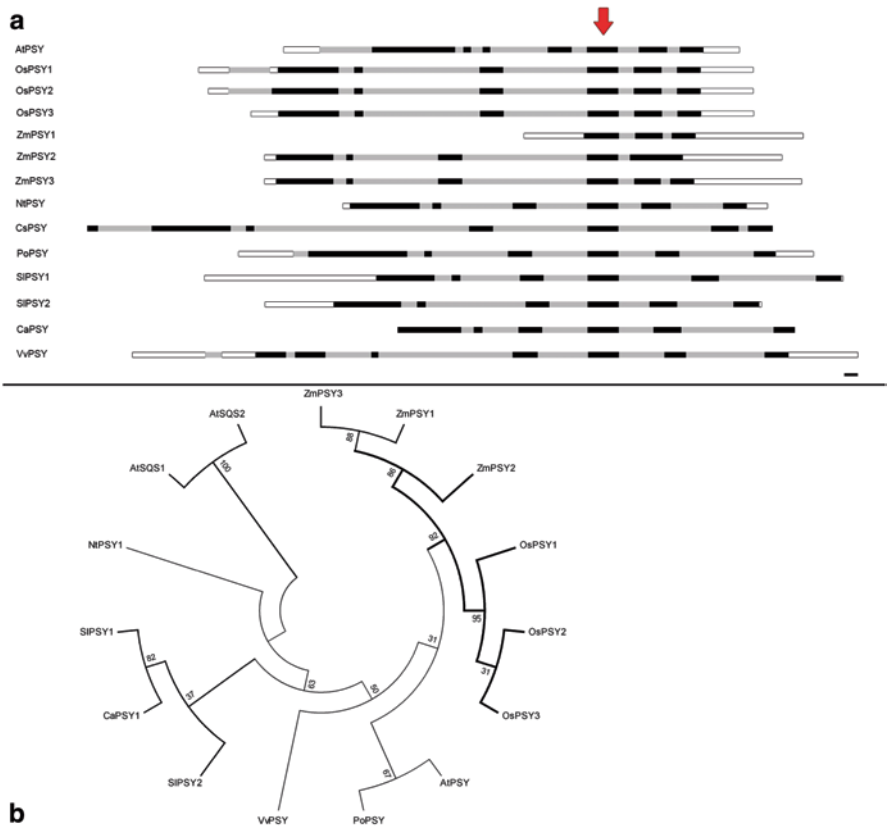

Fig. 2.3 *PSY* gene structure and phylogeny. **a** Exon/intron structure of PSY in different plant species. *Red arrow* denotes the catalytic site of PSY. *White*, *gray*, and *black thin bars* indicate UTR, intron and exon regions, respectively. **b** Similarity dendrogram of plant PSYs and squalene synthases (SQSs). Amino acid sequences were aligned using ClustalW and a neighbor-joining tree was constructed with a 1000-bootstrap replication support. Abbreviations for the name of plant species are as follows: At *Arabidopsis thaliana*, Os *Oryza sativa*, Zm *Zea mays*, Nt *Nicotiana tabacum*, Cs *Citrus sinensis*, Po *Populus trichocarpa*, Sl *Solanum lycopersicum*, Ca *Capsicum annum*, Vv *Vitis vinifera*, *PSY* phytoene synthase, *UTR* untranslated region

5′-untranslated region (UTR) of all *PSY* genes analyzed show differences in length and sequence, even among the different genes within a species, such as *OsPSY1* (227 bp), *OsPSY2* (147 bp), and *OsPSY3* (193 bp; Fig. 2.3a). In contrast, the lengths of exons 3, 4, 5, and 6 are comparable among the different plant species, with 45–51, 173, 236, and 193–211 bp, respectively. Such conservation may be associated to the presence of nucleotide sequences encoding conserved domains of enzyme, such as the catalytic site located at the exon 5 of *Arabidopsis*. The dendrogram of similarity reveals that PSYs of rice and maize clustered together, in a separated clade from PSYs of dicots, which includes the tomato PSY1 and PSY2. It suggests that the duplication event of *PSY* occurred separately in Poaceae and Solanaceae (Fig. 2.3b). Furthermore, the proximity between AtPSY and OsPSY1 represents the existence of an ancient PSY constituting a common ancestor of monocots and dicots [29].

Desaturases

Colorless phytoene undergoes a series of four desaturation reactions in plants that results in the formation of the red-colored carotenoid lycopene (Fig. 2.1a). These reactions are catalyzed by two related enzymes in plants: phytoene desaturase (PDS, EC 1.3.5.5) and ζ-carotene desaturase (ZDS, EC 1.3.5.6). PDS converts phytoene in phytofluene and then in ζ-carotene, while ZDS converts ζ-carotene in neurosporene and then in lycopene [10, 19]. In contrast with plants, bacteria and fungi contain only a single desaturase, *CrtI*, which catalyzes the four desaturation steps [19].

PDS and ZDS are found to be associated with other enzymes of carotenoid biosynthetic pathway, forming multimeric complexes of about 350 kDa [12, 31]. It has been proposed that two molecules of each PDS and ZDS, associated with one molecule of lycopene β-cyclase (β-LCY) and another of lycopene ε-cyclase (ε-LCY), form a multienzymatic complex responsible for the synthesis of α-carotene [12, 32]. A similar association of PDS and ZDS with two molecules of β-LCY would be responsible for the synthesis of β-carotene [12, 32]. PDS may be also associated with chloroplastic chaperonins (Cpn60) or heat-shock proteins (Hsp70) when located in stroma [31, 33, 34].

The active form of PDS is tightly bound to thylacoid membranes, whereas the stroma free form is inactive [31, 33]. Besides the association with membranes, the desaturases require cofactors for their complete activity. The removal of two hydrogen atoms during each desaturation step suggests the involvement of an electron transport chain for the regeneration of reductants [10]. A plastid terminal oxidase (PTOX) was identified in *Arabidopsis* mutants as one component of this electron transport chain required for the desaturation of phytoene [35]. PTOX is a plastoquinone oxidoreductase that regenerates the reduced plastoquinone formed during the desaturation of phytoene and ζ-carotene, using oxygen (O_2) as a terminal acceptor. Thus, PTOX and O_2 are considered as the main cofactors involved in the desaturation of phytoene, in both photosynthetic and nonphotosynthetic tissues [35].

The genomes of higher plants apparently contain only one copy of desaturase genes, *PDS* and *ZDS* [12, 26, 33, 36, 37]. A unique exception has been recently

discovered in sweet orange (*Citrus sinensis*), in which 2 *PDS* and 12 *ZDS* members were found to be clustered, respectively, at one and three loci [38]. A high degree of similarity is observed in the deduced amino acid sequences of all plant desaturases [10]. All contain a conserved dinucleotide (FAD/NADP)-binding site domain at the amino terminus (Fig. 2.2). The carboxy terminus contains another conserved region, the carotenoid-binding domain.

Isomerases

In higher plants, phytoene occurs predominantly as the 15-*cis* isomer, while the predominant isomer of lycopene is all-*trans*, suggesting the existence of an enzymatic step mediating the *cis–trans* isomerization of lycopene precursors (Fig. 2.1a). Map-based cloning of the gene responsible for the *tangerine* tomato fruit phenotype, which accumulates 7,9,7′,9′-tetra-*cis* lycopene (prolycopene) and traces of its poly-*cis* precursors, resulted in the isolation of carotenoid isomerase (*CrtISO*). *CrtISO* encodes a carotenoid isomerase (CrtISO, EC 5.2.1.13) catalyzing the isomerization of prolycopene to all-*trans* lycopene [39]. A *CrtISO* homolog termed *Ccr-2* was also isolated in *Arabidopsis* [40].

Both tomato and *Arabidopsis* isomerases contain a dinucleotide (FAD/NADP)-binding domain, like PDS and ZDS. However, the isomerases show more identities to bacterial phytoene desaturases (CrtI) than the plant desaturases [39, 40]. In fact, the bacterial desaturase *CrtI* also possesses the function of isomerization in combination with that of desaturation, converting 15-*cis* phytoene to all-*trans* lycopene [10].

Cyclases

Cyclization of the linear carotenoid lycopene marks an important branching point in the carotenoid pathway: one branch leads to β-carotene and its derivative xanthophylls, whereas the other leads to α-carotene and lutein (Fig. 2.1b). These carotenoids differ in the type of cyclic end group that is added. It can be a ε- or β-ionone ring, depending on the position of a double bond within the cyclohexane ring. Carotenoids with two β-rings, such as β-carotene and zeaxanthin, are primarily involved in protection against photooxidative damage and dissipation of the excess of light energy in the photosynthetic membranes [12]. Carotenoids with one β-ring and one ε-ring, such as lutein, act as accessory pigments in light-harvesting antennae of the chloroplasts [12]. Carotenoids with two ε-rings, such as lactucaxanthin in lettuce, are rare [41].

The type of end group produced depends on the nature of cyclase enzyme. In higher plants, there are two major cyclases: β-LCY (EC 5.5.1.19), which introduces β-rings, and ε-LCY (EC 5.5.1.18) that introduces ε-rings. The formation of β-carotene requires the introduction of two β-rings by β-LCY, whereas α-carotene requires the interaction of both β-LCY and ε-LCY (Fig. 2.1b) [32]. In contrast with

β-LCY, ε-LCY is able to incorporate only one ε-ring to the symmetrical lycopene, forming δ-carotene [32]. Lettuce ε-LCY is the only example of cyclase that can introduce two ε-rings to the lycopene molecule [41]. All lycopene cyclases, irrespective of class, proceed via a carbocationic mechanism [19].

A membrane-associated multienzymatic complex involving the association of β-LCY and ε-LCY with PDS and ZDS is postulated to act in the synthesis of α- and β-carotene (see the desaturases section). These carotenogenic complexes possibly are associated to other enzymes and cofactors that regulate their catalytic activity [12]. Flux directing towards the β,β- or β,ε-branch of the pathway seems to be determined by the relative amounts of enzymatic activity and/or substrate specificity of β-LCY and ε-LCY [32, 42].

The lycopene cyclases contain a dinucleotide (FAD/NADP)-binding site domain, apparently involved in allosteric activation, and two characteristic conserved motifs: cyclase I and cyclase II (Fig. 2.4) [43, 44]. The FAD/NADP-binding site domain is composed by a typical secondary structure (β-sheet/α-helice/β-sheet) present in all plant enzymes with lycopene cyclase activity [44]. The cyclization reaction seems to be a simple rearrangement that does not involve any change in the oxidation level of lycopene molecule [19]. Thus, the involvement NAD(P)H in the reaction is not expected. NAD(P)H seems to have an indirect action, participating in the enzymatic reaction as an allosteric activator [44].

The first searches for homology carried out with plant ε-LCYs revealed the existence of a conserved region (VQMQQ), which was termed "ε-cyclase conserved region" (Fig. 2.4) [32]. Similarly, β-LCYs contain a conserved region (PLYD) that has been identified as "β-cyclase conserved region." All lycopene cyclases also contain a conserved region that shows similarity to motifs of β-cyclase [32]. Since this region is present in cyclases that introduce β-, ε- or κ-ring, it has been termed "cyclase activity region" (Fig. 2.4).

Only a copy of *ε-LCY* gene has been identified in the genome of *Arabidopsis* and tomato [32, 45]. *Arabidopsis* also contains a copy of *β-LCY* [32], but two *β-LCY* copies, *Crtl-B* and *Cyc-B*, were identified in tomato [46, 47]. *Crtl-B* is active in photosynthetic tissues, whereas *Cyc-B* functions only in chromoplast-containing tissues [22]. The presence of two *β-LCY* genes, one with a chromoplast-specific expression, has been also reported in carotenogenic fruits other than tomato, including watermelon [48], orange, and grapefruit [49, 50].

Plant lycopene cyclases are also related to two other carotenoid cyclase enzymes: the capsanthin–capsorubin synthase (CCS, EC 5.3.99.8) of pepper [32] and the neoxanthin synthase (NSY, EC 5.3.99.9) of tomato [51] and potato [52]. CCS catalyzes the formation of the unusual five-carbon κ-ring [53], converting antheraxanthin or violaxanthin to capsanthin or capsorubin, respectively. In addition, CCS exhibits a β-LCY activity when lycopene is provided as a substrate [43]. NSY also modifies violaxanthin to the allenic product via a carbocation with a structure similar to the intermediate in the CCS-catalyzed reaction [54]. Although NSY operates mechanistically like CCS, its cryptic LCY activity has not been demonstrated [19].

Conservation of amino-acid sequences and their similar mechanisms of catalysis suggest that all plant cyclases, including CCS and NSY, have evolved from a common ancestor, most probably the cyanobacterial CrtL [55]. Since cyanobacteria do not

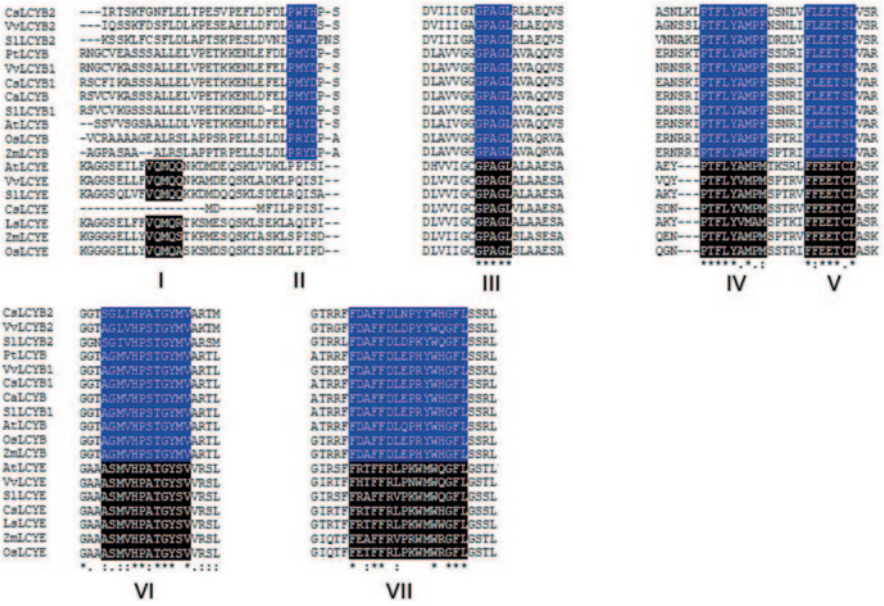

Fig. 2.4 Alignment of the partial amino acid sequences of β-LCY and ε-LCY from different plant species. Conserved amino acid sequences of β-LCY and ε-LCY in a given position are in white text on a blue and black background, respectively. *Dashes* denote a gap in the amino acid sequence. The conserved regions are plant ε-cyclase conserved region (I), plant β-cyclase conserved region (II), dinucleotide (FAD/NADP)-binding site domain (III), cyclase I motif (IV and V), cyclase II motif (VI) and cyclase activity region (VII). *Asterisk* (*), *colon* (:) and *dot* (.) symbols denote identical, conserved and similar amino acids, respectively. Abbreviations for the species names are as follows: At *Arabidopsis thaliana*, Ca *Capsicum annum*, Cs *Citrus sinensis*, Ls *Lactuca sativa*, Os *Oryza sativa*, Pt *Populus trichocarpa*, Sl *Solanum lycopersicum*, Vv *Vitis vinifera*, Zm *Zea mays*, *FAD/NADP* flavin adenine dinucleotide/nicotinamide adenine dinucleotide phosphate, *LCY* lycopene cyclase

contain any ε-LCY activity, it is presumed that this enzyme evolved by gene duplication of a β-LCY in prochlorophytes, where carotenoids with an ε-end group appear. In contrast with higher plants, *Prochlorococcus marinus* can synthesize α-carotene from lycopene by a single enzyme [55]. Thus, the *P. marinus* ε-LCY can be regarded as a premature form of the plant ε-LCY, which has not yet lost its β-cyclase activity [55].

Hydroxylases

Xanthophylls are oxidation products of α- and β-carotene in higher plants [10]. Hydroxylation of the number three carbon of each ring (C-3 and C-3′) of α-carotene and β-carotene results in the formation of lutein and zeaxanthin via α-cryptoxanthin and β-cryptoxanthin, respectively (Fig. 2.1b). These reactions are carried out by two types of enzymes, one specific for ε-rings (ε-hydroxylase, ε-CHY, EC 1.14.99.45) and the other for β-rings (β-CHY, EC 1.14.13.129) [22].