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Biosynthesis and Engineering of Carotenoids and Apocarotenoids in Plants: State of the Art and Future Prospects

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Abstract

Carotenoids and their apocarotenoid derivatives are isoprenoid molecules important for the primary and secondary metabolisms of plants and other living organisms, displaying also key health-related roles in humans and animals. Progress in the knowledge of the carotenoid pathway at the genetic, biochemical and molecular level, supported by successful genetic engineering examples for an increasing number of important plant crops have paved the way for precise molecular breeding of carotenoids. In this review, following a description of the general carotenoid pathway, select examples of plant species able to produce specialty carotenoids and apocarotenoids are illustrated.

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Abbreviations:. **ABA, abscisic acid; ADH, aldehyde dehydrogenase; BADH, bixin ADH; BETA, chromo**plast-specific lycopene β-cyclase; BMT, norBixin methyltransferase; CCD, carotenoid cleavage dioxygenase; CCS, capsanthin/capsorubin synthase; CHY, non-heme carotene hydroxylase; CMK, 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol kinase; CrtB, bacterial phytoene synthase; CrtI, bacterial carotenoid desaturase/isomerase; CRTISO, carotenoid isomerase; CrtW, bacterial carotene ketolase; CrtY, bacterial lycopene β-cyclase; CrtZ, bacterial CHY; CYP97A and CYP97C, cytochrome P450 carotene hydroxylases; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; HDS, 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; LCY-b, lycopene β-cyclase; LCY-e, lycopene ε-cyclase; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; MDS, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MVA, mevalonic acid / mevalonate; MVAP, mevalonate-5-phosphate; MVAPP, mevalonate-5-pyrophosphate; NXS, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZCD, zeaxanthin-specific cleavage dioxygenase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase; Z-ISO, ζ-carotene isomerase.

An update on plant carotenoid engineering is also provided for non-solanaceous crops and members of the Solanaceae family, by means of different strategies and making use of plant and bacterial genes.

Introduction

The visual properties and health benefits of carotenoid pigments have fostered fundamental research and industrial applications for decades. Carotenoids are isoprenoid molecules synthesized by plants as well as by bacteria, fungi, yeast and algae. In plants, the presence of carotenoids is mandatory for photosynthesis, and determines the pigmentation of flower organs, fruits and seeds in many important crops. Carotenoids cover a wide range of functions in plants and animals such as energy transfer and protection against photooxidative damage during photosynthesis, attraction of insects and animals for pollination and fruit/seed dispersal, contribution to plant cross-talk with pathogens, pests, and symbiotic organisms, reduction of degenerative disease risks and prevention of cardiovascular accidents, and provide a source of pro-vitamin A (Gann *et al*., 1999; Hirschberg, 2001 and references therein). To date, hundreds of carotenoid and apocarotenoid derivatives have been described (Britton *et al*., 2003; Winterhalter and Rouseff, 2001). This figure is most likely to increase owing to the continuous progress in analytical technologies and the advances in the knowledge of the pathway.

The existing scientific literature on the carotenoid pathway is vast and exceeds the scope of the present article, which will focus on the most recent publications after the latest reviews (Giuliano *et al*., 2008; Fraser *et al*., 2009) and on the exploitation of plant systems for the spontaneous or engineered production of high-value carotenoid and apocarotenoid compounds.

The carotenoid pathway in plants

CAROTENOID BIOSYNTHESIS

Plant carotenoids are isoprenoid-derived molecules generally synthesized and located in plastids, and their production is driven by nuclear-encoded enzymes (Hirschberg, 2001). In plants, isoprenoids are produced from the distinct but cross-talking cytosolic mevalonate (MVA) and plastidial 2-*C*-methyl-D-erythritol 4-phosphate (MEP) routes, to give rise to isopentenyl phosphate (IPP), the C5 building block for the synthesis of carotenoids and other compounds (*Figure 1*). Phytoene, the first carotenoid, is a C40 molecule synthesized from the condensation of two C20 geranylgeranyl diphosphate units by phytoene synthase (PSY). Subsequent desaturation steps, catalyzed by the phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) enzymes, take to the synthesis of *cis*-lycopene, which is converted into *all-trans*-lycopene either by the carotenoid isomerase (CRTISO) enzyme in non-photosynthetic tissues or spontaneously by light in chlorophyll-containing ones (Giuliano *et al*., 2002; Isaacson *et al*., 2004; Breitenbach and Sandmann, 2005). A recent study characterized the *Y9* locus of maize to code for a ζ-carotene isomerase (Z-ISO), and put forward the existence of such an activity across higher plants (Li *et al*., 2007). The pathway then bifurcates

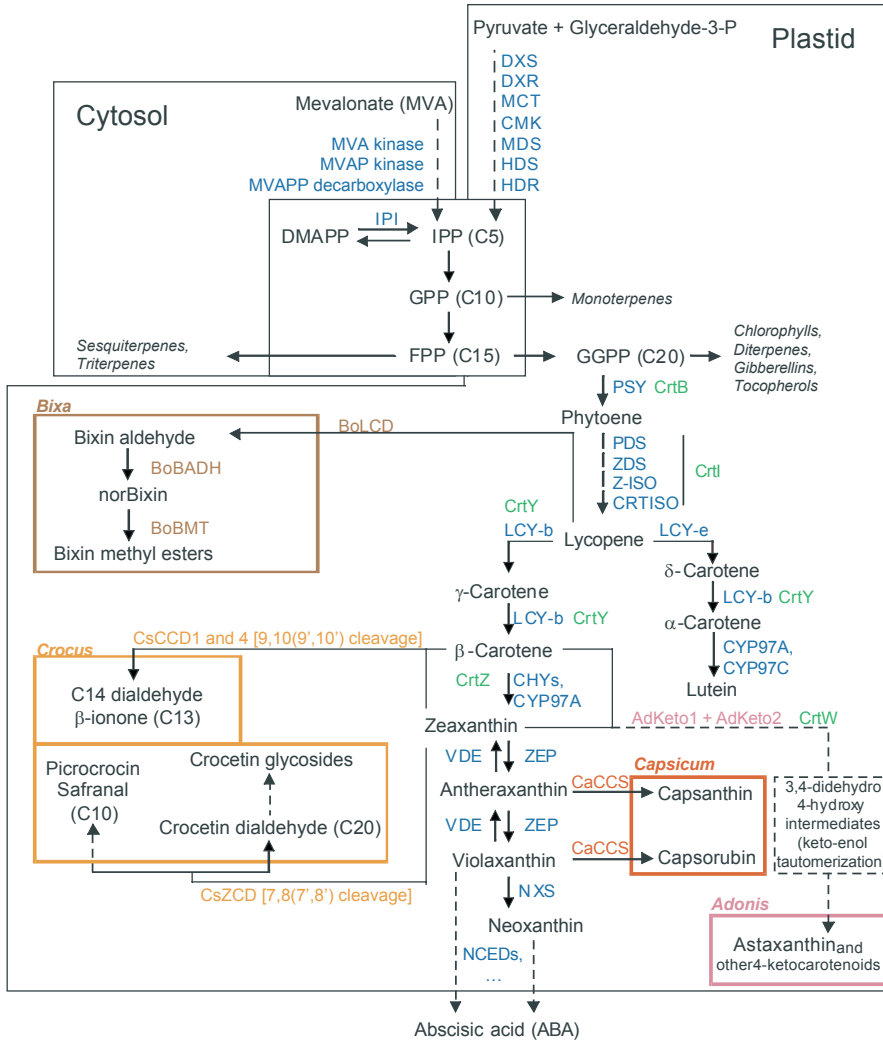

Figure 1. Simplified isoprenoid and carotenoid pathways. Names of plant enzymes are in blue, those of bacterial orthologs in green, while those of plants able to produce particular carotenoids and apocarotenoids are highlighted in brown (*Bixa orellana*), orange (*Crocus sativus*), red (*Capsicum annuum*) and magenta (*Adonis aestivalis*). Steps involving multiple enzymes are indicated with dashed arrows.

into two branches through the action of lycopene β- (LCY-b and chromoplast-specific BETA; Ronen *et al*., 2000) and ε- (LCY-e; Cunningham and Gantt, 2001) cyclases. Two kinds of carotene hydroxylases – cytochrome P450 (CYP97A and C) and non-heme (CHY) enzymes – hydroxylate the β- and ε-rings to produce xanthophylls (DellaPenna and Pogson, 2006). The β-ε-ring lutein is the most abundant natural carotenoid present in photosynthetic tissues, while zeaxanthin is the first xanthophyll of the β-β-ring branch leading to the synthesis of abscisic acid (ABA). The xanthophyll cycle enzymes zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE) regulate the amounts of such compounds during day and night: levels of violaxanthin are high

at night, while those of zeaxanthin, a better quencher of singlet chlorophyll as well as a ROS scavenger, increase during the day when low lumen pH conditions induce the VDE enzyme activity. The late biosynthetic steps to neoxanthin and ABA have to be fully clarified yet. Neoxanthin synthase (NXS) genes, encoding paralogs of BETA β-cyclase in tomato, have been identified to date only in tomato and potato (Al-Babili *et al*., 2000; Bouvier *et al*., 2000). Nevertheless, tomato *beta* mutants are not impaired in neoxanthin biosythesis (Ronen *et al*., 2000). *Arabidopsis* is able to produce ABA, although it lacks a *BETA* ortholog in its genome. The AtABA4 protein is directly involved in neoxanthin biosynthesis, but analysis of *ABA4* mutants and overexpressors could not clarify whether it coincides with NXS or it is part of a NXS multienzyme complex (North *et al*., 2007). The possible multiple routes to ABA formation, involving not only enzymatic cleavage (see below), also await further clarification. Last but not least, the observed redundancy of carotenoid ortholog and paralog genes/enzymes is critical for the spatial and developmental regulation of carotenogenesis among and within species (e.g., Galpaz *et al*., 2006; Li *et al*., 2008).

CAROTENOID–SPECIFIC CLEAVAGE DIOXYGENASES PRODUCE APOCAROTENOID VOLATILES AND ABA PRECURSORS

Plants emit volatile organic compounds (VOCs) with crucial roles in plant-environment crosstalk, e.g. attraction of pollinators, herbivore deterrence and defense against pathogens. The structure similarity of some flower and spice isoprenoid VOCs (e.g., β-ionone and safranal) to carotenoids recently led to the discovery of carotenoid-specific cleavage dioxygenases (CCDs), which belong to a multienzyme family counting nine members in *Arabidopsis* (Tan *et al*., 2003). Biochemical characterization established that most CCDs have wide substrate promiscuity, but are highly specific for the position of the double bond that they cleave (reviewed by Auldridge *et al*., 2006). CCDs also differ for their subcellular localization: some, like CCD1s, are predicted to be cytosolic; others possess transit peptides for plastid or plastoglobule targeting (e.g., Tan *et al*., 2003; Rubio *et al*., 2008). CCDs include 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), which cleave epoxy-xanthophylls to produce xanthoxin, a precursor of ABA (Schwartz *et al*., 1997). Among CCDs, CCD7 and CCD8 are strikingly divergent: they cleave carotenoids and apocarotenoids asymmetrically, and were shown to be involved in the synthesis of a hormone inhibiting lateral branching, as confirmed by selective chemical inhibition, as well as controlling leaf senescence, root growth and flower development (Schwartz *et al*., 2004; Snowden *et al*., 2005; Sergeant *et al*., 2009). CCD genes and enzymes have been characterized in a growing number of species, including fruit, vegetable, flower and field crops (e.g., Simkin *et al*., 2004a and 2004b; Ibdah *et al*., 2006; Garcia-Limones *et al*., 2008; Huang *et al*., 2009; Ilg *et al*., 2009). Because of their economic value, the CCD-derived apocarotenoid products of *Crocus sativus* and *Bixa orellana* will be dealt more in detail in the following section.

Selected plant species produce specialty carotenoids and apocarotenoids

ACHIOTE (*BIXA ORELLANA* L.)

Annatto (bixin; European colorant code E160b) is a pigment extracted from achiote (*Bixa orellana*) seeds, used in food and cosmetic industry. Structure similarity be-

tween bixin and the *Crocus* pigment crocin, as well as the mixture of bixin aldehyde, carboxylated and methylated forms in annatto, indicated the possible involvement of CCD, aldehyde dehydrogenase (ADH) and methyltransferase enzymes in the minipathway (*Figure 1*). The corresponding genes were isolated from a seed cDNA library of *B. orellana* and characterized (Bouvier *et al*., 2003a). The lycopene cleavage dioxygenase (BoLCD) is peculiar in that it cleaves lycopene at the 5,6(5',6') double bonds, but neither β-carotene nor zeaxanthin substrates. The bixin ADH (BoBADH) clustered with cytosolic ADHs, consistently with its putative localization, while the (nor)bixin MT (BonBMT) shared similar function (methylation of carboxyl groups) with jasmonic and salicylic acid MTs (Giuliano *et al*., 2003). Expression of the *Bixa* minipathway enzymes in tomato might represent a biotechnological alternative for annatto production.

SAFFRON CROCUS (*CROCUS SATIVUS* L.)

Saffron is an expensive spice obtained from the dried styles of saffron crocus (*Crocus sativus*), whose distinctive flavor and color are due to picrocrocin, safranal and crocetin-derived apocarotenoids. The biosynthesis starts from the cleavage of zeaxanthin, to produce cyclic carotenoid VOCs (picrocrocin and safranal) and crocetin, which is eventually glycosylated to crocin (*Figure 1*). Some of the genes and enzymes involved in these steps have been studied. The *Crocus* CCDs so far characterized are similar to CCD1 and CCD4 enzymes, are differentially expressed in flower organs and CCD4s only contain predicted transit peptides for plastid (plastoglobule) localization (Rubio *et al*., 2008). The CCD1-like generic CsCCDs possess 9,10 (9',10') cleavage activity on various carotenoid substrates (Bouvier *et al*., 2003b; Rubio *et al*., 2008). CCD4-like CsCCD4a and CsCCD4b proteins (Rubio *et al*., 2008) are very similar (98-100% homology) to the CsZCD enzyme, previously reported to cleave zeaxanthin at the 7,8(7',8') positions and giving rise to crocetin dialdehyde (Bouvier et al., 2003b). CsCCD4 enzymes are longer than CsZCD and carry a plastid transit peptide. They perform a 9,10(9',10') cleavage, and are also able to cleave zeaxanthin, although the expected apocarotenoids could not be detected by neither LC nor GC (Rubio *et al*., 2008). Further investigation is needed to clarify the exact number, protein structure, and enzymatic activity of *Crocus* CCD4/ZCD enzymes. Besides apocarotenoid volatiles, water-soluble crocetin glycosides are presumed to accumulate in vacuoles to confer pigmentation. A UDP-glucose crocetin 8-8'-glycosyltransferase enzyme was purified from cell suspensions and characterized (Côté *et al*., 2000), and the product of the stigma-expressed *UGTCs2* gene was shown to glucosylate crocetin aglycones and glycosides *in vitro* (Rubio Moraga *et al*., 2004).

PHEASANT'S EYE (*ADONIS SPP*.)

The ability to synthesize ketocarotenoids is usually exclusive in some bacteria and marine organisms. Few plants are known to produce ketocarotenoids, but only two of them accumulate these pigments at high levels: *Adonis aestivalis* and *Adonis annua*, whose ketocarotenoid content reaches 1% of dry weight in flower petals of the latter (Renstrøm *et* al., 1981). Astaxanthin, the high-value ketocarotenoid widely used as

a feed supplement in aquaculture and poultry farming, is the major ketocarotenoid accumulated in *Adonis* flowers: *AdKeto* genes involved in ketocarotenoid formation have been isolated and characterized (Cunningham and Gantt, 2005). Interestingly, both AdKeto1 and AdKeto2 enzymes display high identity (>90%) to CHY enzymes, but, contrary to the latter, do not possess 3-hydroxylase activity, nor directly synthesize 4-keto carotenoids. Surprisingly, the Keto enzymes were shown to act as either 3,4-desaturase or 4-hydroxylase: in this context, a keto-enol tautomerization mechanism would rather be responsible for ketocarotenoid synthesis. More recently, a novel approach based on the use of several carotenoid-producing *E. coli* strains has been developed in *Adonis aestivalis* to characterize carotenoid and isoprenoid biosynthesis enzymes (Cunningham and Gantt, 2007).

PEPPERS (*CAPSICUM SPP.*)

Pepper and its hot chili varieties have long been cultivated and widely used as food and additives, also owing to the production of several peculiar carotenoids. Capsanthin, capsorubin and capsanthin 5,6-epoxide are the red carotenoids exclusively accumulating in *Capsicum spp*. fruits (Deli *et al.*, 2001). The gene encoding capsanthin-capsorubin synthase (CCS), involved in the synthesis of these pigments, has been isolated and characterized: CCS is a bifunctional cyclase enzyme able to use both antheraxanthin and violaxanthin as precursors and is specifically expressed during chromoplast development in fruits accumulating ketocarotenoids (Bouvier *et al*., 1994). Pepper varieties can be classified according to fruit color: the two main isochromic families include red varieties, synthesizing capsanthin and capsorubin pigments, and yellow ones accumulate a carotenoid pool comprising antheraxanthin, violaxanthin (the precursors of capsanthin and capsorubin) as well as zeaxanthin, α-cryptoxanthin, α-carotene, and cucurbitaxanthin A (Guil-Guerrero *et al*., 2006).

Genetically, three independent loci (*y*, *c1*, and *c2*) have been proposed to control fruit pigmentation. The *Ccs* gene was determined to be tightly associated with or to be encoded by the *y* locus, and yellow fruit phenotypes suggested to result from deletions of this gene (Lefebvre *et al*., 1998 and references therein). A more complex scenario concerns orange-fruited varieties, with two possible genotypes that are responsible for this colour (Popovsky and Paran, 2000). From the molecular and biochemical viewpoint, red-fruited varieties display high *Ccs* and other carotenoid gene transcript levels, and high red-to-yellow (*R/Y*) isochromic pigment fraction and capsanthin-to-zeaxanthin (Caps/Zeax) ratios. Such ratios are very important to select varieties for paprika production, and are inversely related to the total carotenoid content of pepper fruit (Hornero-Méndez *et al*., 2000). In contrast, yellow varieties are generally characterized by low carotenoid gene transcript levels, reduced carotenoid content, and absence, impaired expression or mutations of the *Ccs* gene (Bouvier *et al*., 1994; Popovsky and Paran, 2000; Ha *et al*., 2007). Recently, the analysis of yellow-fruited varieties, containing *Ccs* genes with premature stop-codons or frame-shifts in their coding sequences, indicated that post-transcriptional silencing mechanisms should also be taken into account in the determination of fruit yellow color (Ha *et al*., 2007).

Engineering carotenoids and apocarotenoids in plants

Because of their pro-vitamin A and antioxidant properties, carotenoid biofortification of major food crops *via* either conventional or molecular breeding is an important issue especially in developing countries, where vitamin A deficiency (VAD) poses serious public health concerns and is responsible for various diseases and mortality (Al-Babili and Beyer, 2005 and references therein). For two of the three major cereal crops (in decreasing order of production: maize, rice and wheat), successful carotenoid engineering has been achieved: by *de novo* synthesis in the case of rice, or by increasing/optimizing the carotenoid profile in maize (see below). Novel approaches combining genetic, molecular and bioinformatics analyses open the way to marker-assisted breeding for improved carotenoid content in maize and wheat (Harjes *et al*., 2008; Singh *et al*., 2009; Vallabhaneni and Wurtzel, 2009). The Solanaceae family has been the target of genetic engineering since it includes economically important crops, among which tobacco, tomato and potato are easily amenable to transformation and have been engineered for carotenoid content with diverse constructs and strategies. Solanaceous crops are also being targeted by the SOL genomics network (http://sgn.cornell.edu/), which is expected to unravel the complexity of genomic information in the coming years and to provide new molecular tools for crop breeding. Other major crops engineered for carotenoid production are canola and carrot. Altogether, the six mentioned engineered crops (rice, maize, tomato, potato, canola and carrot) account for almost two billion tons production and 370 million hectares surface worldwide (*Table 1*). These data demonstrate how plant-based carotenoid molecular breeding could provide economic advantages and be a feasible alternative to chemical synthesis.

Table 1. World 2007 production and area harvested data of the six major crops already engineered for carotenoid content (Source: FAO Statistics; http://faostat.fao.org).

	Production (million metric tons)	Surface (million hectares)
Maize	785	157.8
Rice	651	157.0
Potatoes	321	19.3
Tomatoes	126	4.6
Rapeseed	50	30.2
Carrots[a]	27	1.2
TOTAL	1960	370.1

[a] includes turnips

Plant carotenoids have been successfully engineered with either plant or bacterial genes – or combinations of genes from the two sources. Because they display some particular features, bacterial genes have been used to engineer both early (phytoene synthesis, desaturation and isomerization) and late (lycopene cyclization, ketocarotenoid biosynthesis) biosynthetic steps and deserve a particular mention. Engineering of early steps using the *CrtB* and/or *CrtI* genes, encoding respectively PSY and PDS (*Figure 1*), has been obtained in tobacco leaves, tomato fruits, potato tubers,

and seeds of canola, flax, maize and rice (for details and references, see specific sections below). Overexpression of *CrtB* results, depending on the plant species, in an increase of phytoene as well as of compounds further downstream: β- and α-carotene and lutein. Overexpression of *CrtI* results, rather than in an increase of its product lycopene, in a re-direction of synthesis towards the beta-branch (Misawa *et al*., 1994; Römer *et al*., 2000).

Carotenoid biosynthesis enzymes of bacterial origin lack a transit peptide, and when expressed from the nucleus must be fused to an artificial transit peptide, usually derived from the *RbcS* gene, for correct localization in the plastid compartment (Ye *et al*., 2000; Fraser *et al*., 2002; Ducreux *et al*., 2005; Diretto *et al*., 2007a). Alternatively, bacterial enzymes can be expressed directly from the plastid genome. A lycopene cyclase from *Erwinia* has been expressed from the tomato plastid genome, resulting in an elevation of fruit β-carotene levels (Wurbs *et al*., 2007). The polycistronic nature of the plastid transcripts allows also the expression of multiple genes under the control of a single promoter sequence. The *Brevundimonas CrtZ* and *CrtW* genes, encoding respectively β-carotene hydroxylase and ketolase enzymes, have been expressed as a single operon under the control of a single tobacco plastid promoter, for astaxanthin production in tobacco leaves (Hasunuma *et al*., 2008).

Another possibility is the overexpression of bacterial mini-pathways from the nuclear genome. The *CrtI* gene from *Erwinia* is often used in these mini-pathways, since it contains, in a single enzyme, activities corresponding to plant PDS, ZDS, CrtISO and, possibly, Z-ISO (*Figure 1*). This multifunctional bacterial enzyme has been used, in metabolic engineering efforts, to surrogate the plant enzymes in the phytoene desaturation/isomerization pathway (Misawa *et al*., 1994; Ye *et al*., 2000; Paine *et al*., 2005; Römer *et al*., 2000; Ravanello *et al*., 2003; Diretto *et al*., 2007a). As a consequence, we know very little on the consequences of the overexpression of the latter enzymes. Examples of mini-pathway expression are the *PSY-CrtI-CrtY* minipathway overexpressed in rice (Ye *et al*., 2000), and the *CrtB-CrtI-CrtY* minipathway overepxressed in canola and potato (Ravanello *et al*., 2003; Diretto *et al*., 2007a). In some cases, *CrtY*, which encodes a lycopene β-cyclase, has been shown to be dispensable for β-carotene biosynthesis, probably because the endogenous LCY-b enzymes are sufficiently expressed. In an engineering *tour de force*, seven genes involved in ketocarotenoid formation from three different bacterial genera were introduced into canola (Fujisawa *et al*., 2009). Recent combinatorial approaches, based on biolistic transformation and introducing up to five of plant and bacterial gene constructs of interest, produced repertoires of plants with several transgene combinations in maize, showing the potential for pathway investigation and vitamin biofortification (Zhu *et al*., 2008; Naqvi *et al*., 2009).

NON-SOLANACEOUS PLANTS

RICE (ORYZA SATIVA)

Rice dehusked grain is devoid of carotenoids. Overexpression of a *PSY* gene alone produced the accumulation of phytoene but not that of downstream carotenoids (Burkhardt *et al*., 1997). One of the major biotechnological breakthroughs of the last decade has been the molecular breeding of golden rice (GR) in both japonica and

indica backgrounds, whose grain accumulate β-carotene (pro-vitamin A) through the expression of a minipathway composed of a plant *PSY* and a bacterial desaturase/isomerase *CrtI* under different promoters (Ye *et al*., 2000; Datta *et al*., 2003; Hoa *et al*., 2003). The synthesis of β-carotene and xanthophylls was made possible likely by the sufficient expression of endogenous carotene cyclase and hydroxylase genes in the endosperm (Schaub *et al*., 2005). In second-generation GR (GR2), the use of a maize optimized *PSY* coding sequence increased up to 17-fold and 23-fold the synthesis of β-carotene and total carotenoids, respectively (Paine *et al*., 2005). Such levels allow to efficiently fight VAD with diet-compatible amounts of GR2 (Giuliano *et al*., 2008).

MAIZE (ZEA MAYS)

Maize kernels naturally contain β-carotene and other xanthophylls, albeit at levels usually insufficient to cover pro-vitamin A recommended daily allowance (RDA) values. As an alternative to conventional breeding, transgenic maize plants containing the bacterial *CrtB* and *CrtI* genes, controlled by either "normal" or "super" γ-zein promoters, showed increased total carotenoid levels (especially β-carotene) up to 34-fold, contributing the first transgenic maize designed to alleviate VAD in third world countries (Aluru *et al*., 2008). Two recent works showed the dramatic combinatorial potential of biolistic transformation for fast production of repertoires of multitransgenic plants in maize biofortified in carotenoids and vitamins. In the first work, Zhu *et al*. (2008) introduced up to five carotenoid genes (plant *Psy1*, *Lcy*-b and *Chy*; bacterial *CrtI* and *CrtW*), each with a different endosperm-specific promoter, to produce an array of transformants with different combinations of transgenes. Transgenic kernels displayed several phenotypes consistent with the combinations of transgenes integrated. In the second work, Naqvi *et al*. (2009) transformed genes for three independent vitamin pathways (carotenoid, ascorbate and folate) to produce transformants with increased β-carotene (169-fold), ascorbic acid (sixfold) and folate (twofold).

CANOLA (BRASSICA NAPUS)

Overexpression of a bacterial *CrtB* (*PSY*) gene in oilseed rape directed by a seed-specific napin promoter and a plastid target peptide was sufficient to increase total carotenoids 50-fold, mostly α- and β-carotene (Shewmaker *et al*., 1999). Overexpression of *CrtB* with different combinations of other genes (*CrtI, CrtY* and a plant LCY-b) increased β-carotene levels and β- to α-carotene ratios, suggesting the existence of carotenoid multienzyme complexes (Ravanello *et al*., 2003). Downregulation of a LCY-e diverted the carotenoid flux and increased not only the content of β-carotene and β-β xanthophylls, but unexpectedly also that of lutein (Yu *et al*., 2008). More recently, transformation of seven genes for ketocarotenoid biosynthesis from *Erwinia*, *Brevundimonas* and *Paracoccus*, each with its own promoter, terminator and transit peptide, was achieved in canola. Approx. 80% of the regenerated plants retained all seven genes, and formed orange- or pinkish orange-colored seeds. The total amount of carotenoids increased up to 30-fold, with β-carotene and ketocarotenoids making up a significant proportion (Fujisawa *et al*., 2009).

CARROT (DAUCUS CAROTA)

Despite being a traditional and rich source of carotenoids in the diet, molecular research on carrot carotenoid pathway was reported only recently (Just *et al*., 2007). Besides orange-rooted types, carrot germplasm display purple, red, yellow and white root color phenotypes, combinations of them in root tissues, and tissue-specific accumulation of the different carotenoids (Surles *et al*., 2004; Baranska *et al*., 2006). Ketocarotenoid engineering was accomplished by overexpressing an algal β-carotene ketolase *CrtO* gene in an orange-rooted genotype using three different promoters (Jayarai *et al*., 2008). Transgenic roots produced several β-ketocarotenoids but not ketolutein, in quantities as high as 70% the total carotenoid pool (up to 2.4 mg/g root dry weight). Transformants were also more resistant to UV-B radiation and oxidative stress than wt ones (Jayarai and Punja, 2008).

ARABIDOPSIS (ARABIDOPSIS THALIANA)

Arabidopsis has been used in fundamental and proof-of-concept studies, also for carotenoid engineering. Carotenoid engineering in *Arabidopsis* seeds was achieved by up-regulation of an endogenous *PSY* gene, which increased the total carotenoid levels, namely β-carotene, lutein and violaxanthin (Lindgren *et al*., 2003). Seeds also had increased chlorophyll and ABA content, which delayed germination. Comparison of *Arabidopsis* with canola and flax seed-targeted transgenic systems, using different promoters and *PSY* genes, revealed differences in metabolite flux. In *Arabidopsis*, the use of an endogenous *AtPSY* coding sequence directed the flux towards xanthophyll end products lutein and violaxanthin. In the other two systems, bacterial *CrtB* genes preferentially increased the levels of α- and β-carotene (Shewmaker *et al*., 1999; Lindgren *et al*., 2003; Fujisawa *et al*., 2008). Transformed *Arabidopsis* plants more resistant to elevated light and temperature conditions were produced by up-regulating an endogenous *AtCHY* hydroxylase (Davison *et al*., 2002). This increased two-fold the xanthophyll cycle pool and levels of zeaxanthin, which has a photoprotective role in photosynthesis. *De novo* synthesis of ketocarotenoids in *Arabidopsis* seeds was brought by the expression of an oxygenase (ketolase) gene from *Haematococcus* (Stålberg *et al*., 2003). Crossing with *PSY*-overexpressing lines increased total carotenoid levels by almost 5-fold and those of major ketocarotenoids 13-fold, demonstrating the pivotal role of PSY in the governing the metabolite flux in seeds.

SOLANACEAE CROPS

TOMATO (SOLANUM LYCOPERSICUM)

Tomato is the best-investigated species within Solanaceae family, due to its importance as food crop and the nutritional value of fruits accumulating lycopene. Parallel holistic approaches are currently in progress to sequence the gene-rich region of its genome (Mueller *et al*., 2009) and characterize the berry ripening process with transcriptomic (Alba *et* al., 2004), proteomic (Rocco *et* al., 2006) and metabolomic (Moco *et* al., 2006) approaches.

Carotenoid studies in tomato fruits, both using both transgenic plants and natural mutants, have been carried out on several isoprenoid and carotenoid pathway steps. Within the former, tomato transgenic plants overexpressing a plant 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) showed no perturbation of the pathway, while those up-regulated for a bacterial 1-deoxy-D-xylulose 5-phosphate synthase (DXS) had enhanced phytoene and β-carotene contents (Enfissi *et al*., 2005).

In the latter, PSY is the key step in carotenoid biosynthesis, whose modification affects also other pathways. For these reasons, PSY has been extensively investigated in tomato: the first example is the antisense silencing of *PSY1* (preferentially expressed in reproductive organs), resulting in a great reduction in flower and fruit carotenogenesis (Bird *et al*., 1991). In an opposite trial, overexpression of the same gene in a constitutive fashion produced fruits with earlier lycopene production along development, but showing a lower content of this metabolite at maturity (Fray *et al*., 1995). Interestingly, these plants displayed several pleiotropic effects, including albino fruit production, and reduced plant height and chlorophyll content; these phenotypes find an explanation in both endogenous *PSY1* gene silencing and decrease in gibberellin (GA) content, derived from the competition of the two pathways for the geranylgeranyl diphosphate pool. Fruit-specific expression of a bacterial *PSY* gene (*CrtB* from *Erwinia*) produced fruits with higher phytoene, β-carotene and total carotenoid levels, but did not increase lycopene content (Fraser *et al*., 2002). In a more recent attempt, it has been shown that overexpression of endogenous *PSY1* is able to perturb not only carotenoid gene transcripts and metabolites, but also plastid type and development and the accumulation of several metabolites (Fraser *et al*., 2007).

Subsequent carotenoid biosynthetic steps have also been altered through the ectopic expression of a bacterial *CrtI* gene: unexpectedly, β-carotene and xanthophyll levels in transgenic fruits increased (phenotype partially explained by endogenous *LCY-b* up-regulation), while lycopene and total carotenoids were unchanged (Römer *el al*., 2000). Large increases in fruit β-carotene and total carotenoids were achieved manipulating expression of lycopene β-cyclase genes: overexpressing *Arabidopsis*/tomato *LCY-b* genes under the control of chromoplast-specific promoters resulted in higher β-carotene level up to 7- (Rosati *et al*., 2000) and 32-fold (D'Ambrosio *et al*., 2004), respectively. In a more recent study, an *Erwinia CrtY* gene was expressed at the chloroplast level leading to a maximum β-carotene accumulation of 4-fold in transplastomic fruits (Wurbs *et al*., 2007). With a different aim, *LCY-b* was also silenced using an antisense approach in order to obtain, as expected, higher lycopene levels in fruits (Rosati *et al*., 2000).

Tomato fruit has also been investigated as potential target for collecting carotenoid down-stream compounds. Simultaneous and fruit-specific overexpression of *Arabidopsis LCY-b* and pepper *CHY* increased both β-carotene and total xanthophylls, namely zeaxanthin and β-cryptoxanthin, virtually absent in wild-type fruits (Dharmapuri *et al*., 2002). In another attempt, the *CrtW* and *CrtZ* genes from *Paracoccus* encoding, respectively, a 4,4' ketolase and a 3,3' hydroxylase, were assembled as a polyprotein and expressed in a constitutive manner: transgenic leaves accumulated ketocarotenoids, including canthaxanthin, astaxanthin and ketozeaxanthin, while no alteration in ketocarotenoid patterns was revealed in tomato fruits (Ralley *et al*., 2004).

Unintended lycopene accumulation has also been obtained as "secondary effect" in different studies: ectopic expression of pepper fibrillin gene in tomato fruits did not

lead to fibril or fibril-like arising but, contrary to the expectations, total carotenoids and volatiles, lycopene and lycopene-derived apocarotenoids and, in a slighter way, β-carotene increased in transgenic fruits (Simkin *et al*., 2007). The effects derived by the perturbation of pathways not apparently linked to carotenoid biosynthesis are intriguing: for example, modification of polyamine pathway, by the expression of a yeast S-adenosylmethionine decarboxylase (*ySAMdc*) gene (Mehta *et al*., 2002) led to an increase in lycopene content up to 2-fold. A strong cross-link between carotenoid biosynthesis and light perception and signaling has been shown: silencing the expression of *LeDet1* and *LeCOP1LIKE* photomorphogenic repressors (Davuluri *et al*., 2005) or overexpressing the blue light photoreceptor cryptochrome 2 (CRY2) (Giliberto *et al*., 2005) resulted in an enhanced lycopene level, with a maximum of 2-fold with respect to the control. An opposite phenotype (decrease of lycopene content) was reported through silencing of *LeHY5* gene (Liu *et al*., 2004). Very recently, the overexpression of a grape MYB-type transcription factor (TF), producing an increase in fruit β-carotene content, has opened the way for carotenoid engineering through the use of TFs (Mahjoub *et al*., 2009).

An interesting approach in metabolic engineering of carotenoids is represented by the opportunity to modify the apocarotenoid pattern in tomato fruits: antisense down-regulation of the ripening-induced *LeCCD1b* has been shown to reduce the production of both β-ionone (derived from cleavage of α- and β-carotene) and geranylacetone (derived from cleavage of lycopene precursors) in fruits (Simkin *et al.*, 2004a). In a different study, diversion of apocarotenoid and volatile profiles has been obtained expressing, in a fruit-specific fashion, the geraniol synthase gene (*GES*) from basil (Davidovich-Rikanati *et al*., 2007): transgenic fruits showed an increase of monoterpenes (geraniol, nerol, citronellol and citronellic acid) at the expense of lycopene content. These changes deeply altered the flavor of transgenic fruits over control ones.

POTATO (SOLANUM TUBEROSUM)

Potato is the fourth worldwide crop for production and consumption: its tubers are rich in minerals/micronutrients and vitamin C, but not in pro-vitamin A (Brown, 2005); moreover, and unfortunately, there is no available source of β-carotene within potato germplasm, including both current commercial potato cultivars and wild species (*Solanum phureja*, one of the few yellow-flesh wild species, show a high content in total carotenoids, but not in β-carotene) (Morris *et al.*, 2004).

Several attempts have been carried out during last decade to increase the carotenoid potential in potato. Basically, these studies pointed on either "push" or "block" strategies, respectively through the overexpression of heterologous constructs or silencing of endogenous genes. As to "push" approaches, similarly to tomato, constitutive or tuber-specific expression of bacterial genes involved in early steps in the isoprenoid/carotenoid pathway has been attempted: overexpression of a bacterial *DXS* caused an increase in phytoene levels, causing enhanced total carotenoid content, and an early sprouting phenotype, probably derived from concurrent enhanced levels of *trans*-zeatin riboside in tubers (Morris *et al*., 2006b). In a different study, a bacterial phytoene synthase gene (*CrtB*) under the control of *patatin* promoter strongly increased β-carotene and total carotenoid levels in *S. tuberosum* and *S. phureja* tubers (Ducreux *et al*., 2005). Interestingly, the accumulation of these metabolites is more

pronounced in *S. tuberosum* than in *S. phureja* tubers, probably due to the fact that the endogenous carotenoid genes of the latter species are expressed at high levels. In a more extensive approach, a systematic investigation on modulation of potato leaf and tuber carotenogenesis has been performed using three bacterial genes (*CrtB*, phytoene desaturase *CrtI* and lycopene β-cyclase *CrtY*) expressed using different gene/promoter combinations (Diretto *et al*., 2007a). Leaves of all transgenic lines displayed, in an additive way, a strong interference on endogenous carotenogenesis, both at transcript and metabolite levels. Quite the opposite, tuber total carotenoid content increased in all the transgenic plants, but only the simultaneous expression of all three genes in a tuber-specific manner led to a 20-fold increase in transgenic tubers. Furthermore, these tubers showed a great enhancement of β-carotene up to 3600-fold (47μg/gm of dry weight), corresponding to the highest content ever reported for any of the four major crops. These tubers showed a deep-yellow (*Golden*) flesh and also accumulated higher xanthophylls levels, as well as phytoene and α-carotene, which are normally undetected in wild-type tubers.

Within "block" approaches, silencing of genes involved in both ε-β- and β-β- carotenoid branching has been described. Reducing *LCY-e* (Diretto *et* al., 2006) or *CHY* (Diretto *et* al., 2007b) expression in a tuber-specific way increased β-carotene and total carotenoid levels, albeit to a smaller extent than in "*Golden*" tubers. Contrary to expectations, silencing of *ZEP* gene resulted in a dramatic increase in zeaxanthin and total carotenoids, but not in β-carotene (Römer *et* al., 2002). Recently, it has been reported that zeaxanthin from such transgenic tubers is bioavailable to humans. Following ingestion of *ZEP*-silenced tubers, the zeaxanthin concentration incorporated into chylomicrons, the lipoprotein bodies that transport hydrophobic compounds through the body, was significantly increased (Bub *et* al., 2008).

A third and more recent approach is the "sink" strategy, through the ectopic expression of plastid-associated proteins able to increase the total carotenoid pool: a notable example of this kind of manipulation is the overexpression of the *Orange (Or)* locus from cauliflower (Lu *et al*, 2006) which produced yellow-fleshed tubers with dramatically high levels of β-carotene (up to 1600-fold) and total carotenoids (up to 7-fold) with respect to the control. Interestingly, *Or* tubers were characterized by several novel features: they synthesize, *de novo*, phytoene, phytofluene and ζ-carotene, and chromoplasts containing carotenoid-sequestering structures were observed; moreover, as positive trait, they continued to enhance β-carotene and total carotenoids during cold-storage (Lopez *et al*., 2008) according to a not yet elucidated mechanism.

Potato tubers have also been engineered to produce high-value ketocarotenoids. Gerjets and Sandmann (2006) constitutively expressed a β-carotene ketolase gene from *Synechocystis* in *ZEP*-silenced tubers to synthesize ketocarotenoids in both leaves (echinenone, hydroxyechinenone and ketozeaxanthin) and tubers (hydroxyechinenone, ketozeaxanthin and astaxanthin). In a second study, astaxanthin and ketolutein accumulation was achieved through the overexpression of a green alga β-carotene ketolase gene (Morris *et al*., 2006a).

TOBACCO (NICOTIANA SPP.)

The possibility to manipulate the carotenogenesis in several tobacco (*N. tabacum* and *N. benthamiana*) organs has been intensively explored. Expressing a bacterial *DXR*

gene in a transplastomic manner led to a general increase in various isoprenoids such as chlorophyll a, β-carotene, lutein, solanesol and β-sitosterol with no pleiotropic effects on vegetative growth (Hasunuma *et al*., 2008). *CrtI* overexpression was shown to increase β-carotene and β-ring-xanthophyll content, as well as resistance to norflurazon and other bleaching herbicides (Misawa *et al*., 1994).

Ketocarotenoid induction has been the main goal of metabolic engineering of tobacco carotenoids. In a first approach, the pepper *Ccs*, introduced *via* a viral vector, boosted the *de novo* accumulation of capsanthin up to around 36% of total leaf carotenoids in *N. benthamiana*, accompanied by a strong and unexpected increase in light-harvesting processes (Kumagai *et al*., 1998). Another breakthrough is represented by the overexpression of β-carotene ketolase gene from *Haematococcus*, using a chromoplast-specific promoter, which induced a massive ketocarotenoid synthesis in *N. tabacum* nectaries (Mann *et al*., 2000). Subsequent attempts encompassed the simultaneous expression of a 4,4' ketolase (*CrtW*) and a 3,3' hydroxylase (*CrtZ*) from *Paracoccus*, which produced several ketocarotenoids (canthaxanthin, astaxanthin and ketozeaxanthin) in both tobacco leaves and nectaries at less than 5% of the total carotenoids (Ralley *et al*., 2004). Through the use of two ketolase (*CrtO* and *CrtW*) and a β-carotene hydroxylase (*CrtZ*) genes from, respectively, *Synechocystis*, *Nostoc* and *Erwinia* in different combinations, *Nicotiana glauca* and *Nicotiana tabacum* accumulated ketocarotenoids in petal flowers, nectaries and, in a minor fraction, in leaves but without any significant increase in astaxanthin content (Gerjets *et al*., 2007; Zhu *et al*., 2007a). A transplastomic approach was also devised with the *CrtZ* and *CrtW* genes, but from a different source (the marine bacterium *Brevundimonas*): transgenic plants exhibited a reddish leaf color and accumulated extremely high amounts of astaxanthin (>70% of total carotenoids, >0.5% on a dry weight basis) with no adverse effect on vegetative growth or photosynthesis (Hasunuma *et al*., 2008). Interestingly, the new ketocarotenoid 4-ketoantheraxanthin was detected in transgenic leaves.

Tobacco leaves and reproductive organs have also been investigated for the possibility to modify plastid structures: with this aim, pepper fibrillin was constitutively introduced in tobacco and transgenic chloroplasts/leucoplasts showed an increased number of plastoglobules organized in clusters (Rey *et al*., 2000). Fibrillin overexpressors displayed no alteration in vegetative growth under low light conditions, while longer main stem, higher number of lateral stems and faster floral development were observed under higher light intensities, suggesting a role of fibrillin in regulating plant development *vis-à-vis* environmental stresses. The relation between carotenoid pathway and abiotic stress response has been investigated through the overexpression of bacterial β-carotene hydroxylase *CrtZ*, which led to a higher UV tolerance, probably due to the involvement of carotenoid metabolites in the non-photochemical quenching process (Götz *et al*., 2002).

Tobacco has also been used as a model for investigating the relation between carotenoid and ABA metabolism: transgenic plants expressing two *NCED* genes from *Gentiana lutea* were characterized for several ABA-related processes: contrary to expectations, delayed radicle formation and cotyledon appearance were the only phenotypes detected in these plants, albeit no data were reported on carotenoid composition (Zhu *et al*., 2007b).

Conclusions and perspectives

Carotenoid engineering has proven beneficial for improving the nutritional value of important crops. The integration of genetic, molecular and biochemical approaches, and the current genomic initiatives are likely to provide further momentum to the understanding of the (apo)carotenoid pathways in the coming years. Successful carotenoid engineering through diverse transformation (nuclear, plastid and transient) approaches has been achieved by acting on most structural genes of the pathway. The recent modification of carotenoid composition in mutants and transformants for TFs, light signal transduction factors and plastid-associated proteins (e.g., Davuluri *et al*., 2005; Lu *et al*., 2006; Simkin *et al*., 2007; Mahjoub *et al*., 2009) enlarge the palette of tools for molecular breeding. The development of transformation procedures for other carotenoid-accumulating species, such as pepper (Lee *et al*., 2009), potentially expands the number of target species for (apo)carotenoid engineering.

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