# Chapter 2
# Biosynthesis of Carotenoids in Plants: Enzymes and Color

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**Abstract** Carotenoids are the most important biocolor isoprenoids responsible for yellow, orange and red colors found in nature. In plants, they are synthesized in plastids of photosynthetic and sink organs and are essential molecules for photosynthesis, photo-oxidative damage protection and phytohormone synthesis. Carotenoids also play important roles in human health and nutrition acting as vitamin A precursors and antioxidants. Biochemical and biophysical approaches in different plants models have provided significant advances in understanding the structural and functional roles of carotenoids in plants as well as the key points of regulation in their biosynthesis. To date, different plant models have been used to characterize the key genes and their regulation, which has increased the knowledge of the carotenoid metabolic pathway in plants. In this chapter a description of each step in the carotenoid synthesis pathway is presented and discussed.

**Keywords** Carotenoid plant models • Synthesis regulation • Carotenoid gene characterization • Multienzymatic complex • Key enzymes

## 2.1 Introduction

In nature, colors obtained from fruits, flowers, roots, vegetables and microalgae are called biocolors because of their biological origin (Pattnaik et al. 1997). Biocolors are mainly attributed to chemically distinct pigments such as carotenoids, flavonoids, betalains, and chlorophylls, among others, with carotenoids being mainly responsible for yellow, orange and red colors (Grotewold 2006). Carotenoids are synthesized in plastids of all photosynthetic organisms (Cuttriss et al. 2011) as well as in fruits, flowers, seeds and reserve roots (Ruiz-Sola and Rodríguez-

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Concepción 2012). Some bacteria and fungi are also capable of producing carotenoids in response to growth and environmental conditions (Velayos et al. 2000; Iniesta et al. 2008). Other organisms, such as animals do not synthesize carotenoids and must acquire them through dietary intake as these pigments, especially α-carotene, β-carotene and β-cryptoxanthine, are precursors of vitamine A, retinal and retinoic acid, which play essential roles in nutrition, vision and cellular differentiation, respectively (Krinsky et al. 1994). Some curious exceptions exist in nature such as the synthesis of carotenoids in human protist parasites and aphids (Tonhosolo et al. 2009; Moran and Jarvik 2010), which are explained by the existence of a remnant plastid, known as an apicoplast, and as a result of lateral transfer of carotenogenic genes from a fungus respectively. This makes aphids the only known animal to date capable of synthesizing their own carotenoids.

In animals, the regular ingestion of carotenoids also delays aging due to their antioxidant properties (Bartley and Scolnik 1995; Snodderly 1995; Mayne 1996; Giuliano 2000; Delcourt et al. 2006; Rao and Rao 2007; Zu et al. 2014). Birds, fish and crustaceans utilize carotenoids for pigmentation and nutritional purposes too. For example, the cetocarotenoid astaxanthin is responsible for the orange color of salmon meat and lobster shells (Grotewold 2006, see Chap. 3). Carotenoids also have commercial relevance in the food and cosmetic industries (Klaui 1982; Bjerkeng 2000) as well as in the animal feed industry where they are mainly manufactured as poultry and fish feed additives (Bjerkeng 2000).

From a chemical point of view, carotenoids are the second most abundant naturally occurring pigments on earth, with more than 750 structurally different compounds (Nisar et al. 2015). Also, carotenoids are undoubtedly the most important natural polyunsaturated isoprenoids, widely distributed in the world (Britton et al. 2004). These molecules typically consist of a C-40 hydrocarbon backbone, composed of eight isoprene units joined in a head-to-tail fashion except the central unit, which has a reverse connection (Álvarez et al. 2013, see Chap. 3). This means that carotenoids are polyisoprenoid compounds and can be divided into two main groups:

(i) Carotenes or hydrocarbon carotenoids, which are composed of carbon and hydrogen atoms
(ii) Xanthophylls that are oxygenated hydrocarbon derivatives with cyclic and acyclic structures that contain at least one oxygen function such as hydroxyl, keto, epoxy, methoxy, or carboxylic acid groups (Armstrong 1994).

Several prefixes, preceded by the number of the carbon atom(s) that contain the modification, are used for the systematic nomenclature of modified carotenoids. For example, the *apo* prefix designates the shortening of the skeleton by the formal removal of fragments from one or both ends of the carotenoid molecule, *epoxy* is used to indicate oxygen bridges and *hydro* or *dehydro* designates the addition or removal of hydrogens, and applies to the elimination of CH3, CH2, or CH groups (Álvarez et al. 2013).

In plants, carotenoids are synthesized in both light- and dark-grown tissues, such as leaves, endosperm, and roots. The carotenoid biosynthetic pathway occurs

in plastids were they are also stored and accumulated in envelope/thylakoid membranes and plastoglobuli (Jeffrey et al. 1974; Siefermann-Harms 1978; Austin et al. 2006). It has been suggested that the differential distribution of carotenoids between etioplasts and chloroplasts might also require differential localization of their biosynthetic enzymes (Shumskaya et al. 2012, see Chap. 9).

## 2.2 Biological Functions of Carotenoids

Carotenoids play an important role in human health and nutrition. Vertebrates do not synthesize carotenoids and depend on dietary carotenoid sources for making their retinoids, such as retinal (the main visual pigment), retinol (vitamin A) and retinoic acid (a substance controlling morphogenesis) (Fraser and Bramley 2004; Krinsky and Johnson 2005, see Chap. 14). The main precursor of retinoids is β-carotene, also called provitamin A, which contains two unsubstituted beta-ionone rings at the two ends of the molecule (Grune et al. 2010). β-carotene deficiency in human diet causes symptoms ranging from night-blindness to those of xerophthalmia and keratomalacia, leading to total blindness (Berson 1982; Sommer and Vyas 2012). Furthermore, vitamin A deficiency exacerbates afflictions like diarrhea, respiratory diseases and childhood diseases such as measles (Mayo-Wilson et al. 2011). On the other hand, certain carotenoids, such as α- and β-carotene, are good antioxidants and are necessary for human health. Supplementation of the human diet with the carotenoids lycopene or β-carotene has been shown to reduce the risk for the incidence of a number of diseases such as macular degeneration (Delcourt et al. 2006), certain types of cancer (van Poppel and Goldbohm 1995; Zu et al. 2014), neurodegenerative disease (Lu et al. 2010; Obulesu et al. 2011) and coronary heart diseases (Mayne 1996; Karppi et al. 2013).

In plants, carotenoids and their derivatives have an indispensable and prominent role. These molecules endow flowers and fruits with distinct colors and fragrance. Indeed flower color is considered one of the major attractants to pollinators in insect pollinated plants by offering a visual signal (Kevan and Baker 1983). In addition, volatile apocarotenoids mediate plant–animal interactions for pollination or seed dispersal and enhance the flavor characteristics of food crops. Certain carotenoids in the endosperm tissue of some food crops provide nutritional value and have been targets for improvement, especially in cereal crops of the grass family (Kevan and Baker 1983; Harjes et al. 2008; Vallabhaneni and Wurtzel 2009; Yan et al. 2010; Wurtzel et al. 2012, see Chap. 13). Furthermore, carotenoids play essential and multiple roles in photoprotection against photooxidative damage and heat stress to plant cells via energy dissipation and free radical detoxification, which limits damage to membranes and proteins (DellaPenna and Pogson 2006). Therefore, carotenoid antioxidants increase heat and light stress tolerance by protecting membranes from reactive oxygen species and lipid peroxidation (Davison et al. 2002; Havaux et al. 2007; Johnson et al. 2007; Li et al. 2008; Havaux 2014). For example, two molecules of β-carotene in the reaction centre of photosystem II (PSII) participate in singlet oxygen quenching and in the cyclic electron flow along

with Cyt b-559 (Miyake et al. 2002; Telfer et al. 2003, see Chap. 4). In addition, plants with reduced zeaxanthin levels exhibit increased sensitivity to light stress, as zeaxanthin prevents the oxidative damage of the thylakoid membranes (Havaux and Niyogi 1999; Verhoeven et al. 2001). Carotenoids are also involved in photosystem assembly where, from the antenna complex, they harvest light in a broader range of wavelengths in the blue region of the visible light spectrum and subsequently transfer the energy to chlorophyll (Grotewold 2006; Dall'Osto et al. 2007; Walter and Strack 2011).

Carotenoids play a key role as precursors to fitohormones such as ABA and strigolactones which influence processes as diverse as morphogenesis, seed dormancy, and environmental adaptation of plants (Auldridge et al. 2006; Paszkowski 2006; Gomez-Roldan et al. 2008; Messing et al. 2010; Walter et al. 2010; Walter and Strack 2011, see Chap. 12). Particularly, ABA is a $C_{15}$ apocarotenoid derived from the cleavage of xanthophylls (Nambara and Marion-Poll 2005) and it has been linked to several physiological processes in plants, among them the regulation of seed dormancy and germination, and the response to abiotic stress (temperature, light and drought) (Raghavendra et al. 2010). On the other hand, the biosynthetic pathway to strigolactones has recently been shown to involve carotenoid cleavage dioxygenases (CCDs), CCD7 and CCD8, to convert β-carotene to a lactone, given the name carlactone (Alder et al. 2012, see Chap. 12). In addition to their important role as rhizosphere signaling molecules (Akiyama et al. 2005; Bouwmeester et al. 2007), it has been demonstrated that strigolactones also act as a new hormone class that inhibits shoot branching in plants and hence regulates above-ground plant architecture (Gomez-Roldan et al. 2008; Umehara et al. 2008, see Chap. 12).

## 2.3 Vegetal Models as a Tool Box to Study Plant Carotenogenesis

Biochemical and biophysical approaches based on *in vitro* and *in vivo* testing have provided significant advances in our understanding of the structural and functional roles of carotenoids in plants. The use of molecular genetics has been critical in the study of carotenoid synthesis and function by using genetically disruption or over expression of key components of the pathway *in planta.* The identification, characterization and utilization of mutant plant lines, in which the carotenoid composition has been predictably manipulated at the genetic level, has allowed insights into the *in vivo* role of carotenoids and carotenogenic genes in plants. Today, the ongoing research in this field has allowed the study of carotenoid biosynthesis and function in different carotenoid-enriched plants used as models, including *Arabidopsis* (*Arabidopsis thaliana*), pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*), daffodil (*Narcissus sp.*), citrus (*Citrus sp.*), tobacco (*Nicotiana tabacum*), carrots (*Daucus carota*) among others (Dogbo et al. 1988; Beyer et al. 1989; Cunningham and Gantt 1998; Hirschberg 2001; Fraser and Bramley

2004; Stange et al. 2008; Fuentes et al. 2012; Kachanovsky et al. 2012; Ruiz-Sola and Rodríguez-Concepción 2012). Particularly, extensive study of carotenoid biosynthesis in *Zea mays* (maize), a major food crop, has benefitted from the diverse germplasm collection, phenotypic mutants, genetic and physical mapping, and quantitative trait loci (QTL) affecting carotenoid biosynthesis (Wurtzel et al. 2012). Other interesting models for understanding carotenogenesis include saffron (Frusciante et al. 2014) and kiwifruit (Ampomah-Dwamena et al. 2009). However, much remains to be understood about the regulation of carotenoid biosynthesis in the context of the manifold roles of carotenoids in plants.

To date, the knowledge generated has helped to define and characterize the key enzymes for carotenoids production in plants (Farré et al. 2011; Quinlan et al. 2012; Ruiz-Sola and Rodríguez-Concepción 2012) and provided insight into the integration of carotenoid synthesis with components of other metabolic pathways.

## 2.4 Carotenoid Biosynthetic Pathway: Genes, Proteins and Products

Carotenoids, as well as other isoprenoids, are built from the 5-carbon (5C) compound isopentenyl pyrophosphate (IPP). In plants, there are two sources of IPP: The cytosolic mevalonic acid pathway (MVA, see Chap. 1, Fig. 1.1) and the plastid methylerythritol 4-phosphate (MEP, Chap. 1, Fig. 1.1) pathway (Lichtenthaler et al. 1997; Rodríguez-Concepción and Boronat 2002; Eisenreich et al. 2004). In the cytosolic pathway, IPP is formed from three molecules of acetyl-CoA via 3-hydroxy-3-methyl glutaryl-CoA (HMG-CoA), mevalonic acid (MVA), mevalonic acid 5-phosphate (MVAP) and mevalonic acid 5-diphosphate (MVAPP). Hence, this pathway is called the mevalonic acid pathway (MVA). Before chain elongation begins IPP isomerase (IDI) catalyzes IPP conversion into its allylic isomer, dimethylallyl pyrophosphate (DMAPP), which is the initial, activated substrate for the formation of sesquiterpenes (C15) and triterpenes (C30) such as sterols (Laule et al. 2003; Hsieh and Goodman 2005). DMAPP condenses with a molecule of IPP to give the C10 compound, geranyl pyrophosphate.

In contrast to the MVA pathway, the MEP pathway is localized in plastids and is the major source for plant isoprenoids biosynthesis (Bramley 2002, Lichtenthaler 1999). The IPP and DMAPP used for carotenoid biosynthesis in plants are derived from the MEP pathway (Rodríguez-Concepción and Boronat 2002; Eisenreich et al. 2004). In addition, a diverse range of compounds including monoterpenes, tocopherols, phylloquinone, gibberellins (GA), chlorophylls, and plastoquinone are also derived from IPP and DMAPP (Rodríguez-Concepción 2010).

Along the pathway, carotenoids can be cleaved into apocarotenoids by enzymes of the carotenoid cleavage dioxygenase (CCD) family (Auldridge et al. 2006, see Chap. 12). Apocarotenoids are a class of terpenoid compounds, which include some important volatile compounds such as β-ionone, geranyl acetone, pseudoionone,

α-ionone and 3-hydroxy-β-io-none (Simkin et al. 2004). The *Arabidopsis* CCD family has nine members, including four CCDs (CCD 1, 4, 7 and 8) and five 9-cis-epoxycarotenoid dioxygenases (NCED 2, 3, 5, 6 and 9, see Chap. 11). NCEDs specifically catalyze the first step of the ABA biosynthesis pathway and use 9-cis-viola-xanthin and 9-cis-neoxanthin as substrates (Nambara and Marion-Poll 2005).

### *2.4.1 Plastid Localization of the Carotenogenic Pathway*

The core of the carotenoid biosynthetic pathway consists of around 10 enzymes. In plants, the enzymatic formation of carotenoids occurs on plastid membranes and is mediated by nuclear-encoded enzymes (DellaPenna and Pogson 2006; Cuttriss et al. 2011). However, the location of the biosynthetic pathway as a complete entity for controlling the unique spatial distribution of carotenoids remains unclear, even though biochemical studies made clear that carotenoid enzyme location is critical for activity. This suggests that carotenoids destiny may be different on the plastid envelope (e.g. conversion to apocarotenoids involved in mediating signaling) as compared to carotenoids on thylakoid membranes, where these pigments function as structural components for photosynthesis or photoprotection. According to Cunningham and Gantt (Cunningham and Gantt 1998) the carotenoid biosynthetic enzymes are likely to be organized in protein complexes in plants. Evidence of *in vivo* channeling (De la Guardia et al. 1971; Candau et al. 1991), and detection of high molecular weight complexes containing biosynthetic enzymes (Al-Babili et al. 1996; Lopez et al. 2008) support the fact for the limited detection of pathway intermediates (Wurtzel 2004; Quinlan et al. 2012).

Mass spectrometry studies of the chromoplast proteome found individual carotenoid enzymes mostly in plastoglobuli (Ytterberg et al. 2006) or in membrane fractions (Wang et al. 2013). Plastoglobulies attached to thylakoid membrane of chloroplasts contain PSY (with the exception of the “yellow endosperm” allele for maize PSY1), which would suggest that PSY is physically separated from the rest of the enzymes of the pathway (Shumskaya et al. 2012). The envelope localization of carotenoid enzymes can be explained by the need to supply carotenoids for abscisic acid (ABA) synthesis, which occurs outside of the plastid (Cutler et al. 2010). In particular, in recent proteomic studies on *Arabidopsis thaliana* chloroplasts (Joyard et al. 2009; Ferro et al. 2010), many, but not all carotenoid biosynthetic enzymes are found in envelope membranes, while only a few carotenoid enzymes are identified in thylakoids (Ytterberg et al. 2006; Joyard et al. 2009; Ruiz-Sola and Rodríguez-Concepción 2012). These enzymes include xanthophyll cycle enzymes and phytoene desaturase (PDS). In pepper (*Capsicum annuum*) fruit chromoplasts, most carotenoid enzymes are localized to plastoglobuli. In maize (*Zea mays*), through proteomic studies, PDS and ZDS were the only carotenoid enzymes detected in membrane fractions of bundle sheath and mesophyll cells, respectively (Friso et al. 2010). Although, carotenoids are found in both cell types, no other carotenoid biosynthetic enzymes were detected. In maize PSY, isozymes differ in

chloroplast suborganellar localization and can be found either in plastoglobuli or in the stroma and thylakoid membranes (Shumskaya et al. 2012). Even though all of the pathway enzymes are known today and most OMIC approaches have reported the location of individual enzymes, the three-dimensional structure of the "pathway" and a putative metabolomic organization is not yet understood (Shumskaya and Wurtzel 2013).

### *2.4.2 MEP Pathway to GGPP*

The plastid-localized MEP pathway combines glyceraldehyde-3-phosphate and pyruvate to form deoxy-D-xylulose 5-phosphate (DXP), a reaction catalysed by DXP synthase (DXS) (Fig. 2.1). MEP is subsequently formed via an intramolecular rearrangement and reduction of DXP by the enzyme DXP reductoisomerase (DXR). Both DXS and DXR are important in carotenoid flux regulation. In Arabidopsis, both enzymes are encoded by single genes and appear to be rate-determining enzymes. Indeed, the Arabidopsis *Cla1* mutant, in which the DXS gene of the MEP pathway is disrupted, present photo bleaching due to the absence of protective carotenoids (Araki et al. 2000; Estévez et al. 2000). In contrast, over expression of DXS and DXR in *Arabidopsis* seedlings increases carotenoid production (Estévez et al. 2001; Carretero-Paulet et al. 2006) and the expression of *PSY* resulted in increased carotenoid accumulation and a concomitant accumulation of the DXS enzyme (Rodríguez-Villalón et al. 2009). Moreover, Recent work has also suggested the post-transcriptional regulation of DXS activity mediated by the involvement of J-protein (J20) and heat shock protein 70 (Hsp70) chaperones. Indeed, mutants defective in J20 activity exhibit reduced DXS enzyme activity and accumulate DXS protein only in an inactive form (Pulido et al. 2013). It is suggested that plastidial J20 protein appears to assist Hsp70 chaperone in the proper folding and assembly of DXS and participate in the regulation of MEP-derived isoprenoid biosynthesis (Pulido et al. 2013). In poplar, a feedback inhibition of DXS by DMAPP reveals an important regulatory mechanism of the MEP pathway and thus carotenoid biosynthesis (Ghirardo et al. 2014). The enzymes of the MEP and carotenoid pathways are preferred targets for herbicides (Withers and Keasling 2007) because these pathways are not present in animals and the blockage of any of the steps preceding the formation of lycopene inhibits carotenoid synthesis. The herbicide clomazone inhibits DXS (Müller et al. 2000, Fig. 2.1) by an indirect mechanism once it is absorbed into the plant. Clomazone is oxidized by cytochrome P450 monooxygenases (P450s) to form the putative active herbicidal form (Müller et al. 2000), ketoclomazone. This requirement for metabolic activation was shown in plants treated with P450s inhibitors such as phorate, where the herbicidal effect of clomazone was prevented (Ferhatoglu et al. 2005; Ferhatoglu and Barrett 2006). Although the antibiotic fosmidomycin is not used as an herbicide, this compound inhibits DXR (Singh et al. 2007) (Fig. 2.1), the second step of the MEP pathway (Rohmer 1998; Rohmer et al. 2004). The potential benefits of developing new

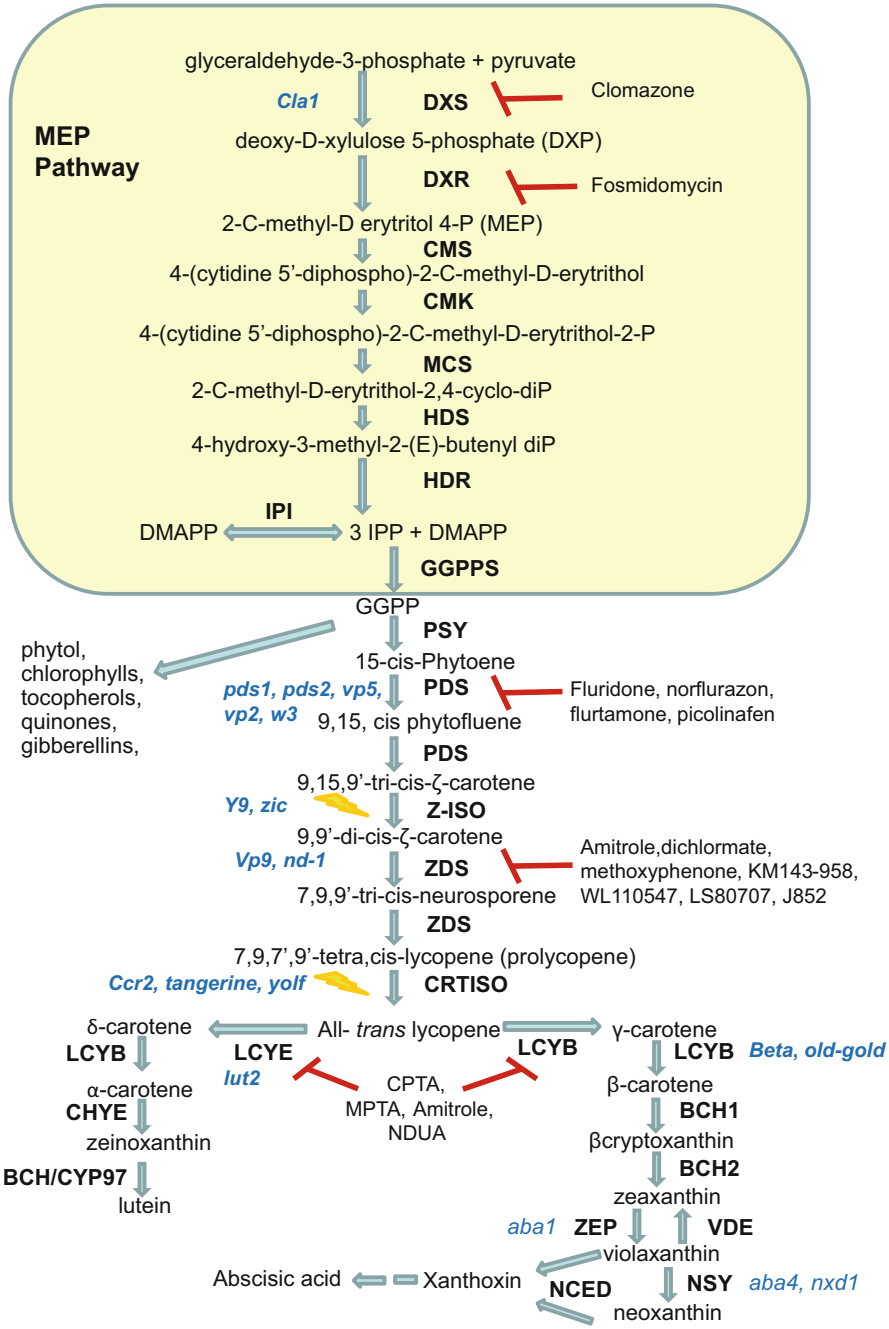


**Fig. 2.1** (continued)

herbicides which interfere with pathways leading to carotenoids, such as the MEP and isoprenoid pathways, has been addressed recently (Hale et al. 2012; Corniani et al. 2014) taking into account the emergence of resistance to herbicides as an increasing problem facing agriculture (Service 2007, 2013). A number of steps are then required in the condensation between IPP and its allylic isomer DMAPP (Fig. 2.1), to form the C10 compound, geranyl pyrophosphate (GPP). The further addition of two IPP units results in the formation of C20 geranylgeranyl pyrophosphate (GGPP) the precursor of carotenoid biosynthesis (Bramley 2002; Cunningham 2002; Fraser and Bramley 2004). The sequential addition of three IPP molecules to a DMAPP molecule is catalyzed by GGPP synthase (GGPPS).

### *2.4.3 GGPP to Phytoene: Phytoene Synthase*

Phytoene synthase (PSY) is the first dedicated enzyme of the carotenoid biosynthesis pathway and catalyzes the condensation of two GGPP molecules into phytoene as a 15-*cis* isomer by introducing a central 15-Z double bond during hydrogen elimination (Cunningham and Gantt 1998). Due to the fact that PSY is the enzyme that catalyzes the first committed step in the plant carotenoid pathway, it has been postulated to be rate-limiting, regulating the reaction flux. In fact, dosage effect of the maize *Y1* allele upon seed carotenoid content was noted as early as the 1940s (Randolph and Hand 1940), where three copies of the dominant *Y1* allele in the triploid endosperm conditioned the most yellow seeds (endosperm) in contrast to homozygous white *y1* seeds. Cloning and subsequent sequence analyses identified the *Y1* gene as the gene coding for *PSY1* (Buckner et al. 1996). Considering PSY as rate-limiting, it has been a preferred target for genetic manipulation. Fray et al. (1995) were the first to report ectopic quantitative and qualitative changes in

**Fig. 2.1** (continued) Isoprenoid and Carotenoid pathway in plants. Schematic representation of the plastidial MEP (2-C-methyl-D-erythritol-4-P) pathway: DXS: deoxyxylulose 5-phosphate synthase, DXR: deoxyxylulose 5-phosphate reductoisomerase, DXP: Deoxy-D-xylulose-5-P, CMS: 2C-methyl-D-erythritol 4-phosphate cytidyltransferase, CMK: 4-(cytidine 5-diphospho)-2-C-methyl-D-erythritol kinase, MCS: 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, HDS: 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HDR: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, IPP (isopentenyl pyrophosphate), GGPPS: geranylgeranyl pyrophosphate synthase, DMAPP: dimethylallyl pyrophosphate, IPI: isopentenyl pyrophosphate isomerase, GGPP: geranylgeranyl pyrophosphate. The relevant genes for carotenoid synthesis are: PSY: phytoene synthase, PDS: phytoene desaturase, Z-ISO: ζ-carotene isomerase, ZDS: ζ-carotene desaturase, CRTISO: carotene isomerase, LCYB: lycopene β-cyclase, LCYE: lycopene ε-cyclase, BCH: carotenoid β hydroxylase, CHYE: carotenoid ε-hydroxylase (CYP97C1, CYP97A3), ZEP: zeaxanthin epoxidase, VDE: violaxanthin deepoxidase, NSY: neoxanthin synthase, NCED: 9-cis-epoxycarotenoid dioxygenase. Reported mutants in isoprenoid and carotenogenic genes are written in blue. Chemical inhibitors of some enzymes are included with a red T symbol. Light, referred as a yellow ray can replace Z-ISO and CRTISO activity in photosynthetic organs

carotenoids through fruit-specific expression of a phytoene synthase cDNA under a CaMV 35S promoter in tomato. Interestingly, the overexpression lines present a dwarf phenotype with a 30-fold reduction in gibberellin A1 (GA1) and chlorophyll levels. The dwarf character was inherited with an inverse relationship between plant height and expression of *PSY*, which redirects GGPP to phytoene and diverts this intermediate away from the gibberellin (GA) and phytol biosynthetic pathways. On the other hand, the overexpression of an exogenous daffodil *PSY* in Japonica rice model variety Taipei 309 leads to phytoene accumulation in endosperm, which was the first instance of carotenoid engineering in rice (Burkhardt et al. 1997). The regulation and rate-limiting role of PSY was also evident in *Brassica napus* expressing an additional *PSY* gene from the bacterium *Erwinia uredovora*, crtB (Shewmaker et al. 1999). The transgenic plants resulted in a 50-fold increase in total carotenoid content, predominantly α-carotene, β-carotene and phytoene. A similar study was reported in transgenic tomato carrying the additional *E. uredovora crtB* gene under the control of fruit-specific polygalacturonase promoter (Fraser et al. 2002). The PSY activity in these transgenic plants was increased five to ten-fold. This was accompanied by a two- to four-fold increase in the total carotenoid content of primary transformants whereas phytoene was increased 2.4 fold. In another study, *E. uredovora* crtB was overexpressed in potato (Ducreux et al. 2005, see Chap. 13). In addition, constitutive *PSY* overexpression has enhanced total carotenoid contents and substantially increased synthesis of β-carotene in a variety of many other vegetal species such as linseed flax, carrots, maize, and cassava roots (Fujisawa et al. 2008; Maass et al. 2009; Naqvi et al. 2009; Welsch et al. 2010, see Chap. 13). It is important to take into account that most of those results obtained after over expression of the *PSY* gene in carotenoid-producing fruits may result in a shift in the regulatory control point within the pathway. Furthermore, the bigger increase in the carotenoid content in those cases of using a plant *PSY* instead of the bacterial *crtB* could be due to the less effective protein-protein interaction in the multienzymatic complexes produced with CRTB compared with those formed with plant *PSY*s.

Recently, a phytoene synthase-RNAi construct was delivered into the *Oncidium* hybrid orchid as the first report using RNAi approaches to break down carotenogenesis in this plant (Liu et al. 2014). Transgenic lines displayed a semi-dwarf phenotype and brilliant green leaves as a consequence of changes in the carotenoid, gibberellin, abscisic acid and chlorophyll biosynthetic pathway (Liu et al. 2014).

The localization of PSY within the chloroplast is another highlight finding reported recently (Shumskaya et al. 2012; Shumskaya and Wurtzel 2013). While the majority of phytoene synthases, such as maize (PSY2 and PSY3), rice and Arabidopsis PSYs, are found to localize to plastoglobuli, maize PSY1 isozymes are localized to distinct chloroplast suborganellar sites (e.g. globular or fibrillar) based on their allelic variation, suggesting that PSY1 sequence variation can affect suborganellar localization of carotenoid storage and bioavailability.

### 2.4.4 *Lycopene Synthesis: Teamwork of Desaturases and Isomerases*

In plants and cyanobacteria, phytoene undergoes four successive dehydrogenations and isomerizations to produce lycopene. The first two desaturation steps are catalyzed by phytoene desaturase (PDS), while the latter two reactions are catalyzed by (zeta) ζ-carotene desaturase (ZDS). These desaturation reactions introduce a series of carbon–carbon double bonds that constitute the chromophore in carotenoid pigments, and they transform the colorless phytoene into the red-colored lycopene. These reactions lead to a poly-cis pathway in contrast to the all-trans pathway carried out by CRTI in bacteria and fungi (Sandmann 1994; Armstrong 1997, see Chap. 1). Therefore, in concert to these steps, ζ-carotene isomerase (Z-ISO) and carotenoid isomerase (CRTISO) catalyze *cis–trans* reactions to isomerize the four cis-bonds introduced by the desaturases (Fig. 2.1). Z-ISO activity occurs upstream of CRTISO and catalyzes the *cis* to *trans* conversion of the product of PDS to form the substrate of ZDS (Li et al. 2007) whereas CRTISO is involved in the conversion of poly-*cis* lycopene to *trans*-lycopene (Figs. 2.1 and 1.3. Isaacson et al. 2002; Park et al. 2002). Reactions performed by Z-ISO and PDS precede ZDS which requires substrates with desaturated 11 and 11′ bonds (Albrecht et al. 1996) and 15-15′ in *trans* (Breitenbach and Sandmann 2005). The isomerization reaction carried out by CRTISO is the last reaction since it requires substrates to have adjacent 7- and 9-*cis* bonds (Isaacson et al. 2004). It is not understood why bacteria and fungi accomplish these reactions with a single enzyme, CRTI. The most likely explanation refers to possible roles of the *cis*-intermediates in regulatory signaling pathways (Kachanovsky et al. 2012, see Chap. 1)

#### 2.4.4.1 Desaturases

Two sequential desaturations of phytoene, catalyzed by PDS, result in the formation of phytofluene, followed by the formation of ζ-carotene. Additional desaturations, catalyzed by Z-ISO and ZDS, give rise to 7,9,9′-*cis*-neurosporene. The deduced peptide sequences of PDS and ZDS show a high degree of similarity between different plant species. Nevertheless, PDS shows 33% to 35% identity to ZDS, they can be grouped together in a phylogenetic tree, indicating a common ancestor for both proteins (Beyer et al. 1989; Albrecht et al. 1995; Hirschberg et al. 1997). The desaturases require FAD and the association with a membrane to be active (Nievelstein et al. 1995; Bonk et al. 1997). The FAD appears to feed an electron transport chain involving quinones and the plastid terminal oxidase (PTOX). Particularly, quinones act as electron acceptors for PDS and ZDS desaturation reactions, as demonstrated in daffodil and Arabidopsis (Beyer 1989; Mayer et al. 1990; Norris et al. 1995), in which the impaired plastoquinone biosynthesis in *pds1* and *pds2* mutants resulted in albino phenotypes and phytoene accumulation (Norris et al. 1995; Norris et al. 1998). The cloning of the *IMMUTANS* gene encoding PTOX also

revealed a link between phytoene desaturation and redox poise of the chloroplast (Al-Babili et al. 1996; Josse et al. 2000; Carol and Kuntz 2001). The disruption of the *IMMUTANS* gene prevents oxidation during chlorophyll biosynthesis and results in the accumulation of phytoene (Carol et al. 1999; Wu et al. 1999; Aluru et al. 2001).

Phytoene desaturase is also a rate-limiting enzyme in carotenoid synthesis (Chamovitz et al. 1993). Maize mutants viviparous5 (*vp5*), *vp2* and *white3* (*w3*) are associated with the function of PDS by the accumulation of phytoene both in leaf and seed (Treharne et al. 1966; Neill et al. 1986; Hable et al. 1998) The *vp5* locus was established as the structural gene for PDS. A soybean cDNA encoding PDS was cloned using a heterologous probe derived from the *Synechococcus PDS* gene (Bartley et al. 1991). The tomato *PDS* cDNA was cloned by a similar approach and its identity was confirmed by expression in *E. coli*, which resulted in the formation of ζ-carotene (Pecker et al. 1992). The pepper *PDS* coding sequence, isolated using antibodies raised against the purified protein, showed high homology to soybean and tomato *PDS* genes and active PDS recombinant enzyme was obtained in *E. coli* (Hugueney et al. 1992). Transgenic tobacco plants overexpressing *PDS* had no phenotypic or metabolic effects (Busch et al. 2002; D'Haeze 2002). However, *PDS* down-regulation led to a dramatic accumulation of phytoene (Busch et al. 2002; D'Haeze 2002), a lethal phenotype in homozygous seedlings (Busch et al. 2002) and albinism (Travella et al. 2006; Kirigia et al. 2014). Transgenic tobacco plants showing different degrees of albinism caused by *PDS* silencing presented a decrease in total carotenoid, chlorophyll and PSII efficiency (Wang et al. 2009).

The protective function of carotenoids depends on the complete conjugated double-bond system. As a consequence, any step in carotene biosynthesis up to lycopene formation is a potential target for a herbicidal inhibitor (Böger and Sandmann 1998). PDS is the molecular target site for several herbicides such as fluridone, norflurazon, flurtamone and picolinafen (Dayan and Duke 2003). These herbicides compete for the binding site of plastoquinone (Breitenbach et al. 2001) as an essential cofactor for this enzyme (Norris et al. 1995). ZDS is essential for cell growth, stress responses and carotenoid biosynthesis, providing a precursor for cyclization reactions (Bartley et al. 1999). Coding sequences for *ZDS* have been isolated and characterized in maize (Matthews et al. 2003), pepper (Breitenbach et al. 1999), common fig (Araya-Garay et al. 2012) and goji berry plants (Li et al. 2015). Maize ZDS did not function efficiently in a heterologous complementation system where PDS did (Matthews et al. 2003). Suggested reasons for the low activity of carotenoid desaturases include: End product inhibition of ZDS (Sandmann and Böger 1989; Breitenbach et al. 1999), failure to reconstitute the interactions of an assembly complex responsible for substrate channeling (Breitenbach et al. 1999), the absence of a carotene isomerase (Bartley et al. 1999), among others.

Mutation of the *ZDS* gene from Arabidopsis resulted in impaired carotenoid biosynthesis and subsequent spontaneous cell death from the increased content of superoxide (Dong et al. 2007).

Moreover, it has been shown that loss of function of the ζ -carotene desaturase encoded by *ZDS*/CLB5/SPC1/PDE181 arrests chloroplast biogenesis at a very early

stage of development and Arabidopsis plants also exhibit dramatic alterations in leaf morphology and in the expression of a variety of chloroplast proteins and genes required for leaf development. These phenotypes were not observed in mutants deficient in the closely related desaturase *PDS* (Avendaño-Vázquez et al. 2014). The *vp9* maize *ZDS* mutant has reduced or suppressed ABA and carotenoid accumulation in both endosperm and vegetative tissues, even when it accumulates ζ-carotene (Matthews et al. 2003), while the non dormant-1 (*nd*-1) mutant of sunflower (*Helianthus annuus* L.) was characterized by an albino and viviparous phenotype (Conti et al. 2004).

Compared to inhibitors of phytoene desaturation, fewer compounds interfere with the ZDS reaction. The following compounds have been reported to accumulate ζ-carotene in whole plants: Amitrole, Dichlormate (Burns et al. 1971), Methoxyphenone (NK049) (Fujii et al. 1977), KM 143-958 (Chollet et al. 1990), WL 110547 (Sandmann et al. 1996; Kerr and Whitaker 1987), LS 80707 (Sandmann et al. 1985), J852 (Chollet et al. 1990).

The modes of action of J852 and LS 80707 (including related compounds) in carotene biosynthesis are well defined. Inhibitors treatment causes ζ-carotene accumulation and the concurrent bleaching of plants (Sandmann and Böger 1989; Chollet et al. 1990). Pepper leaves treated with the herbicide J852 showed an accumulation of phytoene and ζ-carotene. Unexpectedly, none of the genes coding for PSY, PDS, ZDS or the Terminal Oxidase associated with phytoene desaturation were induced upon herbicide treatment in pepper leaves or seedlings (Simkin et al. 2000).

#### 2.4.4.2 Isomerases

Plants do not only require desaturases to convert 15-*cis*-phytoene into all-*trans* lycopene. In fact, while PDS is active it prefers 15-*cis* substrates (Breitenbach and Sandmann 2005). ZDS is only active against substrates with a 15-*trans* conformation, (Breitenbach and Sandmann 2005) indicating the requirement of two types of isomerase enzymes. Particularly 15-*cis* phytoene is transformed by PDS into 15,9′-di-*cis*-phytofluene, and eventually 9,15,9′-tri-*cis*-ζ-carotene, which is isomerized at the 15-*cis*-double bond to form the substrate of ZDS, 9,9′-di-*cis*-ζ-carotene (Bartley et al. 1999; Matthews et al. 2003; Breitenbach and Sandmann 2005). The isomerization step of the 15-*cis* bond of ζ-carotene to *trans*, can be mediated either by light or by the 15-*cis*-ζ-carotene isomerase (Z-ISO) occurring in the chloroplasts of photosynthetic tissues or in other plastid types, respectively (Breitenbach and Sandmann 2005; Li et al. 2007, Fig. 2.1). However, the loss-of-function phenotypes detected in mutants of maize and Arabidopsis strongly suggest that Z-ISO activity is required even in the presence of light. Plants with insufficient Z-ISO also grow poorly under the stress of fluctuating temperature. Z-ISO is predicted to be an integral membrane protein with several membrane-spanning domains, but there is no available information about its sub organellar localization, showing whether the protein is found in the envelope or the thylakoid membranes

(Janick-Buckner et al. 2001, Ruiz-Sola and Rodríguez-Concepción 2012; Beltrán et al 2015). Because climatic variations alter the need for photosynthetic and non-photosynthetic carotenoids, Z-ISO facilitates plant adaptation to environmental stress, a major factor affecting crop yield. It was also described that Z-ISO exists in a high-molecular-weight protein complex of about 480 kDa (Shumskaya and Wurtzel 2013), similarly to other carotenoid enzymes (Zybailov et al. 2008; Ishikawa et al. 2009). Z-ISO homologues can be found in plants and cyanobacteria. A mutant in the Z-ISO gene of maize, referred to as pale yellow 9 (*y9*), in addition to the isolation of Arabidopsis *zic* mutant, showed 9,15,9′-tri-*cis*-ζ-carotene accumulation in the dark, leading to the identification of 15-*cis*-ζ-carotene isomerase (Z-ISO). Expression of the *Z-ISO* transcript is highly correlated with the expression of other carotenogenic genes. Single copy genes appear to encode *Z-ISO* in most plants (reviewed in Ruiz-Sola and Rodríguez-Concepción 2012), but no evidence of alternate transcripts was found in plants such as maize or rice, except for Arabidopsis (Chen et al. 2010). The single Arabidopsis gene encoding Z-ISO (At1g10830) produces two alternate transcripts, *Z-ISO*1.1, which is highly expressed and encodes a functional enzyme, and a shorter *Z-ISO*1.2, which encodes a truncated protein that lacks the predicted C-terminal transmembrane domain and shows no Z-ISO activity. Therefore, the effect of the deletion suggests that the C-terminal TM domain is important for the function of Z-ISO, either for activity or proper folding (Chen et al. 2010). Screening for impaired carotenoid formation in dark germinated seeds of maize (*Zea mays*) and Arabidopsis mutants were performed to identify the gene by functional complementation assays in *E. coli*, expressing bacterial *GGPPS* and *PSY*. In maize Z-ISO is an enzyme related to nitrite and nitric oxide reductase U (NnrU), which is normally present in denitrifying bacteria. Considering that the bacterial denitrification pathway produces nitrogen oxides as alternate electron acceptors for anaerobic growth, it has been suggested that plant carotenogenesis evolved by recruitment of genes from noncarotenogenic bacteria (Li et al. 2007; Chen et al. 2010).

Based on the central location of the double bond in the substrate of Z-ISO, it is predicted that this enzyme recognizes the carotenoid molecule differently in comparison to the mechanism proposed for CRTI, PDS, ZDS or CRTISO, which act on one half of the molecule. The lack of sequence homology of Z-ISO to any known carotenogenic enzyme also suggests a different mechanism of reaction. Recently, it was shown that Z-ISO is a bona fide enzyme, which independently catalyzes the *cis-trans* isomerization of the 15–15′ carbon-carbon double bond in 9,15,9′-*cis*-ζ-carotene through a unique mechanism requiring a redox-regulated heme β cofactor that undergoes redox-regulated ligand switching between the heme iron and alternate Z-ISO amino acid residues (Beltrán et al. 2015). Reduction of the heme iron switches coordination of the heme to *bis*-histidine and exposes the active site for substrate binding. These results let to the proposal that Z-ISO as a metalloprotein, representing a new prototype for heme β proteins that potentially uses a new chemical mechanism.

Following the production of 9,9′-di-*cis*-ζ-carotene by Z-ISO, the enzyme ZDS carries out the stereospecific abstraction of protons from 7 and 7′, which results in

the formation of the 7 and 7′ *cis* bonds in 7,9,9′-tri-*cis*-neurosporene (Breitenbach and Sandmann 2005; Sandmann 2009) followed by the synthesis of 7,9,9′,7′-tetra-*cis*-lycopene (also called pro-lycopene) with very low efficiency (Bartley et al. 1999). Because 7,9,7′,9′-tetra-*cis*-lycopene is not a substrate for β or ε-cyclases (Schnurr et al. 1996; Yu et al. 2011), the formation of the poly-*cis* intermediates requires the recruitment of an additional isomerase to generate the all-trans-lycopene, a suitable substrate for β and ε-cyclases (Breitenbach and Sandmann 2005). In chloroplasts, the isomerization of pro-lycopene to all-*trans*-lycopene can occur non-enzymatically in the presence of light whereas the activity of CRTISO (Fig. 2.1) fulfills a critical role in converting the *cis*-double bonds introduced by PDS and ZDS to all-*trans* in tissues receiving no light exposure and in non-photosynthetic tissues (Isaacson et al. 2002; Park et al. 2002). Therefore, CRTISO-deficient plants can still synthesize carotenoids but at slower rate and only in specific tissues. The isomerase activity of CRTISO requires the presence of membranes and the flavin adenine dinucleotide (FAD) binding motif, which bonds to the reduced form of the cofactor (FADred) to catalyze a reaction without net redox changes (Isaacson et al 2004; Yu et al. 2011). The regional specificity and the kinetics of the isomerization reaction were recently determined (Yu et al. 2011).

CRTISO is thought to be evolved from the bacterial-type desaturase (CRTI) considering the high sequence homology between both proteins (Isaacson et al. 2002; Park et al. 2002; Isaacson et al. 2004; Chen et al. 2010). Nevertheless, in terms of mechanisms of reaction related to the reduced flavin requeriment for its activity, it is especially interesting that CRTISO seems to be more related to CRTY (bacterial lycopene cyclase) than to its possible ancestor CRTI (Mialoundama et al. 2010; Yu et al. 2010; Yu et al. 2011).

The Arabidopsis, tomato, and melon mutants of *CRTISO* (*ccr2*, *tangerine*, and *yolf*, respectively) accumulate *cis*-carotenes in etioplasts of seedlings or chromoplasts of fruits (Isaacson et al. 2002; Park et al. 2002; Galpaz et al. 2013). However, it is interesting to note that the CRTISO activity can partially be substituted by exposure to light in green tissues via photoisomerization (Isaacson et al. 2002; Park et al. 2002). Photoisomerization of the *cis* bonds facilitates carotene synthesis in the chloroplasts of the *ccr2* mutant, but delays greening and chlorophyll accumulation during photomorphogenesis. Similarly, loss of CRTISO causes partial inhibition of lutein biosynthesis in light-grown tissues and varying degrees of chlorosis in newly developed leaves of tomato and rice (Isaacson et al. 2002; Masamoto et al. 2004; Fang et al. 2008; Wei et al. 2010; Chai et al. 2011). In contrast to green tissues, CRTISO activity cannot be substituted by light in non-photosynthetic tissues. The etiolated tissues of *ccr2*, *tangerine*, and *yolf* fruits exhibit an orange color due to the accumulation of *cis*-lycopene (Isaacson et al. 2002; Park et al. 2002; Galpaz et al. 2013). Such accumulation is associated with a metabolite-dependent feedback regulation of early carotenoid synthesis genes. The feedback regulation of early tomato carotenoid genes (*PSY1*, *PDS*, and *ZDS*) observed in *tangerine* elucidates the recently discovered epistasis effect of *tangerine* over the *r* mutation in *PSY*, which partially restores *PSY1* expression by *cis*-carotene accumulation in *tangerine* (Kachanovsky et al. 2012).

Even though most carotenoids found in nature are primarily in the more stable all-*trans* configurations (Britton 1995), a small proportion of *cis* isomers are encountered. Interestingly, they have different biological potency than their *trans* counterparts (e.g. lower pro vitamin A activity); they are constituents of the light harvesting complex (LHC) such as 9-*cis*-neoxanthin (Liu et al. 2004) and supply substrates for the biogenesis of plant hormone, ABA such as 9-*cis*-epoxyxanthophylls (Schwartz et al. 1997).

### *2.4.5 Lycopene to Cyclic Carotenes: Cyclases*

Lycopene is the starting compound of various end group modifications that produces a large variety of carotenoids with different physical properties. In higher plants two enzymes, lycopene β-cyclase (LYCB) (Pecker et al. 1996) and lycopene ε-cyclase (LYCE) (Cunningham and Gantt 2001, Fig. 2.1) compete for lycopene as substrate, leading to carotenoids with either β or ε-ionone rings. Therefore, cyclization of lycopene represents the first branching point in the carotenogenic pathway (Fig. 2.1).

The amino acid sequence of lycopene cyclases from plants and cyanobacteria are polypeptides with around 400 amino acids and have a molecular mass of 43 kDa (Schnurr et al. 1996). The enzymes from plants also have an additional N-terminal transit sequence of 100 amino acids (Krubasik and Sandmann 2000).

In addition, the N terminus of plant LCYB and CCS contains dinucleotide-binding motifs characteristic of an N-terminal Rossmann fold of FAD-dependent oxidoreductases (Rossmann et al. 1974; Cunningham et al. 1994; Hugueney et al. 1995). Indeed, it has been reported that CCS-bound FAD is required for enzyme activity in the presence of NADPH, which functions as a reductant of FAD (Cunningham et al. 1994). *Erwinia uredovora* LCYB, which cyclizes lycopene into β-carotene, strictly requires NADPH but proceeds without any net redox change (Schnurr et al. 1996; Hornero-Méndez and Britton 2002).

LCYB and LCYE share significant similarities in their amino acid sequences, suggesting that they have originated from a common ancestor through gene duplication (Sandmann 2002). There are also two other related carotenoid cyclase enzymes: the capsanthin-capsorubin synthase (CCS) of pepper (Cunningham et al. 1996) and the neoxanthin synthase (NSY) of tomato (Bouvier et al. 2000) and potato (Al-Babili et al. 2000). CCS catalyzes the formation of the unusual five-carbon k ring (Bouvier et al. 1994), converting antheraxanthin or violaxanthin into the k-cyclic carotenoids capsanthin or capsorubin, respectively, by a similar mechanism to lycopene cyclization (Bouvier et al. 1997). Interestingly, CCS exhibits LCYB activity when lycopene is provided as a substrate (Hugueney et al. 1995).

The LCYB enzyme catalyzes the cyclization of one end of the linear lycopene molecule to produce γ-carotene. Subsequently, the enzyme forms a second β-ring at the other extreme to yield β-carotene (Fig. 2.1). LCYE introduces a single ε-ring into lycopene to produce δ-carotene and a subsequent cyclization of the ψ- acyclic

end of δ-carotene by LCYB generates α-carotene. Carotenoids containing two ε-rings are rarely found in plants and algae (Goodwin 1980; Cunningham et al. 1996) with the notable exception of *Lactuca sativa* (lettuce), in which a unique LCYE can cyclize both ψ-ends of lycopene to generate ε,ε-carotene and the dihydroxy derivative lactucaxanthin (Siefermann-Harms et al. 1981; Phillip and Young 1995; Cunningham and Gantt 2001). Several specific domains and amino acid residues have been identified which determine whether LCYE introduces one or two ε-ionone rings (Cunningham and Gantt 2001).

Lycopene cyclases, LCYB and LCYE, are involved in determining carotenoid content and composition in different plants. Some plants fine-tune carotenoid content using tissue-specific isoforms of lycopene cyclases such as those chromoplast-specific *LCYB* genes expressed in the fruits and flowers of tomato, papaya and saffron that correlate with the accumulation of β-carotene and/or downstream xanthophylls (Ronen et al. 2000; Ahrazem et al. 2010; Devitt et al. 2010). Two color mutations in tomato, *Beta* and *old-gold* (og) affect the fruit and flower-specific *LCYB* which is encoded by the B gene (Ronen et al. 2000). *Beta* is a partially dominant, single-locus mutation that causes an orange color in the fully ripened fruit because of the accumulation of β-carotene at the expense of lycopene due to an important increase in the transcription of the B gene. By contrast, the *old-gold* (og) mutant carries a null allele in the locus B resulting in an elevated concentration of lycopene and a reduction of β-carotene (Ronen et al. 2000).

A mutation in the papaya *LCYB2* dramatically reduces its expression and is responsible for the difference between red- and yellow-fleshed fruits as consequence of lycopene accumulation (Blas et al. 2010; Devitt et al. 2010).

Transcript analysis of homo- and heterozygous Arabidopsis *lut2* mutants demonstrates that *lut2* is semidominant and their biochemical phenotype is consistent with a disruption of epsilon ring cyclization (Pogson et al. 1996). In particular, *lut2* mutation has significantly higher levels of the xanthophyll cycle pigments (violaxanthin, antheraxanthin, and zeaxanthin) as well as β-carotene. In tomato, expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant Delta where a single dominant gene, Del, changes the fruit colour to orange as a result of accumulation of delta-carotene at the expense of lycopene (Ronen et al. 1999). Metabolic engineering to manipulate the lycopene cyclase expression and enzyme activity can bias the flux toward one or the other branch of the carotenogenic pathway (Yu and Beyer 2012; Giorio et al. 2013). Enhanced levels of β-carotene were observed in *LCYE* tuber-specific silencing of potato (Diretto et al. 2006). Similarly, *LCYE* downregulation in canola results in the enhanced accumulation of β-carotene, zeaxanthin, violaxanthin, and lutein (Yu et al. 2008). Suppression of *LCYE* in sweet potato and tobacco showed increased synthesis of a β-branch–specific pathway and enhanced tolerance to abiotic stress (Kim et al. 2013; Shi et al. 2014). Maize endosperm tissues lacking LCYB activity accumulate ε,ε-carotene, δ-carotene, and ε-carotene produced by a dual mono- or bicyclic LCYE enzyme activity (Bai et al. 2009).

The expression levels of *LCYB* and *LCYE* in tomato, rice, and Arabidopsis affect the relative cyclase activities as well as the synthesis of cyclic carotenoids playing

a critical role in determining the β-carotene/α-carotene ratio (Yu and Beyer 2012; Giorio et al. 2013).

Inhibition of these enzymes produces growth abnormalities as well as herbicidal effects. The only specific non-competitive inhibitors of lycopene cyclases are N,N-diethyl-N-[2-(4-chlorophenylthio)ethyl]amine (CPTA) and N,N-diethyl-N-[2-(4-methylphenoxy)ethyl]amine (MPTA) (Yokoyama et al. 1977; Spurgeon, 1983; Schnurr et al. 1998). CPTA exhibited preferential inhibition of the LCYB over LCYE, resulting in an accumulation of the monocyclic carotenoid precursor δ-carotene as well as lycopene (at the expense of β-carotene and xanthophylls) (La Rocca et al. 2007). MPTA belongs to a group of substituted triethylamines that have a number of effects including inhibition of lycopene cyclases (Phillip and Young 2006; Liu and Puckhaber 2011). Another inhibitor of carotenoid biosynthesis on the step of cyclization of lycopene is the bleaching herbicide amitrole (AM) (Agnolucci et al. 1996; La Rocca et al. 1998). It has been reported that amitrole (AM) treatment decreased salt tolerance of *Salicornia Europaea* by inhibiting lycopene cyclization (Chen et al. 2011). However amitrole also has other effects in plants such as inhibition of geranylgeraniol hydrogenation to phytol in chlorophyll synthesis of wheat seedlings that have been transferred from darkness to light (Rüdiger and Benz 1979).

The bleaching herbicidal compound N,N-diethyl-N-(2-undecynyl) amine (NDUA) was also identified as an inhibitor of lycopene cyclase in *Lepidium sativum* plants. Plants under NDUA treatment showed both lycopene accumulation and chlorotic effects similar to CPTA and MPTA treatments (Fedtke et al. 2001).

### *2.4.6 Cyclic Carotenes to Xanthophylls Cycle: Dynamic Role of Hydroxylases, Epoxidases and More*

From a chemical point of view, xanthophylls are oxygenated hydrocarbon derivatives of carotenoids that contain at least one oxygen function, such as carboxylic acid, epoxy, hydroxyl, keto or methoxy groups. Xanthophylls are among the main carotenoid pigments in the photosystems of plants. The oxygen-containing xanthophylls are produced from either α- or β-carotene and require ring-specific hydroxylation reactions. β–Hydroxylase catalyzes two hydroxylation reactions, converting β-carotene to zeaxanthin via β-cryptoxanthin whereas α-carotene is twice hydroxylated by two different enzymatic reactions catalyzed by the ε- and β–hydroxylases. In *Arabidopsis thaliana*, these reactions are catalyzed by a set of four enzymes (Kim et al. 2009). Two non-heme di-iron enzymes (β-carotene hydroxylase-1 (BCH1) and -2 (BCH2)) are primarily responsible for β-ring hydroxylation of β-carotene and produce zeaxanthin, while two heme-containing cytochrome P450 enzymes (CYP97A3 and CYP97C1) preferentially hydroxylate the ε- and β-ionone rings of α-carotene, yielding lutein (Fig. 2.1). In carrot,

unusually high levels of α- and β-carotene are accumulated in leaves due to the presence of a defective carotene hydroxylase CYP97A3 (Arango et al. 2014).

Zeaxanthin is further epoxidized by the enzyme zeaxanthin epoxidase (ZEP), leading to antheraxanthin, violaxanthin and neoxanthin, a reversible reaction carried out by violaxanthin de-epoxidase (VDE), representing the xanthophyll cycle (Niyogi et al. 1998). Six types of xanthophyll cycles have been described. Four of them are based on β-xanthophylls and two on α-xanthophylls (Garcia-Plazaola et al. 2007). The common factor in all xanthophyll cycles is the light-dependent transformation of epoxidized xanthophylls to de-epoxidized ones, which facilitates the dissipation of excitation energy, and their reversion to epoxidized xanthophylls in low light (Muller et al. 2001; Latowski et al. 2004). Two of the xanthophyll cycles have been described for land plants: The violaxanthin cycle, in which violaxanthin is reversibly converted to zeaxanthin via antheraxanthin (Sapozhnikov et al. 1957; Yamamoto et al. 1962; Jahns et al. 2009) and the lutein epoxide cycle, in which lutein epoxide is reversibly converted into lutein (Bungard et al. 1999; Garcia-Plazaola et al. 2007). Both cycles are involved in the light-regulated switching of PSII from a light-harvesting state (with epoxidized xanthophylls, violaxanthin and lutein epoxide present in low light or darkness) to an energy dissipating state (with de-epoxidized xanthophylls, antheraxanthin, zeaxanthin and lutein, present in high light). Therefore, these cycles facilitate the short- and long-term acclimation of plants to varying light conditions. Although the violaxanthin cycle is present in all land plants, the lutein epoxide cycle is restricted to some species only (Esteban et al. 2009). The most commonly occurring type of xanthophyll cycles in plants and the most intensively studied is the violaxanthin cycle, also called the xanthophyll cycle, where the main product of strong light-stimulated de-epoxidation is zeaxanthin. The violaxanthin or xanthophyll cycle, first described in lettuce by Yamamoto et al. (1962), consists of the light-driven de-epoxidation of the diepoxide violaxanthin through the intermediate monoepoxide antheraxanthin into zeaxanthin, and the dark epoxidation of zeaxanthin via antheraxanthin into violaxanthin. The cycle is catalyzed by VDE and ZEP that act in two successive steps. These enzymes are differentially located in chloroplast thylakoids; VDE is located in the thylakoid lumen and ZEP is on the stromal side (Hager 1980; Pfundel et al. 1994). Low pH and ascorbate are required by VDE that converts violaxanthin into zeaxanthin via the intermediate antheraxanthin (Pfundel et al. 1994; Bratt et al. 1995; Kramer et al. 1999). Under low light and relatively alkaline conditions (optimum pH of 7.5), ZEP hydroxylates β-rings of zeaxanthin in two consecutive steps to yield antheraxanthin and then violaxanthin, thus forming an integrated cycle. This cycle is present in all higher plants studied to date, as well as in ferns, mosses, lichens, and some algae (Phaeophyta, Chlorophyta and Rhodophyta).

Finally violaxanthin is converted to neoxanthin by neoxanthin synthase (NSY), which also represents the final step in the core carotenoid biosynthetic pathway.

The lutein epoxide cycle was reported in green tomato fruits (Rabinowitch et al. 1975) and was later found in the photosynthetic stems of the parasitic plant *Cuscuta reflexa* Roxb. (Bungard et al. 1999). It involves the de-epoxidation of lutein epoxide (monoepoxide) to lutein and the epoxidation of lutein to lutein epoxide.

For the lutein epoxide cycle, two different types have been described:

(i) A complete lutein epoxide cycle, in which lutein Lut is reconverted to lutein epoxide overnight
(ii) A truncated lutein epoxide cycle, in which lutein Lut reconversion is not observed overnight (Garcia-Plazaola et al. 2007).

Essential photoprotective functions have been assigned to lutein and the xanthophyll cycle pigments antheraxanthin and zeaxanthin, particularly related to the heat dissipation of excess light energy (Non-Photochemical Quenching).

The xanthophyll precursor pool plays an important role in the biosynthesis of the phytohormone abscisic acid (ABA) (Fig. 2.1, Li and Walton 1990; Seo and Koshiba 2002; Wasilewska et al. 2008). *De novo* synthesis of ABA requires ZEP-catalyzed epoxidation of zeaxanthin to violaxanthin. Subsequently, the violaxanthin-derivatives neoxanthin and xanthoxin are converted into ABA through a series of isomerization and dehydrogenation reactions (Milborrow 2001). In the ABA-deficient mutant *aba1* (an allele of *npq2*), ZEP is not functional and causes accumulation of zeaxanthin in parallel with the decrease in the epoxy-xanthophylls antheraxanthin, violaxanthin and neoxanthin (Duckham et al. 1991; Marin et al. 1996). In the xanthophyll cycle, VDE requires ascorbate as reductant to convert violaxanthin to zeaxanthin (Bratt et al. 1995). As a result, reduced levels of ascorbate in the *Arabidopsis vtc1* (vitamin C1) mutant stimulate ABA production (Pastori et al. 2003). In contrast, enhanced VDE activity can reduce ascorbate levels and antagonize ABA synthesis (Pastori et al. 2003). Therefore, the regulation of the xanthophyll cycle allows ABA levels to be modified.

### *2.4.7 Neoxanthin Synthase*

The final step of the β,β-branch in the classic carotenoid pathway is the conversion of yellow-colored violaxanthin into a xanthophyll carrying an allenic double bond, named neoxanthin which represents the classical final step in plant xanthophyll formation (Li and Walton 1990; Parry and Horgan 1991). Neoxanthin together with violaxanthin can further be used for the production of the apocarotenoid hormone, ABA. Interestingly, in tomato violaxanthin is a sufficient precursor for ABA production *in vivo* (Neuman et al. 2014).

Even though, neoxanthin is synthesized from violaxanthin in plants (Li and Walton 1990; Parry and Horgan 1991; Rock and Zeevaart 1991; Galpaz et al. 2008; Qin et al. 2008), the identity of the gene coding for neoxanthin synthase (NSY) and the mechanism of formation of this xanthophyll, are still not known. Two previous reports have suggested that NSY is encoded by a gene of the lycopene cyclase gene family (Al-Babili et al. 2000; Bouvier et al. 2000). Indeed NSY enzyme was practically identical to the chromoplast-targeted lycopene β-cyclase from tomato (LCYB2/CYC-B isoform) (Ronen et al. 2000). However, the ortholog of this gene in pepper codes for capsanthin–capsorubin synthase,

(CCS) an enzyme involved in the production of ketocarotenoids in pepper fruit chromoplasts (Bouvier et al. 1994). Genetic and molecular analyses of two alleles of *nxd1*, a recessive neoxanthin-deficient mutation in tomato, identified a gene of unknown function, which is necessary for neoxanthin synthesis in tomato (Neuman et al. 2014). Two unrelated mutations in the gene *NXD1* were identified in tomato and a loss-of-function mutation in the orthologous *NXD1* gene of Arabidopsis showed an identical phenotype of lack of neoxanthin in leaves, but the study was unable to demonstrate any neoxanthin synthesis activity by NXD1 in a functional expression assay in *E. coli* (Neuman et al. 2014). Although, no NSY homologous gene has been identified in the genome of Arabidopsis, the ABA4 (At1g67080) gene of Arabidopsis (North et al. 2007) led to increased accumulation of trans-neoxanthin when overexpressed, whereas the loss-of-function in aba4 mutant was defective in this xanthophyll, indicating that the ABA4 protein has a direct role in neoxanthin synthesis. However, in vitro activity of the cloned gene has never been demonstrated.

Considering the results obtained at present, the identification of a functional NSY has several hindrances in view of that this reaction is not readily accessible by classical methods of protein purification, since it has not been possible until now to perform the reaction *in vitro* starting from exogenous synthetic violaxanthin. The high degree of lipophilicity of the substrate and product, suggest that the enzymatic activity could be dependent on membrane structures. Also the incapability of transformed *E. coli* to synthesize epoxidated xanthophylls *in vivo* and the very high spectral similarity of violaxanthin and neoxanthin (Marin et al. 1996) rule out color complementation methods in *Escherichia coli* even though this strategy have proven to be successful with other carotenoid biosynthetic enzymes. Therefore, the identification of a mutant defective in this biosynthetic step is expected to give better insights on the identification and characterization of NSY.

## 2.5 Concluding Remarks

The updated information provided in this chapter aims to confirm that until now the plant carotenoid pathway and the characterization of each enzymatic step is ongoing. Several efforts have been done to understand the metabolic flux in different plant models. Although most knowledge has been obtained from *Arabidopsis thaliana* and mutants obtained thereof, we may conclude that each plant is a whole and unique specie for carotenoid synthesis regulation.

At present most of the genes and enzymes have been identified and characterized, which has been very useful and amenable to continuously improve the carotenogenic metabolism of many different vegetal species by genetic engineering.

This obviously provides opportunities for the improvement of the nutritional value of various food plants and it also opens up the possibility for the metabolic engineering of compounds further downstream in the carotenoid pathway (Chap. 13), for which naturally accumulating mutants or genotypes are not currently

available. Those of more than 750 structurally different yellow, orange, and red colored molecules found in both eukaryotes and prokaryotes that were classified in simplified terms in this chapter comprised an estimated global market totaled $1.5 billion in 2014. This market is expected to reach nearly $1.8 billion in 2019, with a compound annual growth rate (CAGR) of 3.9% (According to "The Global Market for Carotenoids" from PR Newswire Association LLC). This projected market size reflects the multiple functions and uses of carotenoids as cells protectors against photooxidative damage involving important applications in disease control, environment, food and nutrition, and as potent antimicrobial agents.

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