Chapter 2
Carotenoid Biosynthesis Genomics

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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)

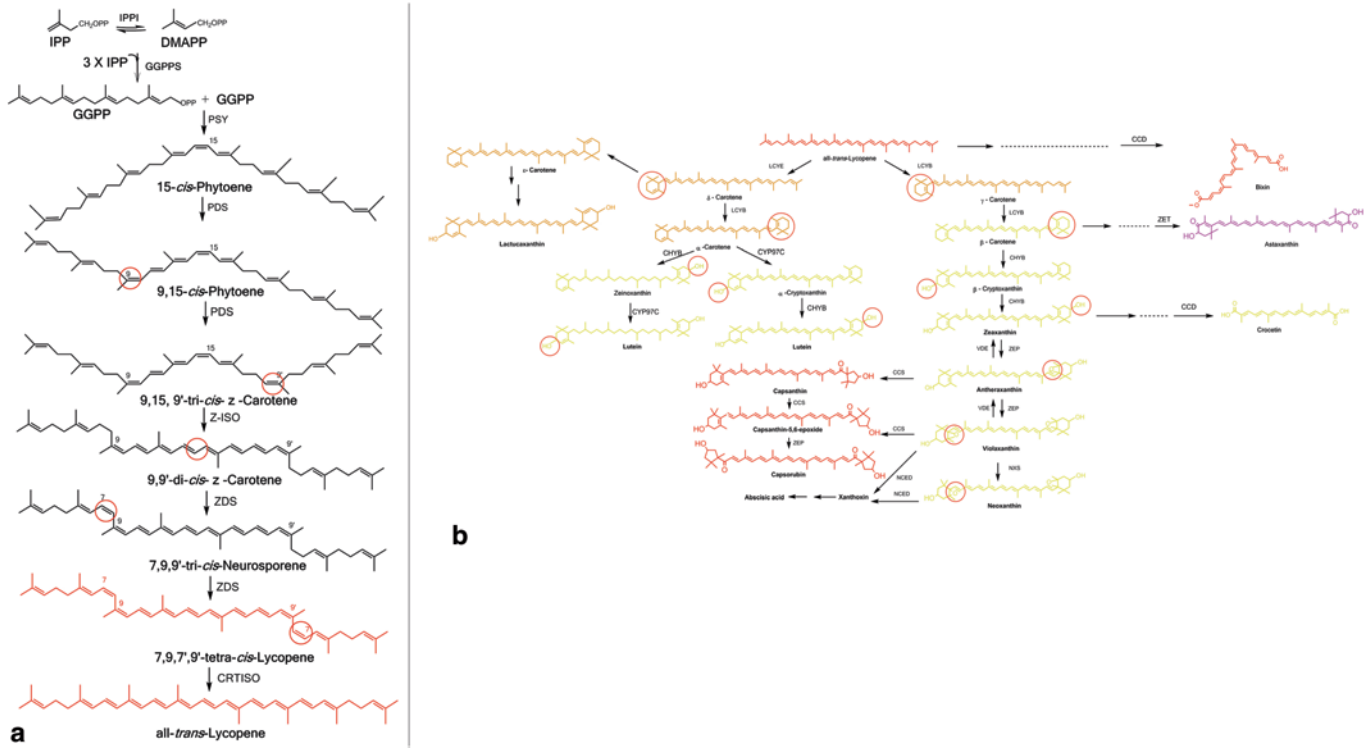

Fig. 2.1 The general isoprenoid carotenoid biosynthetic pathway in plants. **a** Formation of acyclic carotenoids from the precursor GGPP. **b** Formation of cyclic carotenoids from the precursor lycopene. Enzyme abbreviations are as follows: *IPPI* isopentenyl diphosphate isomerase, *GGPPS* geranylgeranyl 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

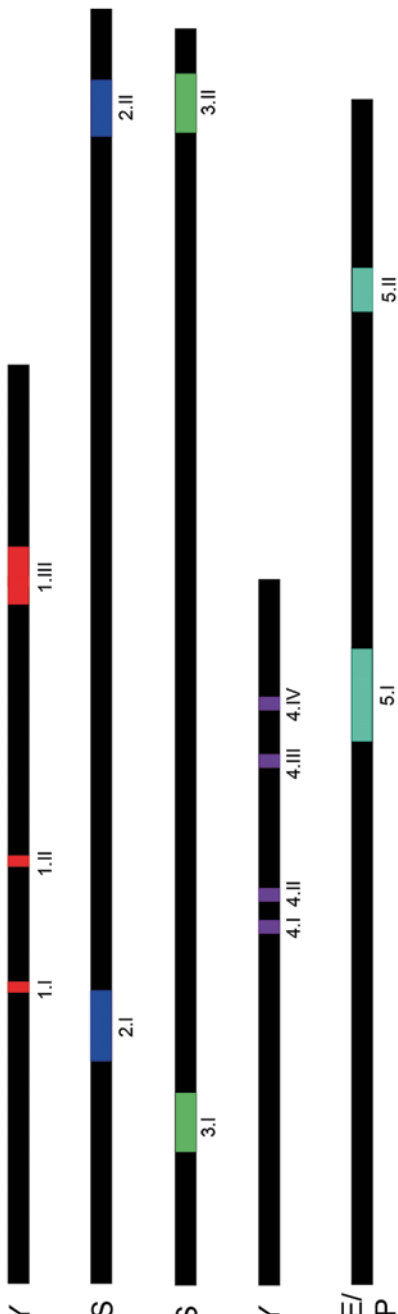

Fig. 2.2 Major functional domains present in some carotenogenic enzymes. 1.I, Active site of PSY; 1.II, squalene synthase domain type; 1.III, catalytic site of PSY. 2.I, Dinucleotide (FAD/NADP)-binding site domain of PDS; 2.II, carotenoid-binding domain of PDS. 3.I, Dinucleotide (FAD/NADP)-binding site 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

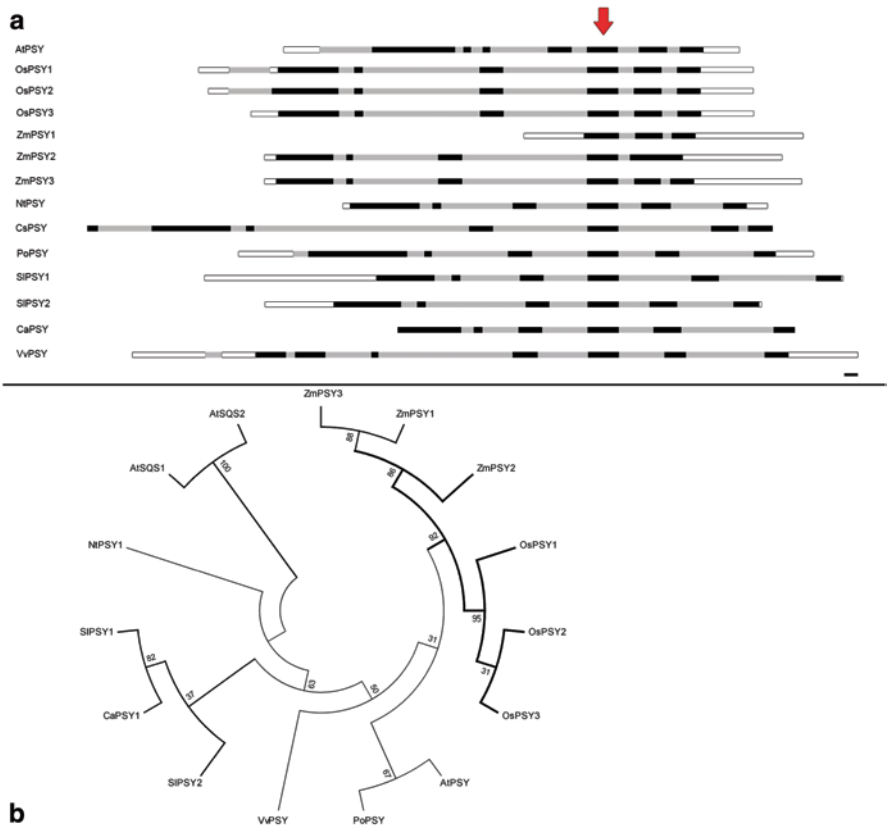

Fig. 2.3 *PSY* gene structure and phylogeny. **a** Exon/intron structure of PSY in different plant species. *Red arrow* denotes the catalytic site of PSY. *White*, *gray*, and *black thin bars* indicate UTR, intron and exon regions, respectively. **b** Similarity dendrogram of plant PSYs and squalene 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

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