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Carotenoid Biosynthesis in Arabidopsis: A Colorful Pathway

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Plant carotenoids are a family of pigments that participate in light harvesting and are essential for photoprotection against excess light. Furthermore, they act as precursors for the production of apocarotenoid hormones such as abscisic acid and strigolactones. In this review, we summarize the current knowledge on the genes and enzymes of the carotenoid biosynthetic pathway (which is now almost completely elucidated) and on the regulation of carotenoid biosynthesis at both transcriptional and post-transcriptional levels. We also discuss the relevance of Arabidopsis as a model system for the study of carotenogenesis and how metabolic engineering approaches in this plant have taught important lessons for carotenoid biotechnology.

INTRODUCTION

Carotenoids are a group of isoprenoid molecules generally regarded to as pigments because of their characteristic color in the yellow to red range. This physical property is due to a polyene chain with a number of conjugated double bonds that functions as a chromophore. Carotenoids are synthesized by all photosynthetic organisms (including plants) and some non-photosynthetic bacteria and fungi. Plant carotenoids are tetraterpenes derived from the 40-carbon isoprenoid phytoene. With only a few exceptions (Moran and Jarvik, 2010), animals cannot synthesize carotenoids but take them in their diets as an essential source of retinoids (including vitamin A). Ingested carotenoids are also used as pigments that furnish many birds, fish and invertebrates with their characteristic colors. In humans, dietary carotenoids have been shown to act as health-promoting phytonutrients. Although hundreds of carotenoid structures exist in nature, they can be grouped in two major classes: carotenes (hydrocarbons that can be cyclized at one or both ends of the molecule) and xanthophylls (oxygenated derivatives of carotenes) (Figure 1).

Carotenoids take their name from carrot (*Daucus carota*), a plant that accumulates high levels of these pigments in the root. However, the accumulation of carotenoids in underground organs such as tubers and roots is an exception among plants. Most commonly, plant carotenoids color flowers, fruits, and seeds. For example, carotenoids provide the yellow color of corn and bananas, the orange color of pumpkin and oranges, and the red color of tomato and watermelon. In these organs, carotenoids accumulate in specialized plastids (the chromoplasts) and act as visible signals for animals that contribute to pollination or seed dispersal. Chromoplasts sequester large amounts of carotenoids in plastoglobules or/and carotenoid storage structures of globular, tubular, fibrillar, membranous, or crystalline shapes composed of lipids and proteins (Deruere et al., 1994; Vishnevetsky et al., 1999; Simkin et al., 2007; Walter and Strack, 2011). Besides chromoplasts, all other plastid types synthesize carotenoids. However, the level of carotenoid accumulation varies widely among different plastid types. Chromoplasts typically accumulate the highest amounts of carotenoids, but high carotenoid levels are also found in the chloroplasts of photosynthetic tissues. Unlike chromoplasts, which show highly diverse carotenoid profiles depending on the organ, species, and genetic variety, chloroplasts have a remarkably similar carotenoid composition in all plants, with lutein (45% of the total), β-carotene (25-30%), violaxanthin (10-15%) and neoxanthin (10-15%) as the most abundant carotenoids (Britton, 1993). The presence of violaxanthin and other carotenoids confers a yellow color to the chloroplast envelope membrane (Douce et al., 1973; Jeffrey et al., 1974; Costes et al., 1979; Markwell et al., 1992). However, most chloroplast carotenoids are located, together with chlorophylls, in functional pigment-binding proteins embedded in photosynthetic (thylakoid) membranes (Demmig-Adams et al., 1996). Carotenes (mainly β-carotene) are enriched in the photosystem reaction centres, whereas xanthophylls are most abundant in the light-harvesting complexes (Niyogi et al., 1997; Pogson et al., 1998; Davison et al., 2002; Dall'Osto et al., 2007b). The massive presence of chlorophylls masks the distinctive colors of carotenoids in chloroplasts. Only when chlorophylls are degraded carotenoids become visible in the leaves, resulting in the autumn colors of many plants in temperate areas of the Earth. Although much lower levels of carotenoids are found in the etioplasts of dark-grown (etiolated) seedlings, these are high enough to be perceived as the characteristic yellow color of their

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cotyledons (Park et al., 2002; Rodriguez-Villalon et al., 2009a). The amount of carotenoids in plastid types such as leucoplasts is so low that they are colorless to the human eye (Howitt and Pogson, 2006).

But carotenoids are much more than just pigments. Chloroplasts carotenoids act as membrane stabilizers and play two major roles in photosynthesis: light collection (which requires passing energy on to chlorophylls) and photoprotection (which involves channeling energy away from chlorophylls). Carotenoids act as accessory light-harvesting pigments by absorbing in the range of 450-570 nm (where chlorophylls do not absorb efficiently) and then transferring the energy to chlorophylls (Demmig-Adams et al., 1996). However, the essential role of carotenoids in photosynthesis is the protection against photooxidative damage. Plants, like all other oxygenic photosynthetic organisms, live with a continual paradox: light is required for photosynthesis but an excess of light generates inevitable byproducts which are highly reactive photooxidative species. Carotenoids protect the photosynthetic apparatus in different ways, including the quenching of chlorophyll triplets and singlet oxygen, and the dissipation of excess light energy absorbed by the antenna pigments by non-photochemical quenching of chlorophyll fluorescence (Demmig-Adams et al., 1996; Niyogi et al., 1997; Pogson et al., 1998; Baroli and Niyogi, 2000; Kim and DellaPenna, 2006; Dall'Osto et al., 2007b; Kim et al., 2009a). In etioplasts of dark-grown seedlings, carotenoids facilitate the transition to photosynthetic development upon illumination (Park et al., 2002; Rodriguez-Villalon et al., 2009a). Furthermore, in these and other plastid types such a leucoplasts, carotenoids serve as precursors of important hormones such as abscisic acid (ABA) and strigolactones that regulate plant development and the interaction of plants with their environment (Nambara and Marion-Poll, 2005; Van Norman and Sieburth, 2007; Xie et al., 2010).

ARABIDOPSIS AS A VERY USEFUL TOOL TO STUDY PLANT CAROTENOGENESIS

The first major advances in the plant carotenoid field were initially accomplished using plant systems that accumulate massive amounts of these pigments in chromoplasts, such as the ripe fruit of pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*) and the flowers of marigold (*Tagetes sp.*) and daffodil (*Narcissus sp.*) (Cunningham and Gantt, 1998; Hirschberg, 2001; Fraser and Bramley, 2004). Because *Arabidopsis thaliana* lacks chromoplasts, it cannot be used to directly investigate processes related to this particular type of plastid. However, the amenability of Arabidopsis to molecular and genetic approaches is a major advantage for the study of plant biology in general and carotenoid biosynthesis in particular. The use of Arabidopsis mutants with altered carotenoid profiles has facilitated a deeper understanding of the function of etioplast carotenoids in greening (Park et al., 2002; Rodriguez-Villalon et al., 2009a) and chloroplast carotenoids in photosynthesis (reviewed in this issue by Hirschberg, Bassi, Dall'Osto). In particular, the characterization of Arabidopsis (and *Chlamydomonas*) *lut*, *npq* and *aba* mutants with specific defects in xanthophyll biosynthesis has significantly helped to clarify the role of individual xanthophylls for photoprotection

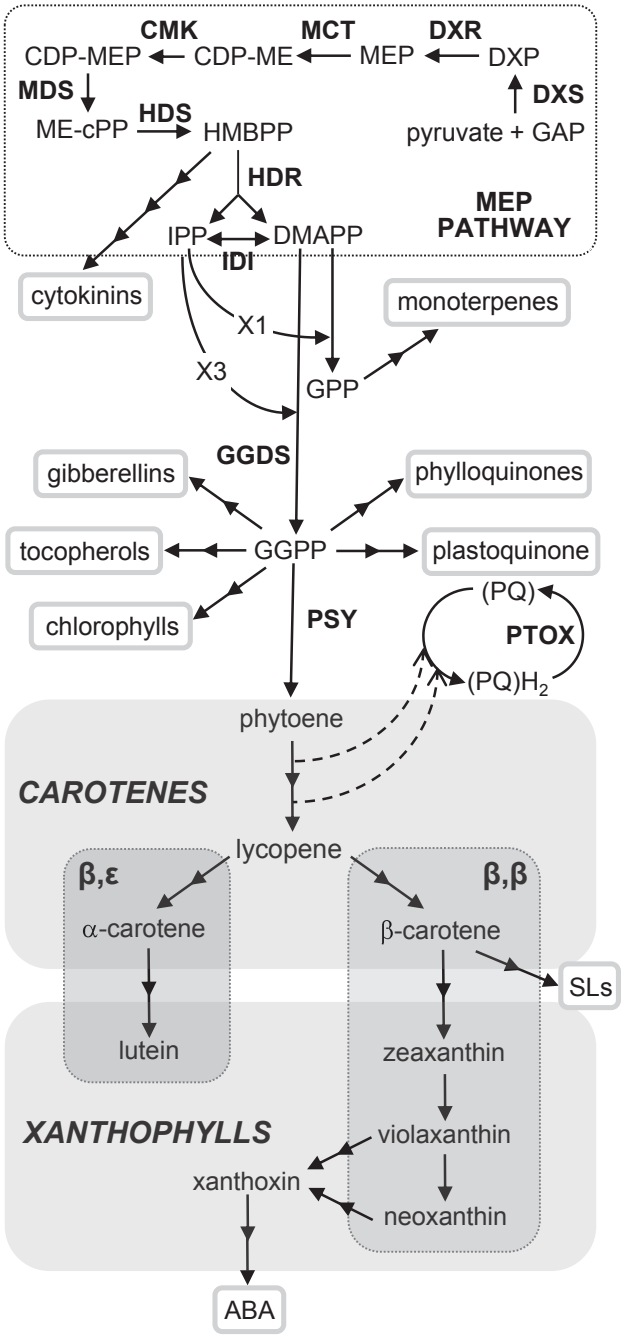


Figure 1. Carotenoid biosynthesis and related pathways in Arabidopsis.

GAP, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; CDP-ME, 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol; CDP-MEP, CDP-ME 2-phosphate; ME-cPP, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP, 4-hydroxy-3-methylbut-2-enyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; ABA, abscisic acid; SLs, strigolactones. Enzymes are indicated in bold: DXS, DXP synthase (EC 2.2.1.7); DXR, DXP reductoisomerase (EC 1.1.1.267); MCT, MEP cytidylyltransferase (EC 2.7.7.60); CMK, CDP-ME kinase (EC 2.7.1.148); MDS, ME-cPP synthase (EC 4.6.1.12); HDS, HMBPP synthase (EC 1.17.4.3); HDR, HMBPP reductase (EC 1.17.1.2); IDI, IPP isomerase (EC 5.3.3.2); GGDS, GGPP synthase (EC 2.5.1.29); PSY, phytoene synthase (EC 2.5.1.32); PTOX, plastidial terminal oxidase.

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in plants (and algae) (Pogson et al., 1996; Pogson et al., 1998; Niyogi, 1999; Pogson and Rissler, 2000; Tian et al., 2004; Kim and DellaPenna, 2006; Dall'Osto et al., 2007a). As described in the next section, screenings for Arabidopsis mutants impaired in specific steps of the carotenoid pathway have additionally led to the identification of several biosynthetic genes, including a candidate for neoxanthin synthase, the last core pathway enzyme to be identified (Pogson et al., 1996; Pogson et al., 1998; Park et al., 2002; Tian et al., 2004; Kim and DellaPenna, 2006; North et al., 2007; Chen et al., 2010). Furthermore, the knowledge and tools acquired in Arabidopsis regarding developmental processes or environmental responses that involve changes in the production of carotenoids represents a dramatic advantage over other plant systems for studying the regulation of the pathway. For example, the identification of Phytochrome-Interacting Factors (PIFs) as the first class of transcription factors shown to directly regulate the pathway was possible thanks to the knowledge available in Arabidopsis on the molecular mechanisms controlling deetiolation, a light-triggered process associated with a burst of carotenoid production (Rodriguez-Villalon et al., 2009b; Toledo-Ortiz et al., 2010). Transgene-mediated fluctuation in the levels of other factors identified in Arabidopsis to have a role in light signaling (including HY5, COP1 and DET1) has been shown to be effective in increasing the levels of carotenoids in tomato fruit (Liu et al., 2004; Davuluri et al., 2005).

The powerful genomic/proteomic tools available in Arabidopsis have also been extremely useful to the study of the core carotenoid biosynthetic pathway (which is similar in all plastid types) and its regulation. Soon after the sequencing of the Arabidopsis genome (AGI, 2000), extensive information about candidate Arabidopsis genes and enzymes involved in the biosynthesis of isoprenoids (including carotenoids) could be assembled by combining homology searches, algorithm predictions, and available experimental data (Lange and Ghassemian, 2003; Lange and Ghassemian, 2005). Based in part on this pioneer work, a comprehensive web-based database has been recently constructed (Vranova et al., 2011). The *Arabidopsis thaliana* Isoprenoid Pathway Database, AtIPD (www.atipd.ethz.ch), was compiled using information on isoprenoid pathway models and genes from public metabolic pathway databases such as BioPathAt (Lange and Ghassemian, 2003: Lange and Ghassemian, 2005), AraCyc (www.arabidopsis.org/biocyc), KEGG (www.genome.jp/kegg), and SUBA (suba.plantenergy.uwa.edu.au), and from the literature (Vranova et al., 2011). The database was manually curated to remove redundancy and to improve comprehensiveness and annotation quality of the genes and pathways involved in isoprenoid biosynthesis. AtIPD can be searched or browsed to extract data and external links related to isoprenoid pathway models, enzyme activities, and subcellular enzyme localizations. AtIPD is a particularly valuable resource to investigate the regulation of carotenoid pathway within the network of related isoprenoid pathways by using quantitative technologies and systems biology.

Proteomics is another critical tool to understand plant processes at the molecular level. A wide array of proteomic data and resources available to the Arabidopsis community has been recently put together in the MASCP Gator web portal (gator.mascproteomics.org), which can be searched for information retrieved from a series of proteomics online databases on the fly (Joshi et al., 2010). One of such databases that is particularly interesting to researchers interested in the biosynthesis of isoprenoids in general and carotenoids in particular is the AtCHLORO database (www.grenoble.prabi.fr/at_chloro). AtCHLORO compiles extensive proteomics data (peptide sequences and molecular weight, chromatographic retention times, MS/MS spectra, spectral count) corresponding to hundreds of proteins identified in highly purified leaf chloroplasts and their subfractions (envelope, thylakoids and stroma) (Joyard et al., 2009; Ferro et al., 2010). Based on this information, experimental data on the subplastidial compartmentation of most of the enzymes specifically involved in the biosynthesis of carotenoids was provided for the first time (Joyard et al., 2009). This is particularly relevant because the study of carotenoid enzyme compartmentation has been neglected for a long time. Together, an impressive collection of state-of-the-art tools and basic knowledge has been generated for Arabidopsis that can be used to better understand and manipulate the molecular mechanisms controlling carotenoid biosynthesis in plants.

BIOSYNTHETIC PATHWAY: GENES, PROTEINS AND PRODUCTS

The main carotenoid biosynthetic pathway was elucidated in the latter half of the 20th century using biochemical (from the 1960s) and molecular (from the 1980s) approaches. Major advances in the identification of genes and enzymes of the pathway have been made from the 1990s. In particular, the isolation and characterization of carotenoid-defective mutants from plants (Arabidopsis, tomato), algae (*Chlamydomonas sp.*), and cyanobacteria (*Synechocystis sp.*) and the development of *Escherichia coli* strains engineered with carotenogenic genes from bacteria (*Erwinia sp.*, renamed *Pantoea sp.*) substantially contributed to the identification of many of the pathway genes before the end of the century (Cunningham and Gantt, 1998). In the first decade of the 21st century, more biosynthetic genes have been identified despite the research focus moved to regulatory issues. Today, we have an almost complete picture of the genes and enzymes that catalyze the core reactions of the carotenoid biosynthetic pathway in plants, including Arabidopsis (Table 1).

Like all isoprenoids, carotenoids are synthesized from the five-carbon units isopentenyl diphosphate (IPP) and its double-bond isomer dimethylallyl diphosphate (DMAPP). Two independent pathways exist in plant cells for the production of these prenyl diphosphate precursors, but carotenoids are mainly synthesized from IPP and DMAPP produced by the plastidial 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Rodriguez-Concepcion, 2010), as shown in Figure 1. Addition of three IPP molecules to DMAPP generates geranylgeranyl diphosphate (GGPP), a precursor for several groups of plastidial isoprenoids including carotenoids (Lichtenthaler, 1999; Bouvier et al., 2005a). The first committed step of carotenoid biosynthesis is the production of 40-carbon phytoene from condensation of two GGPP molecules (Figure 1). This reaction, catalyzed by the enzyme phytoene synthase (PSY), is considered the main bottleneck in the carotenoid pathway (Hirschberg, 2001; Fraser and Bramley, 2004; Bouvier et al., 2005a; Sandmann et al., 2006; Cazzonelli and Pogson, 2010). Phytoene is then desaturated to create the chromophore-bearing chain of conjugated double bonds that forms the back-

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Table 1. Arabidopsis genes and encoded enzymes of the carotenoid biosynthesis pathway.

			Plant Proteome Database (PPDB)						AT-CHLORO
			Size (aa)			MW (kD)	Transmembrane domains		Subplastidial localization in
Accession	Gene	Protein	Full	TP[1]	Mature	Mature	TMHMM	Aramemnon	Arabidopsis chloroplasts (%)[2]
At5g17230	*PSY*	PSY	422	70	352	39.7	-	-	nd
At4g14210	*PDS3*	PDS	566	75	491	54.8	-	-	ENV (84), THY (16)
At3g04870	*ZDS*	ZDS	558	34	524	58.1	-	-	ENV (90), STR (10)
At1g10830	*ZIC1*	Z-ISO	367	58	309	34.5	5	6	nd
At1g06820	*CCR2/CRTISO*	CRTISO1	595	62	533	58.5	-	-	ENV (100)
At1g57770	*CRTISO2*	CRTISO2	574	30	544	58.3	-	-	ENV (100)
At3g10230	*LYC*	LCYB	501	46	455	50.8	-	-	ENV (100)
At5g57030	*LUT2*	LCYE	524	45	479	53.3	1	2	nd
At4g25700	*BCH1/CHY1*	BCH1	310	51	259	28.7	4	4	nd
At5g52570	*BCH2/CHY2*	BCH2	303	52	251	28.1	4	4	nd
At1g31800	*LUT5*	CYP97A3	595	28	567	63.7	-	-	ENV (100)
At4g15110	*CYP97B3*	CYP97B3	580	52	528	59.6	-	-	ENV (100)
At3g53130	*LUT1*	CYP97C1	539	36	503	56.6	-	-	ENV (100)
At5g67030	*ABA1/NPQ2*	ZEP	667	59	608	67.4	-	-	ENV (75), THY (11), STR (14)
At1g08550	*NPQ1*	VDE	462	82	380	43.1	-	1	THY (100)
At1g67080	*ABA4*	NSY	220	37	183	20.6	4	4	ENV (100)

[1] TP, targeting peptide

[2] nd, not determined in this study; ENV, envelope membranes; THY, thylakoid membranes; STR, stroma.

bone of plant carotenoids and determines their physical and biological properties (Britton, 1995). Desaturation and isomerization of uncolored phytoene eventually results in the production of lycopene, a red carotenoid. The cyclization of the ends of the lycopene polyene chain is the first branch point in the pathway and results in the production of carotenes either with one ß ring and one ε ring (α-carotene) or with two ß rings (ß-carotene). Carotenoids with two ε rings do not exist in Arabidopsis and are uncommon in plants. Hydroxylation of the carotene rings generates xanthophylls such as lutein (from α-carotene) and zeaxanthin (from ß-carotene). Zeaxanthin is epoxidated twice to make violaxanthin, which can be subsequently modified to make neoxanthin (Figure 1). These carotenes and xanthophylls can be further modified to generate the wide diversity of carotenoids found in plants and other organisms (Walter and Strack, 2011). Hundreds of these carotenoids structures can be found online at LipidBank (lipidbank.jp), the official database of the Japanese Conference on the Biochemistry of Lipids. Additionally, carotenoids can be cleaved to generate apocarotenoids, a structurally diverse class of compounds widely distributed in nature (Giuliano et al., 2003; Bouvier et al., 2005b; Auldridge et al., 2006a; Walter and Strack, 2011). Plant apocarotenoids with potent biological activities include the hormones ABA and strigolactones, as well as branch-inhibiting hormone-like compounds not identified yet (Nambara and Marion-Poll, 2005; Van Norman and Sieburth, 2007; Xie et al., 2010). Other apocarotenoids are involved in the interaction of plants with their biological environment, acting as pigments and flavors that attract pollinators or seed-dispersing animals. Additional functions proposed for apocarotenoids have been revised elsewhere (Giuliano et al., 2003; Bouvier et al., 2005b; Auldridge et al., 2006a; Walter and Strack, 2011).

This article focuses on the Arabidopsis carotenoid biosynthetic pathway from phytoene to the production of xanthophylls, although some background information is also provided on the genes and enzymes responsible for the production of GGPP in Arabidopsis. The biosynthesis of apocarotenoids and the mechanisms for the degradation of carotenoids either by enzymatic cleavage or by unspecific oxidation have been extensively reviewed elsewhere (Finkelstein and Rock, 2002; Giuliano et al., 2003; Bouvier et al., 2005b; Nambara and Marion-Poll, 2005; Auldridge et al., 2006a; Van Norman and Sieburth, 2007; Xie et al., 2010; Walter and Strack, 2011) and will not be covered here.

MEP pathway to GGPP

All the enzymes of the MEP pathway have been identified (Figure 1). In Arabidopsis, they are encoded by single nuclear genes (Rodríguez-Concepción and Boronat, 2002; Phillips et al., 2008b) and are targeted to the stroma of plastids, as experimentally demonstrated by fusion to fluorescent reporters (GFP), immunocytochemistry, and proteomic approaches (Carretero-Paulet et al., 2002; Querol et al., 2002; Hsieh et al., 2008; Zybailov et al., 2008; Joyard et al., 2009; Ferro et al., 2010). The first reaction of the pathway is the condensation of glyceraldehyde-3-phosphate with (hydroxyethyl) thiamine derived from pyruvate to produce 1-deoxy-D-xylulose 5-phosphate (DXP), catalyzed by DXP synthase (DXS; At4g15560). The intramolecular rearrangement and reduction of DXP catalyzed by DXP reductoisomerase (DXR;

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At5g62790) yields MEP in the next step of the pathway. MEP is afterwards converted via 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME) and CDP-ME 2-phosphate (CDP-ME2P) into 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (ME-cPP) by the enzymes MEP cytidylyltransferase (MCT; At2g02500), CDP-ME kinase (CMK; At2g26930) and ME-cPP synthase (MDS; At1g63970), respectively. In the last two steps in the pathway, the enzyme 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) synthase (HDS; At5g60600) transforms ME-cPP into HMBPP, whereas HMBPP reductase (HDR; At4g34350) converts HMBPP into a *ca.* 5:1 mixture of IPP and DMAPP (Figure 1).

Unlike Arabidopsis, most plants appear to have small gene families encoding DXS isoforms of at least two classes (Walter et al., 2002; Krushkal et al., 2003; Kim et al., 2005; Cordoba et al., 2009; Vallabhaneni and Wurtzel, 2009; Paetzold et al., 2010; Cordoba et al., 2011). All classes of DXS isozymes have been found to participate in carotenoid biosynthesis. The rest of the MEP pathway enzymes are encoded by a single gene in most plants, with only some recently reported exceptions in the case of DXR and HDR (Kim et al., 2008; Seetang-Nun et al., 2008; Kim et al., 2009b). Overexpression of DXS, DXR or HDR results in an increased production of carotenoids in light-grown Arabidopsis seedlings, whereas increased levels of other pathway enzymes such as HDS do not alter the levels of these plastidial isoprenoids (Estévez et al., 2001; Botella-Pavía et al., 2004; Carretero-Paulet et al., 2006; Flores-Perez et al., 2008a). These results suggest that flux through the MEP pathway to carotenoids can be controlled by DXS, DXR and HDR, whereas other pathway enzymes might not be rate-determining. Further information on the MEP pathway enzymes and their regulation is available elsewhere (Bouvier et al., 2005a; Rodríguez-Concepción, 2006; Hunter, 2007; Phillips et al., 2008b; Cordoba et al., 2009; Tholl and Lee, 2011).

IPP and DMAPP can be interconverted in a reversible reaction catalyzed by the enzyme IPP isomerase (IDI). Unlike the MEP pathway enzymes, IDI is encoded by two genes in Arabidopsis, *IPI1/IPPI1/IDI1* (At5g16440) and *IPI2/IPPI2/IDI2* (At3g02780). Although the simultaneous production of both IPP and DMAPP by the MEP pathway is expected to make the presence of a plastidial IDI activity dispensable, both IDI1 and IDI2 isoforms have been found in chloroplasts by using GFP fusions and proteomic approaches, the latter suggesting the presence of IDI1 in the stroma and IDI2 in membranes (Phillips et al., 2008a; Sapir-Mir et al., 2008; Joyard et al., 2009). A plastidial isomerase might ensure that an appropriate ratio between IPP and DMAPP is maintained for isoprenoid biosynthesis.

The sequential and linear addition of three molecules of IPP to one molecule of DMAPP catalyzed by the enzyme GGPP synthase (GGDS) generates the 20-carbon GGPP molecule, which serves as the immediate precursor not only for carotenoids but also for the biosynthesis of gibberellins and the side chain of chlorophylls, tocopherols, phylloquinones and plastoquinone (Figure 1). The Arabidopsis genome contains a family of more than 10 genes encoding putative GGDS isoforms (Lange and Ghassemian, 2003) (www.atipd.ethz.ch), but only some of them are targeted to plastids (Okada and K., 2000; Joyard et al., 2009). The presence of several genes encoding putative GGDS enzymes in most plants (www.phytozome.net) suggests that different isozymes might be involved in the production of specific groups of isoprenoids (including carotenoids). Consistent with this possibility, multi-enzyme complexes harboring GGDS and PSY activities to directly convert IPP into phytoene have been isolated from chromoplasts (Islam et al., 1977; Maudinas et al., 1977; Camara, 1993; Fraser et al., 2000). Additionally supporting a role for specific GGDS isoforms in channeling precursors to the carotenoid pathway, statistical testing of the correlation between carotenoid content and candidate gene transcript levels in a maize germplasm collection revealed that the expression of genes encoding DXS, DXR, HDR, and only one of the three plastidial GGDS enzymes positively correlated with endosperm carotenoid content (Vallabhaneni and Wurtzel, 2009). Similarly, only one Arabidopsis gene encoding GGDS, *GGDS1* (At4g36810), was found to be highly co-expressed with *PSY* (Meier et al., 2011). Complete loss of GGDS1 function is lethal, but decreased GGDS1 activity in knock-down Arabidopsis mutants results in a lower accumulation of carotenoids (our unpublished results), suggesting a role of this particular GGDS isoform in carotenoid biosynthesis. However, it remains to be established whether other GGDS isozymes are also involved in the production of carotenoids.

GGPP to phytoene: phytoene synthase (PSY)

The first committed step in plant carotenoid biosynthesis is the synthesis of phytoene from GGPP (Figure 1). This reaction is catalyzed by PSY, which is probably the best studied enzyme of the plant carotenoid family. Plant, algal and cyanobacterial PSY protein sequences are similar to the homologous bacterial and fungal crtB enzymes and share amino acid sequence similarity with GGDS and other prenyltransferases (Cunningham and Gantt, 1998; Bouvier et al., 2005a). The PSY enzyme catalyzes a two-step reaction (Dogbo et al., 1988): the head-to-head condensation of two molecules of GGPP to form the reaction intermediate pre-phytoene diphosphate followed by the elimination of the diphosphate group from this intermediate in a complex rearrangement that involves a carbocation neutralization to form phytoene (7,8,11,12,7',8,',11',12'-octahydro-ψ,ψ-carotene). Plant PSY enzymes typically use all-*trans* GGPP as a substrate to synthesize 15-*cis* phytoene, the isomer normally found in living cells (Figure 2). The biochemical properties of PSY enzymes from different plant tissues have been reviewed elsewhere (Fraser and Bramley, 2004)

While only one *PSY* gene (At5g17230) is present in Arabidopsis (Table 1), small gene families appear to encode PSY in most plants (www.phytozome.net). For example, two genes encode PSY in tobacco and three genes in tomato, cassava, rice, and maize (Fraser et al., 1999; Busch et al., 2002; Giorio et al., 2008; Li et al., 2008a; Welsch et al., 2008; Arango et al., 2010). Some isoforms are involved in the biosynthesis of carotenoids in chloroplast-containing photosynthetic tissues, whereas others participate in the production of carotenoids in non-photosynthetic tissues of the fruit (tomato *PSY1*), the seed endosperm (maize *PSY1*) or the root (tomato, maize and rice *PSY3*). The Arabidopsis *PSY* gene is expressed in virtually all tissues, including both photosynthetic and non-photosynthetic (Welsch et al., 2003), and it shows high rates of co-expression with the rest of the genes involved in the carotenoid pathway and in the supply of their isoprenoid precursors (Meier et al., 2011).

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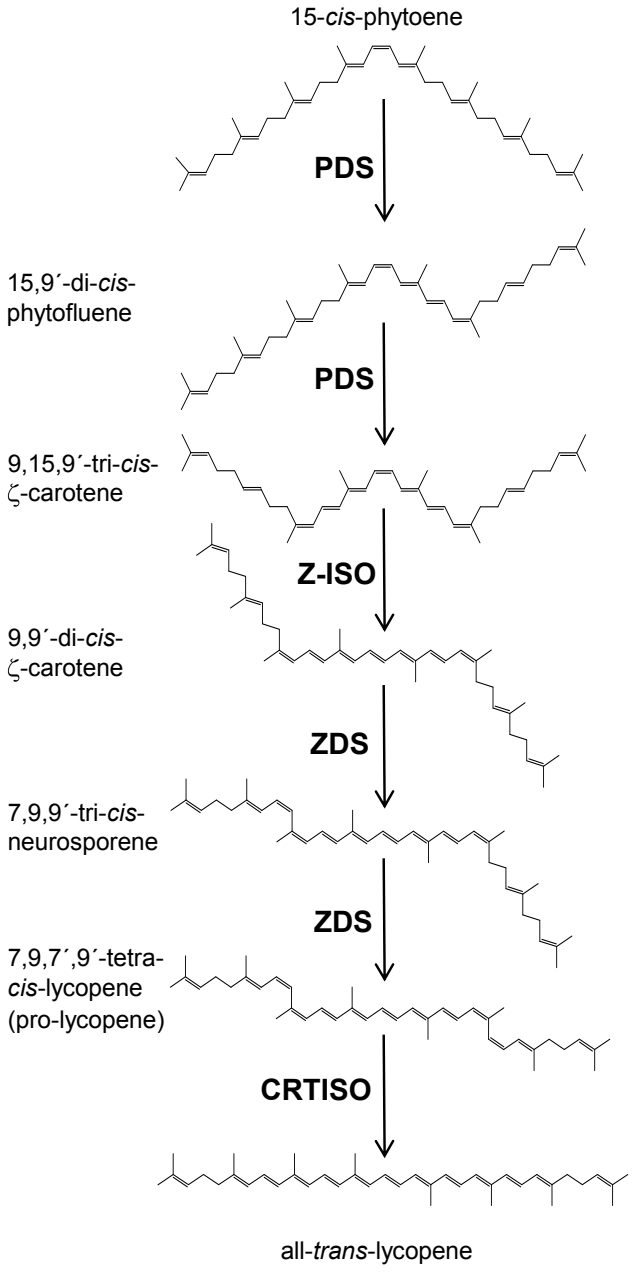


Figure 2. Biosynthesis of lycopene from phytoene.

PDS, phytoene desaturase; Z-ISO, 15-*cis*-ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase.

Studies with tomato and pepper chromoplasts have shown that PSY is integrated into a high molecular mass complex of *ca.* 200 kDa that also contains the enzymes IDI and GGDS (Camara, 1993; Fraser et al., 2000). A multiprotein complex formed by these enzymes might be involved in channeling isoprenoid precursors produced in the stroma (IPP, DMAPP, GGPP) to the biosynthesis of carotenoids *in vivo*. Because phytoene is lipid-soluble and it needs to be delivered to the membranes where downstream enzymes and derived carotenoid products are localized, the complex would be expected to be membrane-associated. IDI and GDPP appear to be soluble enzymes (Joyard et al., 2009), whereas PSY enzymes have been found associated to membranes in all plastid types (Dogbo et al., 1988; Bonk et al., 1997; Fraser et al., 2000; Welsch et al., 2000; Li et al., 2008a). Immunolocalization experiments using a PSY-specific antibody have detected the enzyme in the envelope membranes of maize amyloplasts (Li et al., 2008a). In chloroplasts, early biochemical evidence indicated the presence of appreciable activities of PSY and downstream carotenogenic enzymes in isolated envelope membranes from spinach (*Spinacia oleracea*) chloroplasts, but not in thylakoids (Luetke-Brinkhaus et al., 1982). However, later studies found no PSY protein or activity associated to the envelope membranes of chloroplasts (Bonk et al., 1997; Welsch et al., 2000). In white mustard (*Sinapis alba*), an Arabidopsis relative, PSY protein levels and enzyme activity were estimated in different plastid fractions (envelope membranes, stroma, and membrane pellet) during deetiolation, when carotenoid biosynthesis is normally boosted (Welsch et al., 2000). The composition of the membrane pellet changed depending on the plastid type; in etioplasts of dark-grown seedlings it was reported to consist mostly of the prolamellar body typical of these plastids and to also contain protothylakoid membranes, being progressively enriched in developed thylakoid membranes as etioplasts differentiated into chloroplasts upon illumination. Whereas immunoblot analyses detected PSY polypeptides in stroma and membrane pellet fractions, PSY activity was only found in the membrane pellets, with low levels in etioplasts and higher levels in chloroplasts (Welsch et al., 2000). These results confirmed that membrane association is essential for the activity of the enzyme. The requirement of competent membrane structures for PSY activity has also been shown in chromoplasts (Schledz et al., 1996; Fraser et al., 2000).

Phytoene to lycopene: desaturases and isomerases

Phytoene is an uncolored carotene because only a central conjugated core of three double bonds is present in its hydrocarbon chain. In the following steps of the carotenoid pathway, a series of four desaturation (dehydrogenation) and two isomerization reactions increase the conjugated series of double bonds and transform the 15-*cis* phytoene isomer into all-*trans* lycopene, a pink/red-colored carotenoid (Figure 2).

Desaturases

The desaturation steps sequentially transform phytoene into phytofluene (7,8,11,12,7',8'-hexahydro-ψ,ψ-carotene), ζ-carotene (7,8,7',8'-tetrahydro-ψ,ψ-carotene), neurosporene (7,8-dihydro-ψ,ψ-carotene) and lycopene (ψ,ψ-carotene), increasing the number of conjugated double bonds to five, seven, nine and eleven, respectively. This increase in conjugated double bonds shifts the absorption of the polyene chain towards longer wavelengths, resulting in the pale/yellow color of ζ-carotene, the orange/yellow color of neurosporene, and the pink/red color of lycopene (Cunningham and Gantt, 1998; Tanaka et al., 2008). Two completely unrelated types of phytoene desaturases catalyze these reac-

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tions (Sandmann, 2009; Klassen, 2010). In bacteria (except cyanobacteria) and fungi, a single crtI enzyme catalyzes the entire desaturation process. In plants and cyanobacteria, however, the four desaturations undergone by phytoene are catalyzed in two-steps by two phylogenetically-related enzymes (Figure 2): the two desaturations of phytoene to produce ζ-carotene *via* phytofluene are catalyzed by phytoene desaturase (PDS) and the two desaturations of ζ-carotene to produce lycopene *via* neurosporene are catalyzed by ζ-carotene desaturase (ZDS). Mechanistic differences between the crtI enzymes and the plant desaturases provide an excellent target for herbicides. Actually, Arabidopsis and tobacco plants transformed with a bacterial *crtI* gene acquire multiple strong resistance to bleaching herbicides that target PDS, including norflurazon, but also to inhibitors of ZDS (Misawa et al., 1994) (our unpublished data).

Single genes encode PDS and ZDS in Arabidopsis (At4g14210 and At3g04870, respectively) and most other plants (Dong et al., 2007; Qin et al., 2007; Chaudhary et al., 2010) (www.phytozome.net). Arabidopsis PDS and ZDS enzymes have been found in chloroplast envelope membranes by proteomic analysis of plastidial sub-fractions (Joyard et al., 2009), although PDS was also identified in the thylakoid fraction (Mann et al., 2000) and ZDS in the stroma (Table 1). This is in agreement with early biochemical studies that concluded that the enzymatically active forms of these desaturases are membrane-associated proteins (Beyer et al., 1985; Bramley, 1985; Al-Babili et al., 1996; Bonk et al., 1997). Inactive PDS forms have also been found associated to molecular chaperones in soluble fractions of different plastid types, including Arabidopsis chloroplasts (Al-Babili et al., 1996; Bonk et al., 1997; Lopez et al., 2008). The formation of complexes with chaperones has been proposed to be important for PDS insertion into plastid membranes and therefore for activation (Al-Babili et al., 1996; Bonk et al., 1997). Interestingly, the membrane-bound PDS from chloroplasts and chromoplasts also exists in a large protein complex (Lopez et al., 2008). The size of this complex, *ca.* 350 kDa, is consistent with the proposed existence of a hypothetical membrane-associated multi-enzyme complex containing PDS, ZDS and other enzymes required to synthesize cyclic carotenes from phytoene (Cunningham and Gantt, 1998). Future studies will be necessary to establish whether the membrane PDS complex consists of multiple carotenogenetic enzymes or it contains chaperone proteins as shown for the stroma complex.

In addition to membrane association, the carotenoid desaturase enzymes require several cofactors to be active in the plastid. Evidence for the requirement of oxidized plastoquinone as an electron carrier for the desaturation reactions came from the characterization of the Arabidopsis *pds1* and *pds2* mutants, which accumulate phytoene as a result of impaired plastoquinone biosynthesis (Norris et al., 1995). The study of another phytoene-accumulating Arabidopsis mutant, *immutans* (*im*), allowed the identification of a plastidial terminal oxidase (PTOX) as a second component of the redox chain required for carotenoid desaturation (Wu et al., 1999; Carol and Kuntz, 2001). PTOX is a plastoquinol-O_2 oxidoreductase that regenerates the reduced plastoquinone formed during phytoene and ζ-carotene desaturation (Figure 1). Loss of PTOX function in the tomato *ghost* mutant leads to carotenoid accumulation defects in both leaves (chloroplasts) and fruit (chromoplasts), indicating that PTOX is a major cofactor involved in phytoene desaturation in both photosynthetic and non-photosynthetic tissues (Josse et al., 2000). However, more recent data has shown that PTOX is not absolutely required for carotenoid desaturation in green tissues, which implies the existence of a PTOX-independent pathway for plastoquinol re-oxidation (Carol and Kuntz, 2001; Shahbazi et al., 2007). The NADH dehydrogenase complex can also catalyze the reduction of plastoquinone using soluble (stromal) electron donors (Burrows et al., 1998; Endo et al., 2008). Interestingly, tomato plants deficient in this complex through a mutation in its subunit M show highly reduced carotenoid levels specifically in fruit chromoplasts (Nashilevitz et al., 2010). The possible interaction between the NADH dehydrogenase complex and PTOX and its possible effect on modulating the redox state of plastoquinone (and hence carotenoid desaturation) remains unexplored.

Isomerases

The expression of the bacterial crtI desaturase in *E. coli* results in the conversion of 15-*cis*-phytoene into all-*trans* lycopene, whereas co-expression of Arabidopsis PDS and ZDS only produces poly-*cis* lycopene (also called pro-lycopene) with very low efficiency (Bartley et al., 1999). This is because plants require not only desaturases but also two types of isomerase enzymes to convert 15-*cis*-phytoene into all-*trans* lycopene. As shown in Figure 2, PDS transforms 15-*cis* phytoene into 15,9'-di-*cis*-phytofluene, and eventually 9,15,9'-tri-*cis*-ζ-carotene, which must be isomerized at the 15-*cis*-double bond to form 9,9'-di-*cis*-ζ-carotene, the substrate of ZDS (Bartley et al., 1999; Matthews et al., 2003; Breitenbach and Sandmann, 2005). In the chloroplasts of photosynthetic tissues, this isomerization can occur in the presence of light, but an enzymatic isomerization is required in other plastid types (Breitenbach and Sandmann, 2005; Li et al., 2007). The isolation of maize *y9* and Arabidopis *zic* mutants based on their phenotype of 9,15,9'-tri-*cis*-ζ-carotene accumulation in the dark led to the identification of 15-*cis*-ζ-carotene isomerase (Z-ISO), an enzyme related to nitrite and nitric oxide reductase U (NnrU) from denitrifying bacteria (Li et al., 2007; Chen et al., 2010). The loss-of-function phenotypes observed in mutant maize and Arabidopsis (including a reduced production of carotenoids) strongly suggest that Z-ISO activity is required even in the light. Z-ISO is a plastid-targeted enzyme (Zybailov et al., 2008; Ishikawa et al., 2009) predicted to be an integral membrane protein with several membrane-spanning domains, but no information is available on whether the protein is found in the envelope or the thylakoid membranes. The single Arabidopsis gene encoding Z-ISO (At1g10830) produces two alternate transcripts, *Z-ISO1.1* (which is highly expressed and encodes a functional enzyme) and a shorter *Z-ISO1.2* (which encodes a truncated protein that lacks the predicted C-terminal transmembrane domain and shows no Z-ISO activity). Single copy genes appear to encode Z-ISO in most plants (www.phytozome.net), but no evidence of alternate transcripts was found in plants other than Arabidopsis such as maize or rice (Chen et al., 2010).

Following the production of 9,9'-di-*cis*-ζ-carotene by Z-ISO, the enzyme ZDS transforms it into 7,9,7',9'-tetra-*cis*-lycopene (pro-lycopene) via 7,9,9'-tri-*cis*-neurosporene (Breitenbach and Sandmann, 2005; Sandmann, 2009). In chloroplasts, the isomerization of pro-lycopene to all-*trans*-lycopene can occur non-

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enzymatically in the presence of light. In the dark and in non-photosynthetic tissues, however, the activity of a carotenoid isomerase (CRTISO) is required. The molecular identification of this enzyme came from the characterization of tomato *tangerine* and Arabidopsis *ccr2* mutants, which accumulated pro-lycopene and other poly-*cis* carotene precursors in chromoplasts of ripe fruit and etioplasts of dark-grown seedlings, respectively (Isaacson et al., 2002; Park et al., 2002). The corresponding protein was later shown to convert 7,9,9'-tri-*cis*-neurosporene to 9'-*cis*-neurosporene and 7'9'-di-*cis*-lycopene into all-*trans*-lycopene, confirming the existence of an all-*cis* pathway from ζ-carotene to lycopene in plants (Isaacson et al., 2004). CRTISO activity requires the presence of membranes and an enzyme-bound flavin adenine dinucleotide (FAD) cofactor in a reduced form, despite the catalyzed reaction does not appear to involve 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 enzymes show some sequence similarities to plant desaturases (PDS, ZDS) and, to a higher degree, to the bacterial crtI enzymes. It has been suggested that a crtI ancestor might have evolved into CRTISO by losing its desaturase activity while retaining its function for *cis* to *trans* isomerization (Sandmann, 2009). Two genes have been proposed to encode CRTISO in Arabidopsis (Lange and Ghassemian, 2003): *CCR2/CRTISO1* (At1g06820) and *CRTISO2* (At1g57770). However, only *CCR2/CRTISO1* has been demonstrated to encode a functional carotenoid isomerase (Park et al., 2002). Single copies of both genes are typically found in plant genomes (www.phytozome.net). Both CRTISO1 and CRTISO2 proteins have been localized in the envelope membrane of Arabidopsis chloroplasts (Joyard et al., 2009) (Table 1).

Lycopene to cyclic carotenes: cyclases

Cyclization of one or both ends of the linear C-40 hydrocarbon chain of all-*trans*-lycopene marks the first branching point in the plant pathway (Figure 1). One of the branches leads to carotenoids with two β rings (β-carotene and derived β,β-xanthophylls such as zeaxanthin, violaxanthin, and neoxanthin), whereas the other branch leads to carotenoids with one β ring and one ε ring (α-carotene and derived β,ε-xanthophylls such as lutein). The production of carotenoids with two ε rings is uncommon in plants. An exception is lactucaxanthin, a ε,ε-xanthophyll from lettuce (Phillip and Young, 1995). The two types of cyclic end groups formed only differ in the position of the double bond in the cyclohexene rings (Figure 3). However, the double bond of β rings is in conjugation with the polyene chain resulting in a rigid ring structure with only

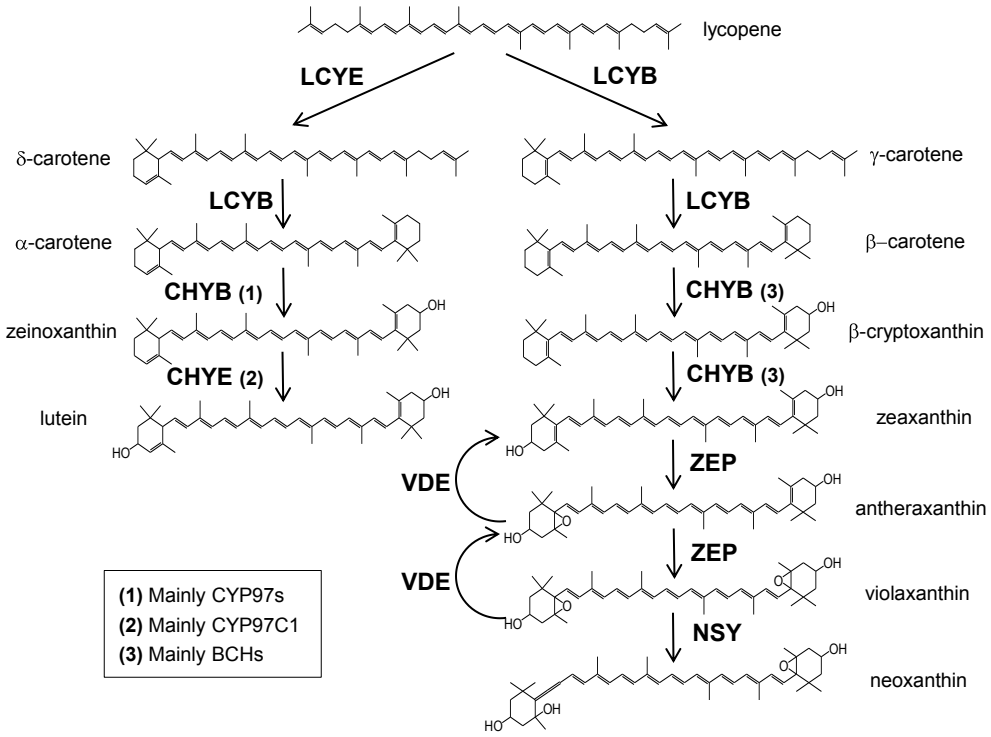


Figure 3. Biosynthesis of xanthophylls from lycopene.

LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; CHYB, carotenoid β-hydroxylase; CHYE, carotenoid ε-hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase. Numbers refer to the type of CHY enzyme that most frequently catalyzes the indicated reactions in Arabidopsis.

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one conformation, whereas the ε ring double bond is not in conjugation, allowing a relatively free rotation. Another important difference is that β rings are ubiquitously found in carotenoid-synthesizing organisms while ε rings distribution is much more restricted to plants, algae, and cyanobacteria, suggesting that ε rings formation and modification evolved only in this subgroup of carotenoid-containing organisms (Kim and DellaPenna, 2006).

In plants, two different lycopene cyclases catalyze the production of end-terminal β- and ε-rings: β-cyclase (LCYB/CRTL-B) catalyzes the formation of β rings (Pecker et al., 1996) whereas ε-cyclase (LCYE/CRTL-E) creates ε rings (Cunningham and Gantt, 2001). Although the similarity of the cyclases from non-photosynthetic bacteria (crtY) with those from cyanobacteria (CrtL) and plants (LCYB and LCYE) is rather low, recent results indicate that, similar to CRTISO, both types of carotenoid cyclases are flavoenzymes that require the reduced form of the FAD cofactor despite catalyzing their reactions with no net redox (Mialoundama et al., 2010; Yu et al., 2010). The existence of some conserved sequence patterns further suggests a common phylogenetic origin (Krubasik and Sandmann, 2000). It is likely that the cyanobacterial CrtL enzyme is the ancestral archetype of plant cyclases, which later evolved, probably by gene duplication, into the closely related LCYB and LCYE enzymes (Klassen, 2010). Consistent with the hypothesis that LCYE arose following duplication of an ancient LCYB gene, LCYE enzymes are only present in plants and some algae and cyanobacteria and, unlike LCYB (which is a high-fidelity enzyme), they show a residual β-cyclase activity (Cunningham and Gantt, 2005; Cunningham et al., 2007; Bai et al., 2009).

LCYE is typically encoded by single genes in most plants, whereas LCYB is encoded by single genes in some plants, including Arabidopsis, maize and rice (Cunningham et al., 1996; Lange and Ghassemian, 2003; Bai et al., 2009; Chaudhary et al., 2010), or by small gene families in others (www.phytozome.net). For example, in tomato there are two highly similar LCYB enzymes, CRTL-B1/LCYB1/ and CRTL-B2/LCYB2/CYCB/ (Pecker et al., 1996; Ronen et al., 2000). While LCYB1 is active in green tissues, LCYB2/CYCB functions only in chromoplast-containing tissues such as ripening fruit (Ronen et al., 2000). In Arabidopsis, lycopene appears to be the preferential substrate of both LCYB (At3g10230) and LCYE (At5g57030), as determined using an *E. coli* expression system (Cunningham et al., 1996). The LCYB enzyme catalyzes a two-step reaction that creates one β ring at each end of the lycopene (ψ,ψ-carotene) molecule to produce the bicyclic β-carotene (β,β-carotene), via the monocyclic γ-carotene (ψ,β-carotene). On the other branch of the pathway, LCYE adds only one ε ring to lycopene, forming the monocyclic δ-carotene (ψ,ε-carotene). Then, δ-carotene is transformed into α-carotene (β,ε-carotene) by LCYB activity (Figure 3). Unlike the Arabidopsis protein, the close homologue LCYE enzyme from lettuce is able to create two ε rings and synthesize ε-carotene (a precursor of lactucaxanthin) using lycopene as a substrate (Cunningham and Gantt, 2001). Surprisingly, when no LCYB enzymes are present, the maize LCYE enzyme can also synthesize monocyclic δ-carotene and bicyclic ε-carotene from lycopene both *in planta* and in *E. coli* (Bai et al., 2009). At least two amino acid residues are responsible for the ability of LCYE to add either only one or two to ε rings to lycopene (Cunningham and Gantt, 2001; Bai et al., 2009). *In planta*, it appears that the proportion of β,β-carotenoids and β,ε-carotenoids synthesized is mainly determined by the relative amounts and/or activities of the LCYB and LCYE enzymes (Pogson et al., 1996; Ronen et al., 1999; Ronen et al., 2000; Harjes et al., 2008; Bai et al., 2009). The existence of hypothetical enzyme complexes containing either LCYB only or both LCYB and LCYE has been proposed to explain the absence of ε,ε-carotenoids in most plants (Cunningham and Gantt, 1998; Bai et al., 2009). Although experimental evidence of such complexes is still missing, proteomic approaches have identified LCYB in envelope membranes, the site where pathway enzymes catalyzing the production of its substrate and the hydroxylation of its products are localized (Joyard et al., 2009).

Cyclic carotenes to xanthophylls: hydroxylases, epoxidases, and more

Cyclic carotenes can be further modified by hydroxylation to generate xanthophylls, a generic name for the oxygenated derivatives of carotenes. Thus, hydroxylation at the C-3 and C-3' positions of each ionone ring of α-carotene (β,ε-carotene) and β-carotene (β,β-carotene) produces the well-known xanthophyll pigments lutein (β,ε-carotene-3,3'-diol) and zeaxanthin (β,β-carotene-3,3'-diol), respectively (Figure 3). Further epoxidations at positions C-5,6 and C-5',6' of the 3-hydroxy β rings of zeaxanthin yields violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol) *via* the mono-epoxidated intermediate antheraxanthin (5,6-epoxy-5,6-tetrahydro-β,β-carotene-3,3'-diol). Violaxanthin can be transformed back into zeaxanthin by de-epoxidation (completing the so called xanthophyll cycle) or converted to neoxanthin (5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro-β,β-carotene-3,5,3'-triol) by opening of the cyclohexenyl 5-6-epoxide ring (Figure 3).

Hydroxylases

Two different types of carotenoid hydroxylases (CHYs) have been found in plants: non-heme di-iron enzymes (BCH type), which are similar to the bacteria crtZ and cyanobacteria CrtR-B enzymes that catalyze the hydroxylation of β rings, and cytochrome P450 enzymes (CYP97 type), which catalyze the hydroxylation of both β and ε rings. The specificities and the degree of functional overlap among the plant enzymes have been best studied in Arabidopsis (Kim et al., 2009a; Kim et al., 2010). At least four genes encode for CHYs in this model species (Table 1): *BCH1/CHYB1/CHY1* (At4g25700) (Sun et al., 1996), *BCH2/CHYB2/CHY2* (At5g52570) (Tian and DellaPenna, 2001), *CYP97A3/LUT5* (At1g31800) (Kim and DellaPenna, 2006) and *CYP97C1/CHYE/LUT1* (At3g53130) (Tian et al., 2004). A fifth Arabidopsis gene phylogenetically related to *CYP97A3* and *CYP97C1* named *CYP97B3* (At4g15110) has also been associated with carotenoid hydroxylation by overexpression in transgenic plants and also in *E. coli* cells (Kim et al., 2010). Rice members of the CYP97 clans A and C have also been shown to function in *E. coli* (Quinlan et al., 2007), underlining the potential of this bacterial system for dissecting the structural basis for ring specificity and other enzymatic mechanisms of CHYs.

Analysis of global co-expression profiles has recently shown a coordinated transcriptional regulation of carotenoid-related genes (Meier et al., 2011). Using the same tools, we show here that the expression of the *CYP97B3* gene is highly correlated with that of other

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carotenoid biosynthetic genes, consistent with a role of the corresponding enzyme in the carotenoid pathway (Figure 4). However, further biochemical characterization of CYP97B3 activity and the analysis of CYP97B3-defective Arabidopsis mutants will be needed to ascertain the role of this enzyme in the carotenoid pathway. Such studies are already available for BCH1, BCH2, CYP97A3, and CYP97C1. The *in vivo* activity of each of these four enzymes was inferred by analyzing the carotenoid profiles of single and multiple mutants. The quadruple mutant showed an albino phenotype and a complete absence of xanthophylls (Kim et al., 2009a). This result suggests that CYP97B3 might require the presence of other CHYs for *in vivo* activity toward carotene substrates or, alternatively, that CYP97B3 might not function in the carotenoid pathway. Analysis of multiple mutants concluded that BCH enzymes are most active in the hydroxylation of the β rings in β-carotene (Figure 3), although they can also hydroxylate the β ring of α-carotene and have a limited activity toward the ε ring. The CYP97 enzymes also show activity toward the two carotene substrates, but in this case their preferred substrate is α-carotene (Figure 3). CYP97A3 is most active on β rings but it shows low activity toward the ε ring of α-carotene, whereas CYP97C1 can efficiently hydroxylate both β and ε rings of α-carotene, showing low hydroxylation activity toward the β rings of β-carotene (Kim et al., 2009a). Overexpression of CYP97 enzymes in a different study has recently confirmed these conclusions, additionally showing that CYP97B3 can hydroxylate the β ring of α-carotene and, with a lower efficiency, β-carotene when overexpressed (Kim et al., 2010).

Phylogenetic analyses suggest that the three lineages of the *CYP97* gene family (A, B, and C) originated before the separation of higher plants and green algae lineages (Bak et al., 2011). In contrast, the duplication of *BCH* genes occurred in higher plants after the monocot-dicot split (Kim et al., 2009a). *BCH* genes are encoded by small gene families in most plant species (Kim et al., 2009a) (www.phytozome.net). They usually show gene-specific expression patterns and, in plants with xanthophyll-accumulating flowers, fruits, or seeds, at least one of the isogenes is typically expressed preferentially in such tissues (Bouvier et al., 1998a; Castillo et al., 2005; Galpaz et al., 2006; Du et al., 2010). It is therefore likely that the differential expression of recently duplicated *BCH* genes provides flexibility to regulate the biosynthesis of downstream xanthophylls or the accumulation of upstream carotenes in a tissue-specific fashion. In agreement, differential expression of BCH-encoding genes in different cultivars has been repeatedly found to correlate with carotenoid levels in maize kernels, potato tubers, and morning glory and orchid flowers (Vallabhaneni et al., 2009; Chiou et al., 2010; Wolters et al., 2010; Yamamizo et al., 2010; Yan et al., 2010; Zhou et al., 2011). Our analysis of gene expression data in Arabidopsis suggest that *BCH2* might be preferentially involved in the production of β,β-xanthophylls for ABA synthesis because it is highly expressed in dry seeds but it shows much lower expression levels in other tissues that produce much higher amounts of carotenoids (Figure 5). Additionally, Figure 4 shows that *BCH2* (but not *BCH1*) is significantly co-expressed with *ZEP/NPQ2/ABA1*, a gene involved in the production of ABA substrates (see below). By analyzing the co-expression of all the Arabidopsis genes with those encoding enzymes specific of either the β,β (ZEP) or the β,ε (LCYE) branch of the carotenoid pathway (Figure 3) as described (Meier et al., 2011), we additionally observed a closer association of the CYP97-encoding genes with *LCYE* (Figure 4B), in agreement with the preferential activity of the encoded hydroxylases in the β,ε branch of the carotenoid pathway. These data together suggest that β,β- and β,ε-xanthophyll synthesis operate relatively independently of each other, which presumably reflects the independent evolution of the two pathway branches in plants (Kim and DellaPenna, 2006).

All three Arabidopsis CYP97 enzymes involved in carotenoid hydroxylation have been localized in chloroplast envelope membranes only (Joyard et al., 2009) (Table 1). Membrane association might be facilitated by the presence of a transmembrane anchoring sequence typical of other eukaryotic P450 enzymes (Chapple, 1998). Although no experimental data are available for the subplastidial localization of BCH hydroxylases, the presence of four transmembrane helices in their sequence (Table 1) suggests that they might be localized to the plastidial envelope or/and thylakoid membranes (Cunningham and Gantt, 1998).

Xanthophyll cycle enzymes

While hydroxylation of α-carotene produces a carotenoid endproduct that accumulates at high levels in chloroplasts (lutein), hydroxylation of β-carotene yields a xanthophyll (zeaxanthin) that under light conditions that do not saturate photosynthesis and in the dark is readily converted to violaxanthin via antheraxanthin in a two-step reaction catalyzed by the enzyme zeaxanthin epoxidase (ZEP). When light is so strong that exceeds the photosynthetic capacity of the leaves, violaxanthin is de-epoxidated back into zeaxanthin (much more efficient than violaxanthin in dissipating the excess excitation energy) by the activity of the enzyme violaxanthin de-epoxidase (VDE) (Demmig-Adams et al., 1996; Cunningham and Gantt, 1998). The interconversion of zeaxanthin and violaxanthin (Figure 3) is known as the xanthophyll cycle and has a key role in the adaptation of plants to different light intensities (reviewed in this issue by Hirschberg, Bassi, Dall'Osto). The Arabidopsis mutants defective in the single *VDE/NPQ1* (At1g08550) and *ZEP/NPQ2/ABA1* (At5g67030) genes were key to define the role of this cycle in photoprotection (Niyogi et al., 1998; Niyogi, 1999). The xanthophyll cycle is uniquely separated on opposite sides of the thylakoid membrane (Figure 6): VDE activity takes place on the thylakoid lumen side of the membrane, whereas ZEP occurs on the chloroplast stromal side (Demmig-Adams et al., 1996; Hieber et al., 2000; Yamamoto, 2006). Consistent with the described distribution, proteomic approaches have identified the Arabidopsis VDE enzyme only in thylakoids, whereas ZEP was localized in both envelope and thylakoid membranes (Joyard et al., 2009) (Table 1). This localization suggests that the envelope membranes (containing ZEP but not VDE) could be the site of violaxanthin biosynthesis whereas the thylakoid membranes (with both ZEP and VDE) would be the site of the xanthophyll cycle (Figure 6). In isolated thylakoid membranes, ZEP was found to be a multi-component FAD-containing monooxygenase (Büch et al., 1995), consistent with the presence of a FAD-binding domain in the enzyme (Marin et al., 1996). Mutants defective in ZEP activity were isolated in a screening for ABA-deficient mutants (named *aba1*) and shown to produce significantly lower ABA levels than the wild type (Koornneef et al., 1982; Rock and Zeevaart, 1991). This illustrates the importance of ZEP activity in controlling the availability of β,β-xanthophyll pre-

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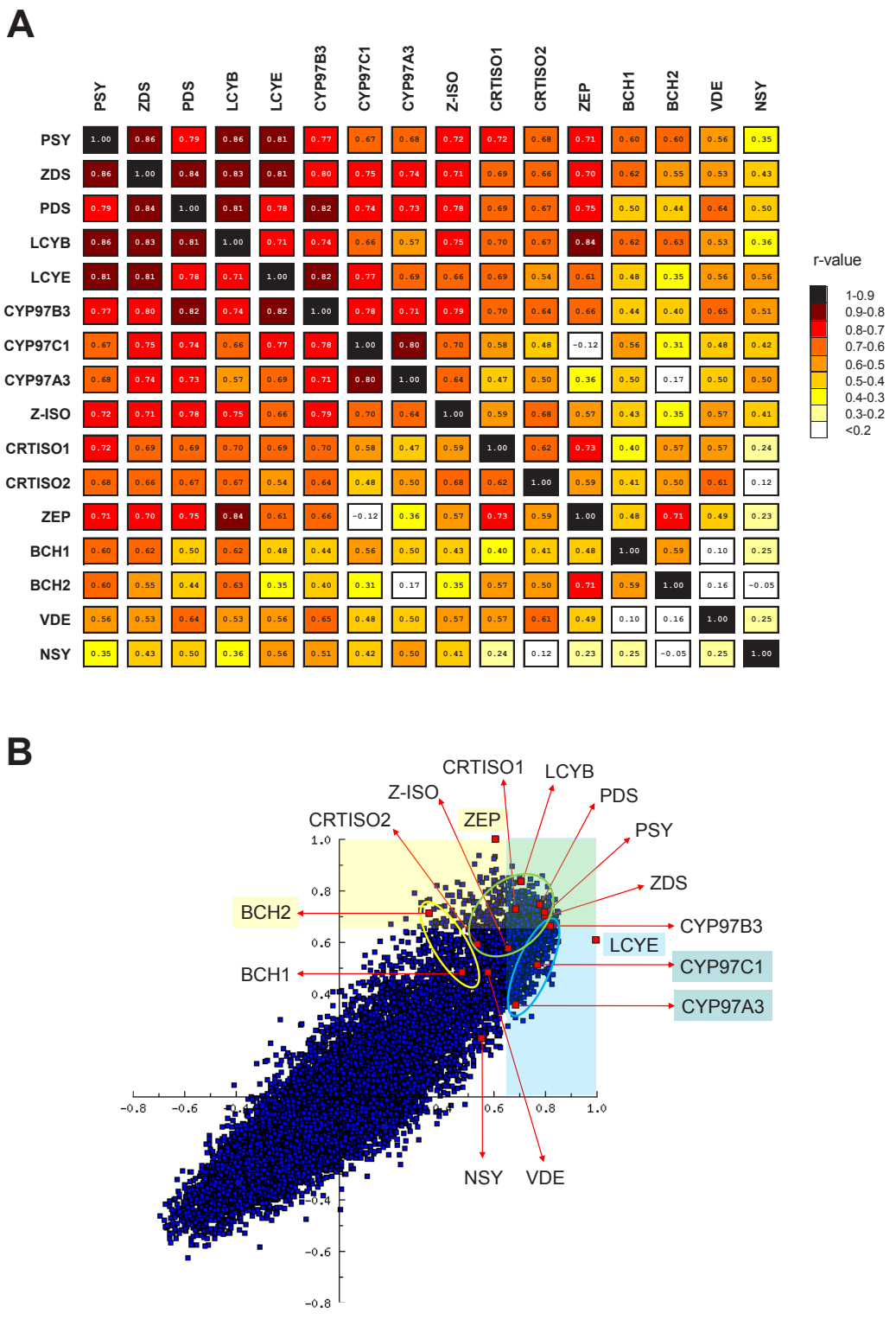


Figure 4. Analysis of co-expression of carotenoid biosynthetic genes.

Data were obtained using the Arabidopsis Coexpression Tool ACT (http://www.arabidopsis.leeds.ac.uk/act/ coexpanalyser.php). Genes are represented by the names of the encoded enzymes (Table 1). A, Heat map representing the level of co-expression among the genes encoding carotenoid biosynthetic enzymes in Arabidopsis. Numbers indicate the Pearson correlation coefficients (r-values): the higher the value, the stronger the co-expression. B, Scatter plot representing the level of co-expression of all Arabidopsis genes (blue squares) relative to those encoding ZEP (y axis) and LCYE (x axis). Genes encoding carotenoid biosynthetic enzymes are marked as red squares. The regions corresponding to highly significant co-expression values (r>0.65, p<5E-40) with *ZEP* (yellow box), *LCYE* (blue box), or both genes (green box) are indicated.

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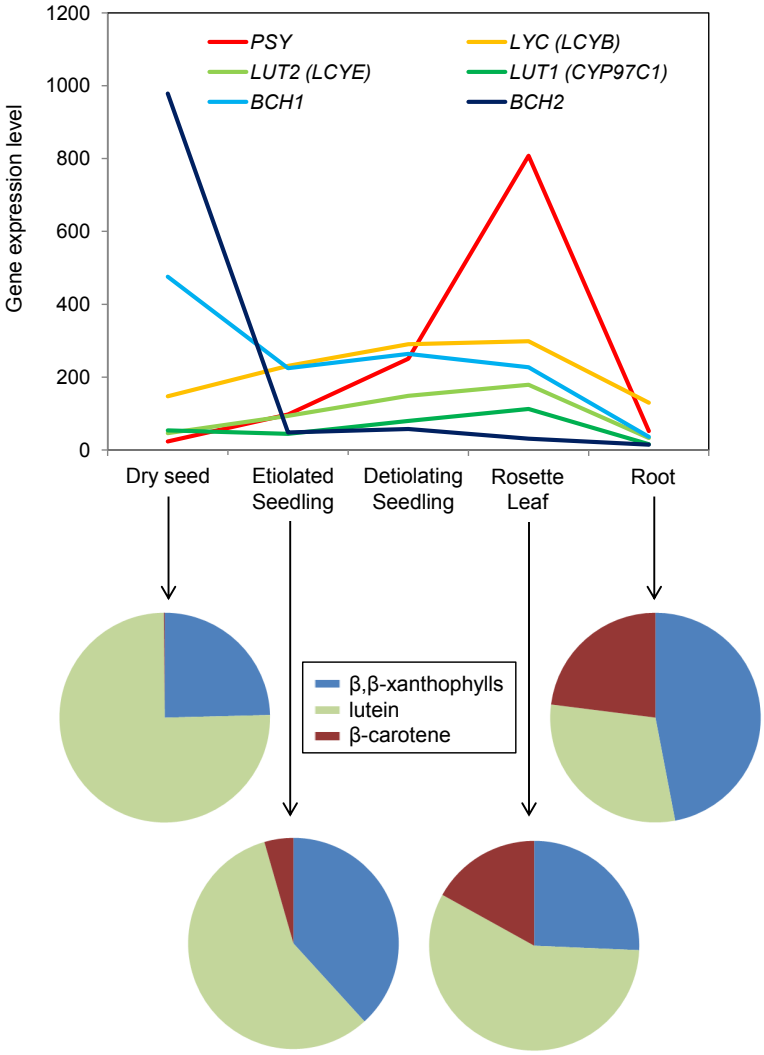


Figure 5. Carotenoid gene expression (upper panel) and end products (lower panel) in Arabidopsis.

Expression data for the indicated genes were obtained from the Arabidopsis eFP browser (http://bar.utoronto.ca). Pie charts represent the proportion of the indicated carotenoid products in dry seeds (Tian et al., 2003), whole etiolated seedlings (Rodriguez-Villalon et al., 2009a), rosette leaves (Tian et al., 2003), and roots (Maass et al., 2009).

cursors for the production of this hormone. Plant VDE is usually encoded by single genes, whereas small gene families encode ZEP in some plants, including maize (www.phytozome.net) (Vallabhaneni and Wurtzel, 2009). Both enzymes have characteristic conserved domains, including a lipocalin signature shared by other proteins that are able to bind hydrophobic molecules such as the apocarotenoid retinal (Hieber et al., 2000).

Neoxanthin synthase

The last step of the β,β branch of the carotenoid pathway in plants is the conversion of violaxanthin into neoxanthin, an allenic xanthophyll that together with violaxanthin can further be used for the production of apocarotenoid hormone ABA (Figure 1). The first genes encoding enzymes with neoxanthin synthase (NSY) activity in transient expression experiments were found to be homologous to LCYB and capsanthin-capsorubin synthase, an enzyme involved in the production of ketocarotenoids in pepper fruit chromoplasts (Al-Babili et al., 2000; Bouvier et al., 2000). Surprisingly, the tomato NSY enzyme was virtually identical to the chromoplast-targeted LCYB2/CYCB isoform (Ronen et al., 2000), suggesting that a single, bi-functional enzyme, might be capable of converting both lycopene to β-carotene and violaxanthin to neoxanthin in tomato. In this case, however, another NSY enzyme should exist because neoxanthin is synthesized in tomato mutants with a defective *CYCB* gene (Ronen et al., 2000). Consistent with the existence of a divergent NSY enzyme in plants, a different gene encoding a novel protein involved in

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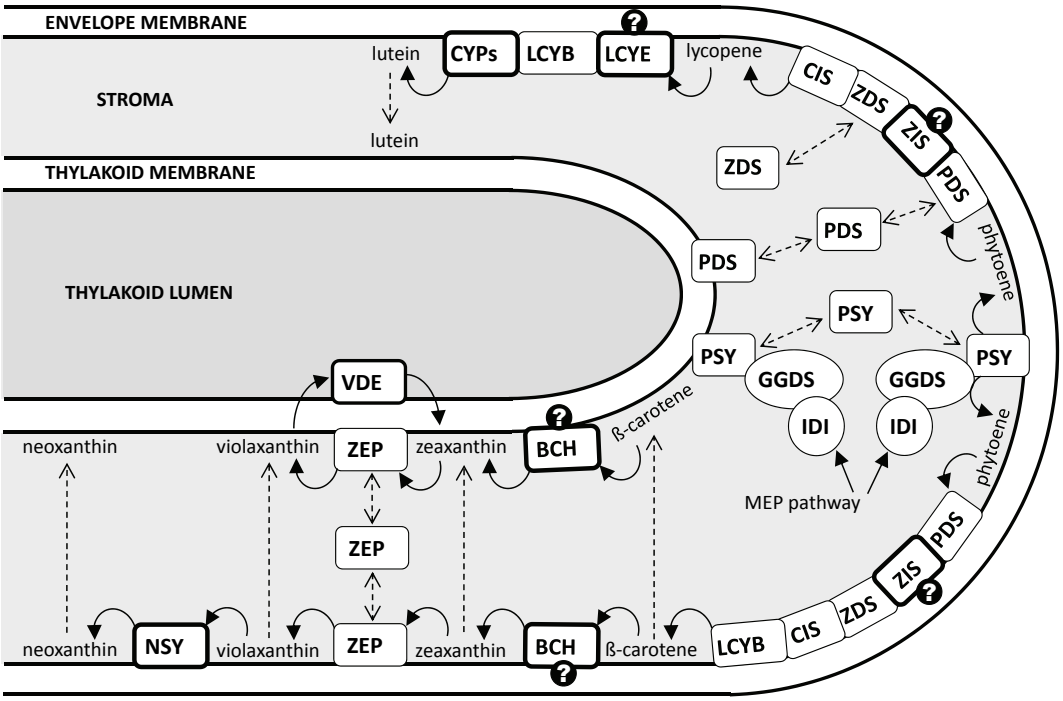


Figure 6. Model for the organization of carotenoid biosynthesis within plastids.

ZIS, Z-ISO; CIS, CRTISO; CYPs, CYP97-type carotenoid hydroxylases. Enzymes harboring domains for membrane attachment (Table 1) are highlighted in bold, and those lacking experimental evidence of their subplastidial localization are indicated with question marks. Dashed arrows indicate implicit transport of the indicated enzymes and carotenoids.

neoxanthin biosynthesis was identified in Arabidopsis based on the ABA-deficient mutant phenotype of the mutant, *aba4* (North et al., 2007). Overexpression of the single *NSY/ABA4* gene (At1g67080) in transgenic Arabidopsis plants led to increased accumulation of neoxanthin, whereas the loss-of-function *aba4* mutant was defective in this xanthophyll. However, the expression of this gene is poorly correlated with other carotenoid biosynthetic genes, including those closely associated to ABA biosynthesis like *BCH2* and *ZEP* (Figure 4). The protein, usually encoded by single genes in plants (North et al., 2007) (www.phytozome.net), has four predicted putative helical transmembrane domains (Table 1) and was found to be localized in the envelope membrane of Arabidopsis chloroplasts (Joyard et al., 2009).

REGULATION OF CAROTENOID BIOSYNTHESIS

Despite the relevance of carotenoids for plant life, our understanding of how plant cells regulate the accumulation of these essential metabolites in their plastids is still limited. It is becoming clearer, however, that the regulation of carotenoid biosynthesis is interconnected with that of related metabolic pathways, with plastid differentiation, and with developmental and environmental responses in which these isoprenoid pigments are involved. Here we will review the current knowledge on how carotenoid biosynthesis is regulated at both transcriptional and post-transcriptional levels in Arabidopsis, comparing it with that observed in other plant systems when appropriate.

Transcriptional regulation of biosynthetic genes

A first level of regulation of carotenoid accumulation in plants is the control of the transcription of biosynthetic genes. This is a major determinant for carotenoid production in classical model systems such as tomato ripening fruit, and increasing evidence indicates that it also has a central role for the control of carotenogenesis in Arabidopsis. This section will review how carotenoid gene expression is regulated by developmental cues, light signaling, the circadian clock, ABA-mediated feedback, and epigenetic mechanisms, with a particular emphasis on the coordination of carotenoid biosynthesis with related pathways.

Developmental regulation

The production of carotenoids is dynamically regulated throughout the plant life cycle by developmental signals but also in response to external environmental stimuli (Hirschberg, 2001; Pizarro and Stange, 2009; Cazzonelli and Pogson, 2010). Both developmental and environmental cues often act together to modulate carotenogenesis. For example, carotenoid biosynthesis

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during tomato fruit ripening is triggered by a developmental program but it can also be strongly influenced by light or temperature changes. Tomato fruit ripening is actually one of the best studied systems for the regulation of carotenoid biosynthesis. During normal development, the changes in the production of carotenoids associated with tomato fruit ripening are mainly controlled at the level of transcription of biosynthetic genes. Mature green fruit contain chloroplasts with a carotenoid composition similar to that of leaves. During ripening, chlorophylls are degraded and a massive accumulation of carotenoids (particularly lycopene) takes place, causing the fruit color to change from green to red. This process is associated with the differentiation of chloroplasts into chromoplasts (Gillaspy, 1993; Giovannoni, 2001). The increased production of carotenoids is preceded by an accumulation of transcripts encoding the fruit isoforms of DXS, and later, PSY, suggesting that an enhanced supply of MEP-derived isoprenoid precursors contributes to the burst in carotenoid biosynthesis observed early during fruit ripening (Lois et al., 2000). While the expression of genes encoding desaturases and isomerases also increases at the onset of carotenoid accumulation, transcripts for both LCYB and LCYE disappear, resulting in the accumulation of lycopene in ripe fruit (Giuliano et al., 1993; Fraser et al., 1994; Pecker et al., 1996; Ronen et al., 1999; Alba et al., 2005). Additional evidence for the relevance of differential gene expression in the regulation of tomato fruit carotenoid biosynthesis was provided by the mutants *Beta* and *Delta*, in which lycopene is converted into δ-carotene or β-carotene due to an increased transcription of the *LCYB2/CYCB* and *LCYE* genes, respectively (Ronen et al., 1999; Ronen et al., 2000). Besides tomato, the controlled transcription of the genes encoding biosynthetic enzymes also appears to be the major regulatory mechanism of carotenoid biosynthesis in most chromoplast-containing tissues of various plant species. However, regulation at the enzymatic level also occurs in flowers and fruit, which explains some of the unpredicted results of several biotechnological approaches (Hirschberg, 2001).

Much less is known about the importance of transcriptional regulation for the control of carotenoid biosynthesis in Aradidopsis. However, our analysis of public microarray-based gene expression databases such as the Arabidopsis eFP brower at http://bar.utoronto.ca (Winter et al., 2007) indicates that transcript levels of key genes correlate with the carotenoid profiles of different organs and developmental stages. For example, the proportion of β-carotene in different plastid types can be explained by the balance between the expression of genes encoding the enzymes involved in its production (PSY, LCYB), and those encoding the BCH enzymes that use it as a substrate for the production of β,β-xanthophylls (Figure 5). Arabidopsis mature seeds contain very low amounts of lutein (about 75% of all carotenoids), β,β-xanthophylls (about 25%) and β-carotene (less than 0.5%) in non-photosynthetic plastids named eoplasts, which derive from the chloroplasts found in young embryos (Lindgren et al., 2003; Tian et al., 2003; Auldridge et al., 2006b; Kim et al., 2009a). The high levels of *BCH2* and, to a lower extent, *BCH1* expression in dry seeds (Figure 5) would be consistent with an active rate of conversion of β-carotene to β,β-xanthophylls, which could be subsequently transformed into ABA (Meier et al., 2011). After germination in the dark, an enhanced production of carotenoids takes place in the etioplasts formed in the seedling cotyledons that will help to protect the photosynthetic apparatus upon illumination (Park et al., 2002; Ghassemian et al., 2006; Rodriguez-Villalon et al., 2009a). The increased level of transcripts encoding PSY and LCYB relative to those encoding LCYE and CYP97C1, together with the drop in the expression of BCH-encoding genes (particularly *BCH2*), can explain why etioplasts accumulate more carotenoids and a higher proportion of β,β-xanthophylls (mainly violaxanthin) and β-carotene than seeds, with lutein remaining the most abundant carotenoid (Figure 5). Illumination triggers a photomorphogenic program of development that results in the differentiation of etioplasts into chloroplasts and a dramatic increase in the production of carotenoids (Frosch and Mohr, 1980; Giuliano et al., 1993; Albrecht and Sandmann, 1994; Park et al., 2002; Woitsch and Römer, 2003; Toledo-Ortiz et al., 2010). In Arabidopsis chloroplasts, *PSY* is expressed at high levels, likely to maintain a high metabolic flux into the carotenoid pathway. The higher proportion of β-carotene and lower proportion of β,β-xanthophylls relative to etioplasts might be explained by a higher expression of genes encoding the enzymes involved in the production of β-carotene (PSY, LCYB) with little or no changes in those encoding the BCH enzymes that use β-carotene to produce β,β-xanthophylls (Figure 5).

Light signaling

Most studies addressing the regulation of carotenogenesis in Arabidopsis have focused on seedling deetiolation, a light-regulated developmental process that is particularly well understood at the molecular level (Quail, 2002; Jiao et al., 2007). Plants develop following a photomorphogenetic program in the light but when seeds germinate in the dark photomorphogenesis is repressed and seedlings develop etiolated to search for light above ground. Etiolated seedlings show long hypocotyls and closed unexpanded cotyledons that contain etioplasts with chlorophyll precursors and relatively low levels of carotenoids. Etioplast carotenoids (Figure 5) are associated to the prolamellar body, a lattice of tubular membranes that facilitates greening when underground seedlings emerge into the light (Park et al., 2002; Cuttriss et al., 2007). Following light perception, photomorphogenesis is derepressed and plants deetiolate. Deetiolation results in decreased hypocotyl elongation, cotyledon expansion and differentiation of etioplasts into chloroplasts.

The light-triggered deetiolation process is tightly associated with a burst in the production of carotenoids in chloroplasts to protect the emerging photosynthetic apparatus and support photosynthetic development. Virtually all the Arabidopsis genes encoding enzymes involved in carotenoid biosynthesis, including those of the MEP pathway, are upregulated during light-triggered deetiolation (Ghassemian et al., 2006; Rodríguez-Concepción, 2006; Meier et al., 2011). The coordination at the gene expression level between the MEP pathway and the carotenoid pathway is consistent with the observation that carotenoid biosynthesis in light-grown plants can be limited by the availability of GGPP precursors, as deduced from the increases observed in carotenoid levels upon overexpression of flux-controlling enzymes of the MEP pathway such as DXS or HDR (Estévez et al., 2001; Botella-Pavía et al., 2004; Enfissi et al., 2005; Carretero-Paulet et al., 2006; Morris et al., 2006; Muñoz-Bertomeu et al., 2006; Vallabhaneni and Wurtzel, 2009). To ensure that the GGPP pre-

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cursors required for carotenoid biosynthesis will be supplied when necessary, an increased expression of genes encoding MEP pathway enzymes has been shown to occur just prior to, or concomitant with, the production of carotenoids not only during during deetiolation but also in other development processes associated with an increased production of carotenoids or apocarotenoids (Bouvier et al., 1998b; Lois et al., 2000; Walter et al., 2000; Botella-Pavía et al., 2004; Hans et al., 2004; Cordoba et al., 2009; Toledo-Ortiz et al., 2010). The close regulatory connection between genes of the MEP pathway and those of the carotenoid pathway has been confirmed by the analysis of Arabidopsis global gene expression profiles (Wille et al., 2004; Ghassemian et al., 2006; Meier et al., 2011). Interestingly, the coordination appears to be strongest with genes encoding flux-controlling steps of both pathways such as *DXS* and *PSY*, which are highly and coordinately up-regulated when carotenoid biosynthesis is boosted during early light-triggered deetiolation in Arabidopsis (Figure 5). A down-regulated accumulation of transcripts encoding *PSY* has also been reported when the MEP pathway is chemically or genetically blocked in Arabidopsis seedlings (Laule et al., 2003), whereas *PSY* expression is induced when DXS expression and/or activity is upregulated in potato tubers and tomato fruit (Lois et al., 2000; Rodríguez-Concepción et al., 2001; Enfissi et al., 2005; Morris et al., 2006). Additional evidence that there is an active communication between the MEP and carotenoid pathways in Arabidopsis and other plant systems is available in other recent reviews (Giuliano et al., 2008; Cordoba et al., 2009; Cazzonelli and Pogson, 2010; Rodriguez-Concepcion, 2010).

To facilitate the transition to a photosynthetic metabolism and minimize the deleterious effects of light on the emerging photosynthetic apparatus during deetiolation, the production of carotenoids is also coordinated with the production of chlorophylls and carotenoid-bearing protein complexes in a process controlled by the phytochrome family of photoreceptors (von Lintig et al., 1997; Welsch et al., 2003; Woitsch and Römer, 2003; Wille et al., 2004; Welsch et al., 2007; Toledo-Ortiz et al., 2010; Meier et al., 2011). A common ATCTA motif found in the promoters of genes encoding photosynthesis-related proteins (including MEP pathway and carotenoid pathway enzymes) was proposed to have a role on this coordination (Welsch et al., 2003; Botella-Pavía et al., 2004). The same motif is also conserved in the promoter of genes responsive to low oxygen availability (Mohanty et al., 2005; Licausi et al. 2011a). The only *trans*-acting factor found to bind to the ATCTA motif, RAP2.2, belongs to a subfamily of ERF transcription factors recently shown to be involved in oxygen sensing for hypoxia acclimation (Welsch et al., 2007; Licausi et al. 2011b). Although it is tempting to speculate that RAP2.2 might regulate the expression of both photosynthetic and anaerobic genes when oxygen supply is compromised, experimental evidence suggests that this transcription factor is not instrumental in the control of carotenoid gene expression or accumulation (Welsch et al., 2007).

Another molecular mechanism for the coordination of chlorophyll and carotenoid biosynthesis with chloroplast development during deetiolation has been recently unveiled (Toledo-Ortiz et al., 2010). In this case, the coordination is mediated by basic helix-loop-helix (bHLH) transcription factors of the Phytochrome-Interacting Factors (PIF) family (Castillon et al., 2007; Bae and Choi, 2008; Leivar and Quail, 2010). PIFs are central mediators in a variety of light-mediated responses, and at least some of them (PIF1, PIF3, PIF4, and PIF5) accumulate in etiolated seedlings to repress photomorphogenic development in the dark (Leivar et al., 2008; Shen et al., 2008; Leivar et al., 2009; Shin et al., 2009). Upon illumination, photoactivated phytochromes directly interact with PIFs, causing their phosphorylation and proteasome-mediated degradation and hence allowing photomorphogenic development to proceed (Castillon et al., 2007; Bae and Choi, 2008; Leivar and Quail, 2010). In particular, a negative role in the regulation of photosynthetic development was deduced for PIF1, PIF3, PIF4 and PIF5 because genes involved in the biosynthesis of chlorophylls and carotenoids and in the differentiation of chloroplasts were found to be up-regulated in defective mutants (Leivar et al., 2009; Shin et al., 2009; Stephenson et al., 2009; Toledo-Ortiz et al., 2010). Among them, PIF1 was shown to be the major regulator of carotenoid biosynthesis during deetiolation and to bind to a G-box motif (CACGTG) in the promoter of the Arabidopsis *PSY* gene to directly repress its expression (Toledo-Ortiz et al., 2010).

Although the genes encoding DXS, LCYE, BCH2 and ZEP were also predicted to be potential PIF targets based on their expression profiles during deetiolation (Leivar et al., 2009; Toledo-Ortiz et al., 2010), none of them was found to be a direct target of PIF1, suggesting that PIF1 specifically targets the *PSY* gene for the control of carotenoid biosynthesis during deetiolation. Consistently, the sole upregulation of *PSY* expression has been demonstrated to be sufficient to increase carotenoid production in various plant systems, including deetiolating seedlings (Maass et al., 2009; Rodriguez-Villalon et al., 2009a; Cazzonelli and Pogson, 2010). Together, the current experimental evidence supports the following model for the regulation of carotenoid biosynthesis during Arabidopsis deetiolation. When Arabidopsis seedlings germinate in the dark, high PIF levels prevent photomorphogenic development and repress carotenoid biosynthesis by direct binding of PIF1 (and possibly other PIFs) to the promoter of the *PSY* gene. The accumulation of PIFs in dark-grown seedlings also represses genes required for chlorophyll biosynthesis and chloroplast development. Immediately after underground seedlings emerge from the soil into sunlight, PIFs are degraded upon interaction with photoactivated phytochromes, causing a common derepression of all these genes (Leivar and Quail, 2010). As a result, carotenoid production increases rapidly in coordination with the biosynthesis of chlorophylls and the assembly of the photosynthetic machinery in differentiating chloroplasts to protect the emerging photosynthetic apparatus from photooxidative damage. Interestingly, PIF1 and other PIFs also repress *PSY* gene expression and carotenoid biosynthesis in fully deetiolated Arabidopsis plants (Toledo-Ortiz et al., 2010), suggesting that these transcription factors regulate the biosynthesis of carotenoids at the transcriptional level throughout the plant's life. Because the expression of the *PSY* gene is still upregulated by light in PIF-deficient seedlings (Leivar et al., 2009; Toledo-Ortiz et al., 2010) and short versions of its promoter lacking the G-boxes appear to remain light-responsive (Welsch et al., 2003), it is likely that other transcription factors besides PIFs participate in the light-mediated regulation of *PSY* expression during seedling deetiolation. It is possible that such as-yet unidentified transcription factors might be involved in the regulation of *DXS* and the other light-induced carotenoid biosynthetic genes for which binding of PIF1 was not observed. Phytochrome-mediated light signals also appear to regulate the expression of carotenoid biosynthesis genes in chro-

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moplast-bearing organs such as tomato fruit, but the coordination with genes involved in photosynthesis observed in leaves is lost (Alba et al., 2000; Giovannoni, 2001; Hirschberg, 2001).

Circadian clock

In both photosynthetic and non-photosynthetic tissues, light and phytochrome signaling pathways are important for setting or entraining the plant circadian clock, a mechanism that produces self-sustained rhythms of *ca.* 24 h to enable anticipation of changes associated with the daily light and dark cycles (Millar, 2004; Gardner et al., 2006). In Arabidopsis, several studies have shown that carotenoid biosynthetic genes (including *PSY* and most MEP pathway genes) are clock-regulated, usually showing a peak phase of transcript abundance at around dawn (Thompson et al., 2000; Woitsch and Römer, 2003; Hsieh and Goodman, 2005; Covington et al., 2008; Facella et al., 2008; Cordoba et al., 2009; Fukushima et al., 2009; Pan et al., 2009). Among the oscillating genes, only that encoding VDE shows peak transcript levels at dusk (Covington et al., 2008). Diurnal oscillations have been found for the accumulation of several groups of MEP-derived plastidial isoprenoids, including the hormones gibberellins and carotenoid-derived ABA (Hedden and Kamiya, 1997; Aharoni et al., 2003; Dudareva et al., 2005; Barta and Loreto, 2006), whereas chlorophyll levels (and plant growth) increase when the circadian clock period is matched to the external light-dark cycles (Dodd et al., 2005). Although these data suggest that carotenoid production might also be controlled by the circadian clock, perhaps allowing the plant to be more efficiently protected against photooxidative damage, experimental evidence is still missing.

Feedback by ABA

In organs such as roots that do not directly depend on light signals for normal development, a positive correlation between Arabidopsis *PSY* expression and carotenoid levels has been observed (Maass et al., 2009). Arabidopsis roots accumulate very low levels of β-carotene (25% of total carotenoids), lutein (30%), and β,β-xanthophylls (45%) in leucoplasts (Figure 5). It is likely that the high proportion of β,β-xanthophylls might act as a reservoir for ABA precursors. However, under abiotic stress conditions requiring an active production of ABA, this β,β-xanthophylls pool might be insufficient. It has been proposed that an increased PSY activity provides carotenoid precursors for the enhanced production of ABA that takes place in roots under drought or saline stress (Li et al., 2008b; Welsch et al., 2008; Arango et al., 2010). Consistent with this role of PSY in roots, in plant species with several genes encoding PSY (such as rice and maize) the *PSY* isogenes involved in the production of root carotenoids are not responsive to light but to abiotic stress and specifically to ABA (Li et al., 2008a; Li et al., 2008b; Welsch et al., 2008; Li et al., 2009a). The only Arabidopsis gene encoding PSY is also expressed in the root, where it is upregulated in response to osmotic stress, which induces the synthesis of ABA (Meier et al., 2011). Our unpublished data indicate that such upregulation is mediated by ABA, confirming the presence of a feedback regulatory mechanism by which *PSY* expression is regulated in the root in response to abiotic stress. PIFs do not appear to have a role in the stress-triggered control of *PSY* expression in the root, in contrast with the pivotal role of these transcription factors for the regulation of *PSY* gene expression and carotenoid accumulation in the cotyledons of etiolated seedlings and the photosynthetic tissues of light-grown plants. The increased expression of *PSY* and many other carotenoid biosynthetic genes that takes place during seed germination following imbibition is also PIF-independent (Meier et al., 2011). During this particular developmental stage, the expression of *PSY* is not induced by ABA (as described in osmotically-stressed roots) but repressed (Meier et al., 2011), illustrating the complexity of the regulation of carotenoid gene expression in plants.

Epigenetic mechanisms

Epigenetic regulatory mechanisms have recently been found to control the expression of carotenoid biosynthetic genes. In particular, the expression of the gene encoding the Arabidopsis CRTISO1 isoform appears to be specifically regulated by CCR1/SDG8, a chromatin-modifying histone methyltransferase enzyme (Cazzonelli et al., 2009). SDG8 is required to maintain the chromatin methylation status and hence the permissive expression of *CRTISO1* during seedling development, in leaves, shoot apex, and some floral organs (Cazzonelli et al., 2010). Microarray analysis of SDG8-defective mutants suggested that no other gene of the carotenoid pathway is targeted by this chromatin-modifying enzyme (Cazzonelli et al., 2009).

Post-transcriptional regulation of carotenoid accumulation

Besides the control of the transcription of genes encoding carotenoid biosynthetic enzymes, a number of post-transcriptional mechanisms have been reported to regulate carotenoid accumulation, explaining the poor correlation between gene expression patterns and the accumulation of carotenoids observed in many cases. These mechanisms affect biological processes that can be grouped in four major areas: modulation of enzyme levels and activities, metabolite channeling by multi-enzyme complexes, sequestration and storage capacity, and carotenoid turnover.

Modulation of enzyme levels and activities

Besides its role in the transcriptional control of the pathway, light also regulates carotenogenesis at multiple post-transcriptional levels. Light-driven processes in functional chloroplasts that result in non-enzymatic isomerization can substitute, at least in part, the activities of the Z-ISO and CRTISO isomerases in photosynthetic tissues (Isaacson et al., 2002; Park et al., 2002; Breitenbach and Sandmann, 2005; Li et al., 2007; Sandmann, 2009). Light also influences the activity of the carotenoid biosynthetic enzymes modulated by photosynthetic redox systems. The MEP pathway enzymes DXR, HDS and HDR appear to be targets of thioredoxin (Balmer et al., 2003; Lemaire et al., 2004), a member of the ferredoxin/thioredoxin system that is chemically reduced in photosynthetically-active chloroplasts to upregulate the activity of

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target proteins through the reduction of specific disulfide groups (Schürmann and Jacquot, 2000; Buchanan et al., 2002). Because the carotenoid desaturases PDS and ZDS use plastoquinone as hydrogen acceptor, their activity is connected to the photosynthetic electron transport chain (Carol and Kuntz, 2001). Both PDS and ZDS, as well as other carotenoid biosynthetic enzymes such as CRTISO, LCYB, LCYE, and ZEP, contain a conserved FAD-binding motif that suggests the involvement of redox balance in the corresponding enzymatic reactions (Hugueney et al., 1992; Büch et al., 1995; Marin et al., 1996; Schnurr et al., 1996; Isaacson et al., 2004; Mialoundama et al., 2010; Yu et al., 2010; Yu et al., 2011). There is also evidence for a redox control of the expression of some carotenoid biosynthetic genes in tobacco chloroplasts and tomato fruit chromoplasts (Woitsch and Römer, 2003; Nashilevitz et al., 2010). Most interestingly, the activities of ZEP and VDE, which control the levels of zeaxanthin and violaxanthin, are tightly regulated by the light and photosynthesis status of the plant. Under light levels that exceed the maximum that can be productively used for photosynthesis, many plants adjust the carotenoid composition of leaves for photoprotection and transform violaxanthin into zeaxanthin to more efficiently dissipate the excess excitation energy. Low light conditions result in the transformation of zeaxanthin back into violaxanthin, in what is known as the xanthophyll cycle (Demmig-Adams et al., 1996; Hieber et al., 2000; Yamamoto, 2006; Li et al., 2009b). Although changes in gene expression in response to modified light conditions might contribute to the described effect in the xanthophyll profile (Bugos et al., 1999; Hirschberg, 2001; Rossel et al., 2002; Woitsch and Römer, 2003), the major driving force for regulating the activities of the enzymes involved in the xanthophyll cycle, VDE and ZEP, appears to be the light-induced changes in the trans-thylakoid pH. Under low light or in the dark, when the pH of the thylakoid stroma is neutral, the VDE enzyme remains soluble (mostly inactive) in the thylakoid stroma. But under high light the photosynthetic proton pump increases the acidity of the lumen, hence stimulating the binding of VDE to the thylakoid membrane and its enzymatic activity, eventually resulting in an enhanced production of zeaxanthin. The reaction catalyzed by ZEP is slow compared to that of VDE but it best functions at neutral pH (Demmig-Adams et al., 1996; Hieber et al., 2000; Yamamoto, 2006; Li et al., 2009b). Together, light not only regulates carotenoid gene expression but it also activates the metabolic flux through the pathway by increasing the activity of biosynthetic enzymes.

In non-photosynthetic tissues, PSY activity appears to be the main limiting step for carotenoid biosynthesis. Thus, transgene-mediated upregulation of PSY levels in Arabidopsis roots is sufficient to increase the production of carotenoids (Maass et al., 2009). The same effect was observed in dark-grown seedlings, whereas overexpression of flux-controlling enzymes of the MEP pathway such as DXS and HDR did not have any effect on etioplast carotenoid levels (Botella-Pavía et al., 2004; Rodriguez-Villalon et al., 2009a). Additionally, derepression of deetiolation in dark-grown seedlings treated with paclobutrazol (an inhibitor of gibberellin biosynthesis) led to an increased production of carotenoids that correlated with increased levels of *PSY* transcripts and enzyme activity but no changes in the expression of most other genes involved in carotenoid biosynthesis, including those of the MEP pathway (Rodriguez-Villalon et al., 2009a). However, the increased PSY activity has been found to initiate a feedback mechanism that eventually results in the post-transcriptional accumulation of higher DXS protein levels (Guevara-Garcia et al., 2005; Rodriguez-Villalon et al., 2009a). It has been hypothesized that a high rate of biosynthesis of carotenoids during the initial stages of Arabidopsis seedling development could lead to a concomitant decrease in the levels of MEP pathway intermediates or products, which in turn would trigger a post-transcriptional accumulation of DXS to upregulate pathway flux (Guevara-Garcia et al., 2005). A similar feedback mechanism might also work in chromoplasts, explaining the increased DXS activity (but not transcript accumulation) detected in tomato fruit with upregulated PSY levels (Fraser et al., 2007). Although the signals and metabolites involved in the feedback communication between the carotenoid pathway and the MEP pathway in Arabidopsis seedlings are still a matter of speculation (Laule et al., 2003; Guevara-Garcia et al., 2005; Rodriguez-Villalon et al., 2009a), it is likely that the upregulation of DXS both transcriptionally (in response to light signals and indirectly mediated by PIFs) and post-transcriptionally (in response to metabolic feedback triggered by PSY upregulation) ensures an appropriate supply of MEP-derived precursors for the biosynthesis of carotenoids during the critical stages of early seedling development in the light. Feedback regulation of carotenoid biosynthetic gene expression and enzyme activities by phytoene, lycopene, or/and other carotenoid metabolites has been proposed in many works (Misawa et al., 1994; Corona et al., 1996; Römer et al., 2000; Romer et al., 2002; Simkin et al., 2003; Diretto et al., 2006; Cuttriss et al., 2007; Qin et al., 2007; Harjes et al., 2008; Apel and Bock, 2009; Bai et al., 2009; Cazzonelli et al., 2009), but clear-cut evidence of the molecular mechanisms and metabolites involved is still missing.

Metabolite channeling by multi-enzyme complexes

A major determinant of the activity of carotenoid biosynthetic enzymes is membrane association. As described in the Biosynthesis section, many of the pathway enzymes (including PSY) need to be in a membrane context for activity. Membrane association might be accomplished *via* membrane attachment or spanning domains or by interaction with proteins harboring such domains that might act as anchors for the whole complex. Among the Arabidopsis carotenoid pathway enzymes, membrane spanning domains are predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and/or ARAMENNON (http://aramemnon.uni-koeln.de/) for Z-ISO, LCYE, BCH1, BCH2, VDE and NSY (Table 1), whereas a single transmembrane anchoring sequence typical of other eukaryotic P450 enzymes is present in the CYP97 type CHYs (Chapple, 1998; Tian et al., 2004). A hypothetical model for multi-enzyme complexes involved in carotenoid biosynthesis in chloroplasts is shown in Figure 6. This model integrates data from biochemical evidence of the presence of membrane-associated plastid complexes containing some of these enzymes (Camara, 1993; Fraser et al., 2000; Lopez et al., 2008), proteomics-based localization of individual pathway enzymes (Joyard et al., 2009), mutant and co-expression data analyses suggesting the involvement of particular enzymes in specific branches of the pathway (Tian et al., 2003; Tian et al., 2004; Kim and DellaPenna, 2006; Kim et al., 2009a; Kim et al., 2010; Meier et al., 2011), and previous models proposing multi-enzyme complexes containing

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enzymes that allow channeling of phytoene to synthesize cyclic carotenes (Cunningham and Gantt, 1998; Bai et al., 2009) and of lycopene to synthesize lutein (Kim and DellaPenna, 2006). This model proposes the existence of protein complexes associated to the envelope membrane that transform IPP and DMAPP into phytoene (formed by IDI, GGDS and PSY enzymes), phytoene into lycopene (PDS, Z-ISO, ZDS and CRTISO), phytoene into β-carotene (PDS, Z-ISO, ZDS, CRTISO and LCYB), and lycopene into lutein (LCYE, LCYB and CYP97s). Individual enzymes attached to either the envelope or the thylakoid membranes would transform β-carotene into β,β-xanthophylls (Figure 6). The model implies metabolic channeling, allowing to explain why only some pathway intermediates accumulate to detectable levels in different plastids. However, a question that arises from this model is how carotenoids that are synthesized in the envelope membrane are transported to the thylakoids, where most of them accumulate associated with the photosystem reaction centres and the light-harvesting complexes.

Because different plastid types show diverse composition of carotenoids and biosynthetic enzymes and unique subplastidial structures, it would be expected that the nature and localization of the hypothetical multi-protein complexes would differ among them. In agreement, the enzymes ZDS, LCYB, BCH1 and BCH2 were found to be part of the plastoglobule proteome in chromoplasts of pepper fruit but not in Arabidopsis chloroplasts (Vidi et al., 2006; Ytterberg et al., 2006). Plastoglobules are lipoprotein particles that participate in the biosynthesis and storage of isoprenoids and other lipids (Brehelin et al., 2007). They are known to store and sequester carotenoids in chromoplasts, but their role in carotenoid biosynthesis remains to be demonstrated. Although it has also been hypothesized that plastoglobules could potentially ferry carotenoids and other lipids between plastid membranes, a recent study found that plastoglobules are closely associated with thylakoids in chloroplasts (Austin et al., 2006). In this work, no plastoglobules attached to or associated with the inner chloroplast envelope membrane or any free-floating stromal plastoglobules were found in Arabidopsis chloroplasts. Future research efforts should provide conclusive experimental evidence of the presence, localization, structure and regulation of putative multienzyme complexes as well as determine whether plastoglobules participate in the intra-chloroplast trafficking of carotenoid intermediates and products.

Sequestration and storage capacity

The type of plastid has profound effects on the accumulation of carotenoids. For example, deetiolation involves the differentiation of etioplasts into chloroplasts, which has a dramatic impact not only in improving the enzyme activities of PSY and other carotenoid biosynthetic enzymes but also in the storage capacity of the plastids (von Lintig et al., 1997; Welsch et al., 2000; Ghassemian et al., 2006). Thus, the assembly of photosynthetic complexes and the build-up of thylakoid membranes and plastoglobules in chloroplasts increase the capacity to sequester the newly synthesized carotenoid molecules. Defects in chloroplast development hence result in a decreased accumulation of carotenoids, even under conditions in which an enhanced supply of their isoprenoid precursors is available (Sauret-Güeto et al., 2006; Flores-Perez et al., 2008b). Differentiation of chloroplasts into chromoplasts involves the development of larger plastoglobules and/or carotenoid-sequestering structures of different shapes, allowing the deposition of massive amounts of carotenoids in a matrix of lipoproteins (Deruere et al., 1994; Vishnevetsky et al., 1999; Simkin et al., 2007; Walter and Strack, 2011). In the orange curds of the cauliflower (*Brassica oleracea* var. *botrytis*) *Orange* variety, chromoplast-like plastids with inclusions of membranous compartments develop due to a mutation in *Or* gene, encoding a plastidial DnaJ-like protein possibly involved in targeting substrates to the DnaK/Hsp70 chaperone (Paolillo et al., 2004; Lu et al., 2006). The mutation results in the accumulation of much higher carotenoid (β-carotene) levels compared to uncolored varieties without changes in the expression of carotenoid biosynthetic genes (Li et al., 2001). Although the biological role of *Or* is still unclear, these studies illustrate how carotenoid accumulation can be boosted by triggering the synthesis of a plastid deposition sink to store carotenoids (Giuliano and Diretto, 2007; Li and Van Eck, 2007). Storage capacity also appears to be responsible for the enhanced accumulation of carotenoids that takes place in the fruit of tomato *high-pigment* (*hp*) mutants *hp1*, *hp2*, and *hp3*, which show an increased plastid volume per cell but no consistent up-regulation of carotenoid gene expression (Benvenuto et al., 2002; Davuluri et al., 2005; Galpaz et al., 2008; Kolotilin et al., 2007; Azari et al., 2010; Enfissi et al., 2010). Genes mutated in *hp1* and *hp2* encode regulators of light signaling at the level of chromatin remodeling, whereas a decreased production of ABA was found to be the cause of the mutant phenotype in *hp3*. Reduced ABA levels in the ZEP-deficient Arabidopsis *aba1* mutant also result in increased numbers of chloroplasts per cell (Rock et al., 1992) and an altered carotenoid profile (Rock and Zeevaart, 1991). These results additionally illustrate that light and ABA can influence carotenogenesis by multiple mechanisms.

Alterations in the production of carotenoids can in turn modulate plastid development. Arabidopsis mutants defective in carotenoid biosynthesis also show defects in chloroplast development (Park et al., 2002; Dong et al., 2007; Qin et al., 2007). Most interestingly, increasing the metabolic flux to the carotenoid pathway by overexpressing PSY-encoding genes is sufficient to trigger the differentiation of chromoplast-like plastids from root and callus leucoplasts in Arabidopsis (Maass et al., 2009) and fruit chloroplasts in tomato (Fraser et al., 2007).

Carotenoid turnover

The steady-state levels of carotenoids are expected to be dependent on the storage (sink) capacity of plastids but also on the balance between biosynthesis and degradation. Recent pulse-chase labeling experiments (Beisel et al., 2010) showed that carotenoid degradation in Arabidopsis leaves occurs at a much higher rate than suspected (a slow rate was assumed based on the persistent carotenoid-derived colors of senescing leaves). Yet the mechanisms controlling carotenoid degradation and turnover are still little known. Carotenoid degradation may occur either by non-enzymatic (photo)oxidation or by enzymatic oxidation. Enzymes than can unspecifically oxidize and degrade carotenoids include peroxidases and lipoxygenases, whereas a family of oxygenases catalyze the specific cleavage of carotenoids (Carail and

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Caris-Veyrat, 2006; Walter and Strack, 2011). Carotenoid cleavage oxygenases (CCOs), also know as carotenoid cleavage dioxygenases (CCDs) in plants, carry out an oxidative cleavage of particular carotenoids and generate products collectively referred to as apocarotenoids (Giuliano et al., 2003; Bouvier et al., 2005b; Auldridge et al., 2006a). An assortment of plant apocarotenoids results from the large number of carotenoid precursors, variations in the site of cleavage, and modifications subsequent to cleavage. As described in the Introduction, apocarotenoids such as the hormones ABA, strigolactones and others yet to be identified serve essential regulatory functions (Nambara and Marion-Poll, 2005; Van Norman and Sieburth, 2007; Xie et al., 2010). Others are involved in the interaction of plants with their biological environment, and some have an important agronomic value as pigments and aromas (Giuliano et al., 2003; Bouvier et al., 2005b; Walter and Strack, 2011).

The contribution of CCOs to carotenoid homeostasis is still a matter of discussion (Walter and Strack, 2011). In photosynthetic tissues, carotenoids can be destroyed by photochemical degradation, but in non-photosynthetic tissues the participation of CCOs might be expected to be more relevant for carotenoid turnover. However, this is not always the case. For example, CCD1 and/or CCD4 levels negatively correlate with carotenoid levels in *Chrysanthemum* and orchid flowers (Ohmiya et al., 2006; Chiou et al., 2010), strawberry fruit (Garcia-Limones et al., 2008), maize endosperm (Vallabhaneni et al., 2010), and potato tubers (Campbell et al., 2010), but not in *Ipomea* flowers (Yamamizo et al., 2010), citrus fruit (Kato et al., 2006) or rice endosperm (Ilg et al., 2010). In Arabidopsis seeds, carotenoid levels might be controlled by the rate of their degradation by CCD1, since mutant seeds defective in this enzyme show increased carotenoid levels whereas transgenic seeds overexpressing CCD1 display reduced amounts of these pigments (Auldridge et al., 2006b). By contrast, altered CCD1 levels did not have an impact on leaf carotenoid accumulation despite the fact that the *CCD1* gene is also expressed in this organ (Auldridge et al., 2006b).

CAROTENOID BIOTECHNOLOGY: LESSONS FROM ARABIDOPSIS

As pigments, carotenoids are responsible for the attractive colors of many fruits, vegetables and flowers, contributing to increase the economic value of crop and ornamental plants. In addition, a number of carotenoids are valuable feed and food additives (natural pigments) and they are widely used in the cosmetics and pharmaceutical industries. Furthermore, dietary carotenoids fulfill essential requirements for human and animal nutrition (Fraser and Bramley, 2004). For example, carotenoids serve as precursors of apocarotenoid retinoids such as retinol (vitamin A) and retinoic acid, which play important functions as visual pigments and signaling molecules (Bollag, 1996). The recent discoveries of the health-related properties of carotenoids and the increasing demand for natural products have spurred an unprecedented interest in the biotechnological overproduction of carotenoids in plants. Indeed, the manipulation of carotenoid biosynthesis in plants might contribute to a carotenoid-enriched diet with improved health benefits. Metabolic engineering could also deliver novel plant systems for the production of natural carotenoids of industrial interest. The achievements of carotenoid biotechnology in bacterial and plant systems, as well as the problems faced by current strategies, are reviewed elsewhere (Sandmann, 2001; Lee and Schmidt-Dannert, 2002; Fraser and Bramley, 2004; Botella-Pavía and Rodríguez-Concepción, 2006; Sandmann et al., 2006; Giuliano et al., 2008).

In Arabidopsis, transgene-mediated carotenoid engineering approaches have significantly contributed to our understanding of the roles of these plastidic isoprenoids in plants but they have also revealed some drawbacks associated with altering carotenoid levels in some tissues. For example, the seed-specific overexpression of the endogenous *PSY* gene in Arabidopsis resulted in increased levels of carotenoids (mainly β-carotene, with lower increases in lutein and violaxanthin) but it also led to an enhanced accumulation of ABA, eventually resulting in delayed germination (Lindgren et al., 2003). The increased flux to the carotenoid pathway in these lines was used to boost the production of carotenoid-derived pigments (ketocarotenoids) in the seeds (Stalberg et al., 2003). It is expected that even higher levels of seed carotenoids (and derived products) could be obtained by down-regulating the levels of CCD1, since overexpression approaches also showed that CCD1 activity is an important for carotenoid degradation in Arabidopsis seeds (Auldridge et al., 2006b).

Metabolic engineering of xanthophyll composition in Arabidopsis, together with genetic evidence, has revealed the key role of these compounds in the protection of plants against oxidative stress (Pogson and Rissler, 2000; Rissler and Pogson, 2001; Davison et al., 2002; Johnson et al., 2007; Johnson et al., 2008). Perhaps the most interesting contribution of these approaches from the biotechnological perspective is the discovery that a specific increase of the xanthophyll cycle pool in Arabidopsis by constitutively overexpressing the *BCH1* gene results in plants that are more tolerant not only to high light but also to high temperature (Davison et al., 2002). The higher BCH activity of the transgenic lines resulted in a 2-fold increase in the amount of violaxanthin (expected after the epoxydation of zeaxanthin by the endogenous ZEP activity), without significantly perturbing the concentration of the rest of carotenoids (or chlorophylls). The extra violaxanthin was available for de-epoxidation after exposure of plants to high light and high temperature, resulting in the accumulation of zeaxanthin in transgenic plants at levels up to 4-fold higher than in the wild-type. Stress protection was probably due to the function of zeaxanthin in preventing oxidative damage of membranes (Davison et al., 2002). An increased salt tolerance has also been reported in Arabidopsis lines overexpressing genes for PSY (Han et al., 2008), LCYB (Chen et al., 2011), or ZEP (Park et al., 2008), likely as a result of an enhanced availability of substrates for ABA synthesis. Together, these studies show that genetic manipulation of the carotenoid pathway represents a potentially powerful tool to improve stress-tolerance in plant crops.

Another important lesson learned from carotenoid engineering in Arabidopsis is the key influence of the plastid type on final carotenoid profiles. For example, Arabidopsis lines constitutively overexpressing the MEP pathway enzymes DXS or HDR showed an increased biosynthesis of carotenoids in chloroplast of light-grown seedlings but not in etioplasts of dark-grown seedlings (Botella-Pavía et al., 2004; Rodriguez-Villalon et al., 2009a). By contrast, constitutive *PSY* overexpression led to enhanced carot-

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enoid levels in seed-derived calli and roots but not in photosynthetic shoots (Maass et al., 2009). The latter work additionally confirmed that the sole overexpression of PSY-encoding genes is sufficient to trigger the differentiation of chromoplast-like plastids in Arabidopsis (Maass et al., 2009). Overexpression studies in Arabidopsis have also established the central role of PSY for the control of carotenoid biosynthesis in plants (Lindgren et al., 2003; Maass et al., 2009; Rodriguez-Villalon et al., 2009a).

CONCLUDING REMARKS

Carotenoids probably arose in primitive organisms as lipophilic molecules that served to reinforce membranes, but today they are synthesized and used by a wide diversity of organisms to serve multiple functions. We now have an almost complete picture of the core carotenoid biosynthetic pathway in plants, but much less is known about how the pathway is regulated despite recent advances in this field. It is expected that novel transcription factors controlling the expression of carotenoid biosynthetic genes will be identified in the near future, taking advantage of the increasing availability of molecular and bioinformatic tools in Arabidopsis. However, understanding the regulation of carotenogenesis as a whole (i.e., as a system) is a major challenge that might be much more difficult to tackle. Quantitative technologies and systems biology approaches, which require a good knowledge of network elements and topologies of the metabolic pathways, might contribute to address tantalizing questions on the global regulation of carotenogenesis that remain open (Fraser et al., 2009). In particular, we still know very little about the intrinsic regulatory mechanisms such as feedback inhibition, forward feed, metabolite channeling, and counteractive metabolic and cellular perturbations that appear to be unleashed when the pathway is manipulated in higher plants, preventing to accurately predict the outcome of carotenoid pathway engineering approaches. The network-based quantitative technologies and systems biology tools currently available in Arabidopsis (including AtIPD) might successfully serve to unveil such regulatory mechanisms.

Future research should also be directed to better understand the regulatory connections of the carotenoid pathway with related metabolic routes, specifically with the pathways that produce and consume GGPP. An in-depth analysis of the localization and organization of the pathway at subplastidial levels (i.e., enzyme localization, composition of multi-enzyme complexes, mechanisms for carotenoid transport among different plastid subcompartments) should also be addressed. Because carotenoid levels are determined by biosynthesis but also by degradation and storage capabilities, efforts should also be directed to unveil the mechanisms controlling carotenoid turnover and sequestration and to integrate this information with that related to the regulation of biosynthesis to build a model reflecting the dynamics of carotenoid accumulation in plant cells. Again, the amenability of Arabidopsis to systems biology and network analysis approaches makes this plant an obvious target for such studies. Insights into these regulatory aspects will eventually facilitate knowledge-based directed biotechnological approaches to modify plant carotenoid metabolism for nutritional or industrial improvement of crops.

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