Review

Metabolic engineering of carotenoid biosynthesis in plants

Giovanni Giuliano[1], Raffaela Tavazza[1], Gianfranco Diretto[1], Peter Beyer[2] and Mark A. Taylor[3]

[1] Ente per le Nuove tecnologie, l'Energia e l'Ambiente (ENEA), Casaccia Research Center, PO Box 2400, 00123 S.M. di Galeria (Roma), Italy
[2] Faculty of Biology, Universität Freiburg, Schänzlestrasse 1, 79104 Freiburg, Germany
[3] Scottish Crop Research Institute, Invergowrie, Dundee, UK, DD2 5DA

Carotenoids are one of the most diverse classes of natural compounds. Plant carotenoids are composed of a C40 isoprenoid skeleton with or without epoxy, hydroxy and keto groups. They have fundamental roles in human nutrition as antioxidants and vitamin A precursors and their consumption is increasingly associated with protection from a range of diseases. They are also used commercially as safe food, feed and cosmetic colorants and they protect plants from photooxidative stress. In the past six years many metabolic engineering efforts have been undertaken in plants aiming to improve the nutritional value of staple crops, to enable the use of plants as 'cell factories' for producing specialty carotenoids and to improve plant resistance to abiotic stress.

The plant carotenoid biosynthesis pathway

It is beyond the scope of this review to analyze the literature on carotenoid biosynthesis and several previous reviews exist on the subject [1–3]. For the sake of clarity, we briefly summarize the main steps involved, as well as some recent papers that have not been previously reviewed.

In higher plants, carotenoids are made from IPP (see Glossary), a C5 prenyl phosphate synthesized in the plastid by the MEP pathway (Figure 1). Four molecules of IPP are converted to GGPP (C20) by the action of IPP isomerase (IPI) and GGPP synthase (GGPS). The condensation of two molecules of GGPP by phytoene synthase (CrtB in bacteria, PSY in plants) gives rise to 15-*cis*-phytoene, the first dedicated compound in the carotenoid pathway. Phytoene is converted into lycopene, the red carotenoid found in tomato and watermelon fruits, by the action of two desaturases (phytoene desaturase, PDS and zeta-carotene desaturase, ZDS). This pathway gives rise to poly-*cis* compounds that are converted to their all-*trans* forms through the action of the carotenoid isomerases CrtISO and ZISO. The existence of ZISO is supported by work in maize [4] but has not yet been demonstrated at the molecular level. The bacterial phytoene desaturase, CrtI, is apparently able to perform all isomerization and desaturation reactions performed by plant PDS, ZDS CrtISO and ZISO (Figure 1). For this reason, it is widely used in metabolic engineering approaches.

Lycopene is the substrate of two competing cyclases: epsilon-cyclase (LCY-e) and β-cyclase (LCY-b), acting together on the two ends of the molecule, lead to the formation of α-carotene, whereas the action of LCY-b alone forms β-carotene (Figure 1). The bacterial homolog of LCY-b is CrtY. BETA, an LCY-b paralog, is responsible for β-ring formation in tomato chromoplasts. Beta- and α-carotene are redundantly hydroxylated by non-heme (CHY1, CHY2) as well as cytochrome P450 (CYP97A and CYP97C) hydroxylases. The level of redundancy varies in different tissues: in *Arabidopsis* leaves, β-carotene is

Glossary

ABA: abscisic acid.
AdKETO: ketolase (plant).
BETA: chromoplast-specific beta-cyclase (plant).
CaCCS: capsanthin/capsorubin synthase (plant).
CHY1, CHY2: non-heme carotene hydroxylases (plant).
COP1: constitutively photomorphogenic 1
CrtB: phytoene synthase (bacterial).
CrtI: phytoene desaturase/isomerase (bacterial).
CrtISO: carotene isomerase (plant).
CrtO: ketolase (algal/cyanobacterial).
CrtW: ketolase (bacterial).
CrtY: β-cyclase (bacterial).
CrtZ: β-carotene hydroxylase (bacterial).
CRY1, CRY2: cryptochrome blue light photoreceptors.
CYP97B, CYP97C: cytochrome P450 carotene hydroxylases (plant).
DDB1: damage-specific DNA binding protein 1.
DET1: de-etiolated 1.
DW: dry weight.
DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase.
DXS: 1-deoxy-D-xylulose 5-phosphate synthase.
FW: fresh weight.
GA3P: glyceraldehyde-3-phosphate.
GGPP: geranylgeranyl diphosphate.
HDR: hydroxymethylbutenyl diphosphate reductase.
HY5: long hypocotyl 5.
IPI: isopentenyl diphosphate isomerase.
IPP: isopentenyl diphosphate.
LCY-b: β-cyclase (plant).
LCY-e: ε-cyclase (plant).
LHC: light harvesting complex.
MEP: 2-*C*-methyl-D-erythritol 4-phosphate.
NPQ: non-photochemical puenching.
Or: cauliflower *Orange* locus.
PDS: phytoene desaturase (plant).
PSY: phytoene synthase (plant).
RDA: recommended daily allowance.
VAD: vitamin A deficiency.
VDE: violaxanthin de-epoxidase (plant).
ZDS: ζ-carotene desaturase (plant).
ZEP: zeaxanthin epoxidase (plant).
ZISO: ζ-carotene isomerase (plant).

Corresponding author: Giuliano, G. (giuliano@casaccia.enea.it).

0167-7799/$ – see front matter doi:10.1016/j.tibtech.2007.12.003 Available online 28 January 2008

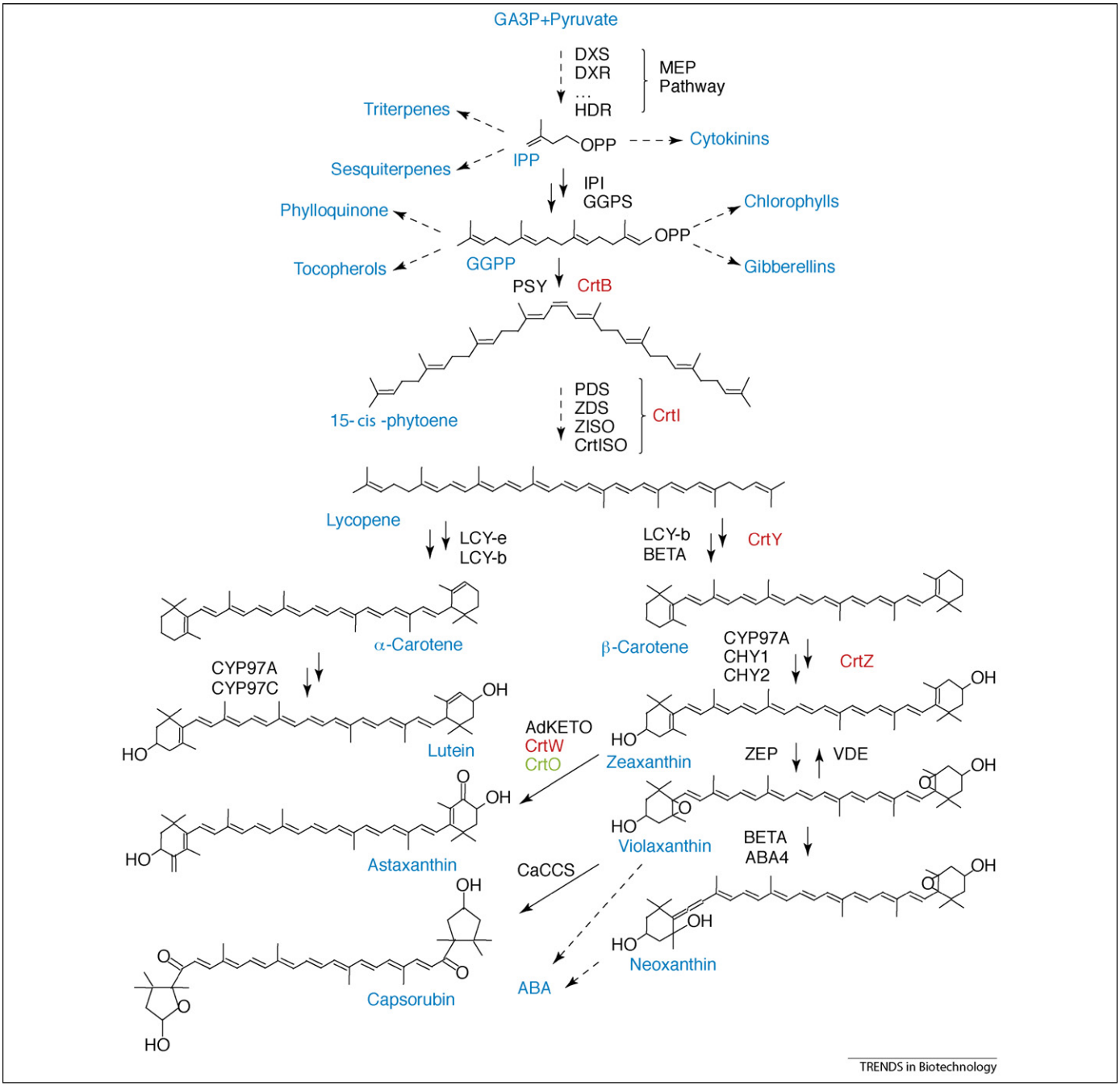

Figure 1. The carotenoid biosynthetic pathway. Names of compounds are in blue, and names of enzymes are in black (plant enzymes), red (bacterial enzymes) and green (CrtO from green algae and cyanobacteria).

hydroxylated by CHY1, CHY2 and CYP97A [5–7], whereas in tomato flowers CHY1 is the main β-hydroxylase [8]. CYP97C hydroxylates the epsilon-ring of lutein [9]. Beta-xanthophylls are epoxidated–de-epoxidated by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), giving rise to the xanthophyll cycle.

Early work suggested that the synthesis of neoxanthin is controlled by β-cyclase paralogs [10,11]. However, mutations in the tomato paralog, *BETA*, affect β-carotene, but not neoxanthin biosynthesis [12]. Furthermore, *Arabidopsis*, which synthesizes neoxanthin, does not possess this paralog. Recently, the *ABA4* gene product has been shown to be necessary, but not sufficient, for neoxanthin accumulation in *Arabidopsis* leaves [13]. This situation is further complicated by the fact that a neoxanthin-less mutant of tomato maps to a position distinct from *BETA* and the tomato ortholog of *ABA4* (J. Hirschberg, personal communication).

Specialized carotenoid metabolism occurs in some plant species: pepper fruits synthesize the ketocarotenoid capsorubin from violaxanthin through the action of capsanthin–capsorubin synthase (CCS), a pepper ortholog of BETA (Figure 1). *Adonis aestivalis* petals synthesize the ketocarotenoid astaxanthin, usually found in marine microorganisms, through the action of AdKETO, a CHY paralog. A lutein epoxidation–de-epoxidation cycle has been described in tropical trees [14], but the underlying enzymes remain undescribed.

Recent work has led to the identification of a transcriptional regulator possibly involved in the control of carotenogenesis: the *Arabidopsis* RAP2.2 transcription factor binds to an ATCTA element found in the *PSY* and *PDS* promoters; its overexpression in *Arabidopsis* calli leads to decreased *PSY* and *PDS* transcription and decreased carotenoid levels [15]. Another mechanism controlling carotenoid levels in plant tissues is their degradation: in *Chrysanthemum* petals the expression of a carotenoid cleavage dioxygenase CmCCD4a correlates inversely with the accumulation of carotenoids [16]. Recent work in tomato showed an inverse correlation between ABA levels and plastid number: the ABA-deficient mutants *flacca*, *sitiens* and *hp3* all show an increased plastid number as well as increased lycopene levels in fruits [17]. The generality of such mechanisms remains to be tested, but they could provide additional approaches for biotechnological improvement of carotenoid synthesis.

Carotenoid engineering for improved nutrition

Along with iron and iodine deficiencies, Vitamin A deficiency is one of the leading causes of micronutrient malnutrition in developing countries. Carotenoids carrying at least one unsubstituted β-ionone ring, such as α- and β-carotene, are nutritional vitamin A precursors [18]. This prompted a series of efforts to engineer high provitamin A levels into staple crops.

Overexpression of the bacterial phytoene synthase gene, *CrtB,* has been a successful strategy: in canola seeds it is sufficient to increase phytoene, α-carotene and β-carotene content dramatically [19]. Co-expression of the *CrtI* gene reduces phytoene levels without improving levels of downstream compounds [20]. Similar results are obtained with co-expression of the *CrtB*, *CrtI* and *CrtY* genes.

Overexpression of *CrtB* in potato tubers results in a similar phenotype with an increase of β-carotene, lutein and violaxanthin [21]. At variance with canola, the co-expression of CrtI and CrtY increases the concentration of α- and β-carotene sixfold, from 10 μg/g to 60 μg/g DW without decreasing phytoene levels [22]. Overexpression of *DXS* results in a sixfold increase in phytoene levels and in a tuber early-sprouting phenotype [23]. Analyses at harvest revealed an increase of *trans*-zeatin riboside, a cytokinin hormone in which the isoprenoid moiety is derived from IPP (Figure 1). Silencing strategies have also been applied to carotenoid engineering in potato tubers: silencing of *ZEP* resulted in increased total carotenoid levels, particularly zeaxanthin [24], whereas silencing of *LCY-e* and *CHY* resulted in increased β-carotene and total carotenoid levels (although this increase was to levels significantly lower than in *CrtB–CrtI–CrtY* overexpressors) [22,25,26].

In contrast to potato and canola, in rice endosperm overexpression of *PSY* induces accumulation of phytoene but not of any downstream products [27]. The simultaneous expression of *CrtI* induces the accumulation of β-carotene and β-xanthophylls, resulting in 'Golden Rice' [28]. In endosperm, the endogenous *PDS*, *ZDS*, *CrtISO* and (at lower levels) *LCY-b* and *CHY* transcripts are expressed [29], providing a rational basis for this result.

The effect of transgenes from various plants has been systematically investigated [30]: *PSY* genes from maize or rice proved more effective in driving β-carotene accumulation in rice endosperm than the gene from daffodil (which was used in the original transformation [28]) resulting in a 17-fold increase in β-carotene, from 1.8 [28] to 31 μg/gm DW [30]. The abovementioned high β-carotene transformants in canola, potato and rice have the potential to reduce vitamin A malnutrition (Box 1).

Whether tissue-specific or constitutive promoters are used to drive transgene expression is important: early experiments showed that constitutive overexpression of *PSY* in tomato causes a dwarf phenotype, probably by diverting the GGPP pool from the gibberellin pathway into the carotenoid pathway [31] (Figure 1). The effect of tissue-specific versus constitutive expression has been systematically investigated in potato tubers [22]. The *CrtB* gene was put under the control of a tuber-specific *Pat* promoter to avoid dwarfism, but the downstream genes, *CrtI* and *CrtY*, were fused to either constitutive (*35S*) or tuber-specific (*Pat*) promoters. A clear negative correlation was observed between tuber and leaf carotenoid levels when *CrtI* was under *35S* promoter control, indicating that high levels of expression of this transgene, needed to achieve high levels of tuber carotenoids, interfered with leaf carotenogenesis.

Tomato fruits accumulate large amounts of the linear carotene, lycopene, which is devoid of provitamin A activity but has a protective effect against prostate cancer [32]. Engineering efforts in tomato have been focused on three goals: the increase of lycopene levels, the increase of β-carotene levels and the synthesis of increased amounts of value-added xanthophylls. Modest (1.5–2-fold) increases in lycopene levels have been seen using several approaches: (i) overexpression of 'early' pathway genes, such as *CrtB* [33] and *DXS* [34]; (ii) silencing of genes involved in lycopene use, such as *LCY-b* [35] or *BETA* [12]; and (iii) overexpression of genes encoding carotenoid-binding proteins [36]. A twofold increase in β-carotene levels has been obtained through the overexpression of *CrtI* [37]. Larger increases (>7-fold) have been seen with overexpression of *LCY-b* [35,38] or *BETA* [12].

Box 1. How much β-carotene enrichment is sufficient to fight vitamin A deficiency?

The US Recommended Daily Allowance (RDA) for vitamin A is 300 μg retinol equivalents/day for children aged 1–3 years (the group at highest risk for VAD) and 800 μg for adults (Institute of Medicine of the National Academies; http://www.iom.edu/Object.File/Master/7/296/0.pdf). The conversion of β-carotene into retinol in the human body (equivalency ratio) is assumed to be 12:1 (Institute of Medicine of the National Academies; http://www.iom.edu/Object.File/Master/7/296/0.pdf). However, in animal systems using β-carotene-fortified maize, this ratio has been shown to be as low as 3:1 [78]. A similarly low equivalency ratio has been obtained in human supplementation studies with 'Golden Rice' (R. Russell, personal communication). Assuming a more conservative ratio (6:1) used in human supplementation studies [79] and considering both α- and β-carotene as vitamin A precursors, 100% of the vitamin A RDA for children and 38% for adults could be reached with ~5 g/day of 'Golden' canola oil [19], 60 g/day (DW) 'Golden Rice 2' [30] and 150 g/day (FW) of 'Golden potatoes' [22].

Because carotenoid biosynthesis enzymes are plastid-localized it is possible to metabolically engineer the pathway through plastid transformation: plastid expression of a bacterial lycopene β-cyclase gene results in fourfold enhanced β-carotene content of tomato fruits [39]. Although there is increasing evidence that plastid transformation guarantees only partial containment of transgenes [40,41], it is an interesting novel approach for the expression of polycistronic constructs.

Plants as cell factories for specialty carotenoids

The global market for carotenoids is estimated at $900 million per year, of which the xanthophylls astaxanthin and canthaxanthin represent >55% [42]. These two xanthophylls are used as feed additives in aquaculture and are responsible for up to 20% of the cost of aquaculture feed (http://en.wikipedia.org/wiki/Astaxanthin). Most astaxanthin available commercially is chemically synthesized. However, the synthetic pigment only contains 25% of the naturally occurring stereoisomer (3S, 3′S). Several metabolic engineering efforts have focused on astaxanthin production in plants. In an early experiment [43] the overexpression of the *CrtO* ketolase gene from the green alga *Haematococcus pluvialis*, under the control of the chromoplast-associated *PDS* promoter, resulted in the accumulation of high levels of astaxanthin and other ketocarotenoids in the nectary, a flower tissue rich in xanthophyll-containing chromoplasts. Overexpression of bacterial CrtZ and CrtW enzymes (encoding, respectively, β-carotene hydroxylase and ketolase) in tobacco induces the accumulation of high levels of ketocarotenoids in nectary, and low levels in leaf tissue [44]. Interestingly, expression of the same enzymes, or of CrtO, in fruits of the tomato *BETA* mutant that accumulate β-carotene does not result in significant transformation of the latter compound into hydroxy- or keto-carotenoids (P.D. Fraser and P.M. Bramley, personal communication). This finding suggests that the β-carotene accumulated by tomato *B* fruits is not accessible to hydroxylation and/or ketolation. By contrast, simultaneous expression of LCY-b and CHY in tomato fruits leads to the biosynthesis of zeaxanthin [45], indicating possible metabolic channeling between LCY-b cyclase and CHY hydroxylase. The exact set of enzymes driving astaxanthin biosynthesis in tomato fruits awaits elucidation.

Evidence for metabolic channeling in astaxanthin production has been obtained in transgenic potato tubers: these tubers, accumulating zeaxanthin as a result of ZEP silencing [24], were co-transformed with a cyanobacterial *CrtO* ketolase gene under a constitutive promoter [46]. Incomplete transformation of β-carotene into astaxanthin was observed, giving rise to 4-ketozeaxanthin and 3′-hydroxyechinenone, together with low levels of astaxanthin. In a different approach [47], the *Haematococcus CrtO* gene was overexpressed in two potato genotypes: *Mayan Gold*, a wild diploid strain accumulating high levels of violaxanthin and lutein in tubers, and Desirée, a low-carotenoid cultivar. In *CrtO*-transformed *Mayan Gold*, high levels of astaxanthin (up to 13 μg/g DW) were accumulated, together with ketolutein. This finding demonstrates that the β-ring of lutein is amenable to ketolation by CrtO. Interestingly, ketolutein is never found in leaves accumulating astaxanthin as a result of ketolase expression [44,46,48] in spite of the fact that lutein is the main xanthophyll in this tissue. This finding could be attributed to the fact that in the LHC complexes of leaves lutein is bound to different sites and is less exposed to the medium than the metabolically active β-xanthophylls, which are subject to epoxidation–de-epoxidation [49].

Astaxanthin overproduction has been obtained through the overexpression of *CrtO* in carrot roots [48]. These roots usually accumulate high levels of α- and β-carotene (>170 μg/g FW for both). In roots overexpressing *CrtO,* the bulk of carotenoids is composed of astaxanthin and other β-ketocarotenoids (>90 and >130 μg/g FW, respectively), without formation of ketolutein. Thus, overexpression of a single ketolase results in efficient re-direction of the vast majority of the carotenoid pool of carrot roots into the formation of β-ketocarotenoids.

Engineering leaf carotenoids for improved stress resistance

Leaf carotenoids have essential photoprotective roles: they scavenge reactive oxygen species, quench dangerous triplet states of chlorophyll and participate in thermal dissipation of excess light energy [50]. Zeaxanthin, which is formed as a result of violaxanthin de-epoxidation under high irradiances, enhances non-photochemical quenching (NPQ) of chlorophyll fluorescence and protects membrane lipids from peroxidation [51]. Thus, it is not surprising that an *Arabidopsis* mutant in which all leaf xanthophylls have been substituted with zeaxanthin is more resistant to photooxidative stress and lipid peroxidation than the wild-type [52]. Overexpression of *LCY-e* in *Arabidopsis* leaves results in increased lutein levels and a marginal increase in the rate of induction of NPQ, despite a reduction of the β-xanthophyll pool size [53], suggesting that in addition to the role of β-xanthophylls, lutein also has a role in NPQ. An increased β-xanthophyll pool size has been obtained through transgenic overexpression of *CrtZ* or *CHY* hydroxylases in leaves of tobacco or *Arabidopsis*, respectively [54,55]. Increased tolerance to UV radiation [54], to high light and high temperature [55] or to high light and low temperature [56] has been reported, and has been attributed to zeaxanthin. However, because these manipulations increase the levels of several β-xanthophylls simultaneously, each of which has distinct photoprotective roles (e.g. [51,57]), it is possible that each of these compounds contributes to the increased stress tolerance phenotype. In agreement with this idea, an *Arabidopsis* mutant lacking all β-xanthophylls is more sensitive to photooxidative stress than a mutant lacking only zeaxanthin or lutein [5,57]. Overexpression of a MEP pathway enzyme, HDR, in *Arabidopsis* resulted in increased leaf carotenoid levels [58], however no effect on stress resistance has been reported.

Engineering regulatory and accessory proteins

Plant carotenoid biosynthesis is under the control of both environmental (e.g. light) and endogenous (e.g. organelle-specific) signals. Phytochrome has been implicated in the

Table 1. Carotenoid metabolic engineering in plants

Target plant or tissue	Strategy	Phenotype(s)	Refs
Canola seed	*CrtB, I, Y* overexpression	Beta-, α-carotene accumulation	[19,20]
Potato plant, tuber	*CrtO* overexpression	Ketocarotenoid accumulation in leaves, tubers	[46,47]
Potato tuber	*CrtB, I, Y* overexpression	Beta-, α-carotene accumulation	[21,25]
Potato tuber	*DXS* overexpression	Enhanced zeatin levels, early sprouting	[23]
Potato tuber	*ZEP* silencing	Zeaxanthin accumulation	[24]
Potato tuber	*LCY-e* silencing	Enhanced β-carotene, total carotenoids	[25]
Potato tuber	*CHY* silencing	Enhanced β-carotene	[80,26]
Potato tuber	*Or* overexpression	Beta-carotene, total carotenoid accumulation	[68]
Rice seed	*PSY, CrtI* overexpression	Beta-carotene, zeaxanthin accumulation	[27,28,30]
Lotus japonicus flower	*CrtW* overexpression	Ketocarotenoid accumulation	[73]
Tomato plant	*PSY* overexpression	Gibberellin depletion, dwarfism	[31]
Tomato plant	*CHRD* silencing	Decreased flower carotenoids, decreased photosynthetic electron flow	[67]
Tomato plant	*DXS* overexpression	Enhanced fruit carotenoids	[34]
Tomato plant	*CRY2* overexpression	Enhanced carotenoids, flavonoids	[61]
Tomato plant, fruit	*DET1, DDB1, COP1-like* silencing	Enhanced carotenoids, flavonoids	[62–64]
Tomato fruit	*CrtB* overexpression	Enhanced lycopene	[33]
Tomato fruit	*FIBRILLIN* overexpression	Enhanced carotenoids, carotenoid-derived volatiles, delayed thylakoid loss	[36]
Tomato fruit	*LCY-b B* silencing	Enhanced lycopene	[12,35]
Tomato fruit	*CrtI* overexpression	Beta-carotene accumulation	[37]
Tomato fruit	*LCY-b, CHY* overexpression	Beta-carotene, zeaxantin accumulation	[35,38,45]
Tomato fruit	*CrtY* overexpression in plastids	Beta-carotene accumulation	[39]
Tobacco, tomato plant	*CrtZ, W* polyprotein overexpression	Ketocarotenoid accumulation in leaves and nectary (flower)	[44]
Tobacco plant, flower	*CrtO* overexpression	Ketocarotenoid accumulation in leaves, nectary, petals	[43,74,81]
Tobacco leaf	*CaCCS* virus-mediated overexpression	Capsanthin accumulation	[82]
Carrot plant	*CrtO* overexpression	Ketocarotenoid accumulation in leaves, roots	[48]
Arabidopsis plant	*LCY-e* overexpression	Enhanced leaf lutein, enhanced NPQ	[53]
Arabidopsis plant	*CrtZ, CHY* overexpression	Enhanced leaf β-xanthophyll pool, resistance to UV and to temperature + light stress	[54–56]
Arabidopsis plant	*HDR* overexpression	Enhanced leaf carotenoids, seed dormancy	[58]
Arabidopsis seed	*CrtO* overexpression	Ketocarotenoid accumulation	[83]

control of carotenoid accumulation and *PSY* gene expression in *Arabidopsis* leaves [59] and in lycopene accumulation in tomato fruits [60]. Overexpression of the blue light photoreceptor CRY2 increases carotenoid and flavonoid levels in tomato fruits and leaves [61]. Transgenic manipulation of downstream components of light signal transduction is also effective: silencing of the negative regulators DDB1, DET1 and COP1-like increases lycopene content of tomato fruits, whereas the opposite effect is seen after silencing of the positive regulator HY5 [62,63]. Fruit-specific silencing of DET1 results in increased fruit carotenoid and flavonoid levels without any adverse plant phenotypes [64]. A similar phenotype is obtained through the constitutive overexpression of CRY1 (L. Giliberto and G. Giuliano, unpublished).

Plant carotenoid-associated proteins provide a metabolic sink for carotenoid sequestration in chromoplasts [65]. Their expression is influenced by perturbations in carotenoid content induced by herbicide treatment [66] or transgenic manipulation [21]. Conversely, transgenic manipulation of carotenoid-associated protein levels influences carotenoid levels: overexpression of the pepper carotenoid-binding protein, fibrillin, in tomato fruits causes an increase in the levels of carotenoids and carotenoid-derived flavor volatiles [36]; silencing of the expression of CHRD, a plastid lipid-associated protein of tomato, suppresses quantum yield of photosynthetic electron flow in leaves as well as carotenoid accumulation in flowers [67].

The *Or* gene of cauliflower induces differentiation of β-carotene-containing chromoplasts in the (usually non-pigmented) curd tissue [68]. This is the first example of a gene product controlling chromoplast differentiation [69]. The OR protein resembles molecular co-chaperones and its overexpression in potato tubers induces the differentiation of β-carotene accumulating chromoplasts [68]. *Or* engineering targets biochemical components different from those affected by engineering of structural genes in the carotenoid pathway [69,70]. Thus, the two engineering approaches could have additive effects.

Conclusions and outlook

In recent years the number of successful efforts to metabolically engineer carotenoid biosynthesis in higher plants has grown at an impressive rate (Table 1). Provitamin A enrichment to levels leading to the delivery of nutritionally significant doses of the vitamin A Recommended Daily Allowance (RDA) has been achieved in canola [19], rice [30] and potato [22]. Important breakthroughs in the engineering of astaxanthin have also been obtained [48]. Engineering of accessory and/or regulatory proteins is increasingly being exploited [61,64,68]. Examples are available of successful engineering in leaves for increased stress protection [55,56]. Our knowledge of the molecular regulation of carotenogenesis in flowers is increasing [16,71,72], and the first examples of engineering in flowers are now available [73] [74]. However, for metabolic engineering to

become a mature technology, several advances in our knowledge are still needed:

(i) Metabolic flux analysis must be applied to the carotenoid pathway, using isotopic labeling coupled with mass spectrometry (see [75] for an example in the general isoprenoid pathway) to pinpoint rate-limiting steps to be engineered as well as precursor abundance and compartmentation.

(ii) The extent of metabolic cross-talk between the carotenoid pathway and other pathways influencing plant development and nutritional value (Figure 1), isoprenoid metabolism and primary metabolism [76] as well as the production of volatiles contributing to flavor [77] must be determined.

(iii) Further elements in the complex regulatory network must be identified, such as transcription factors and their binding partners. The relative importance of carotenoid degradation and biosynthesis in the net accumulation of carotenoids must also be determined.

(iv) Rigorous criteria must be developed for the assessment of the nutritional and agronomic performance of engineered plants.

(v) Development of knowledge in these key areas will help bring carotenoid metabolic engineering out of the trial-and-error era and transform it into a mature technology.

Acknowledgments

Work in G.G.'s laboratory is supported by the EU (Projects EU-SOL and Develonutri) and by the Italian Ministry of Research (FIRB project). M.T. receives support from the EU (EU-SOL) and the Scottish Government Rural and Environment Research and Analysis Directorate. P.B. receives support from the HarvestPlus (www.harvestplus.org) research consortium. We thank Paul D. Fraser, Robert M. Russell and Joseph Hirschberg for communication of results before publication.

References

1 Hirschberg, J. (2001) Carotenoid biosynthesis in flowering plants. *Curr. Opin. Plant Biol.* 4, 210–218
2 Fraser, P.D. and Bramley, P.M. (2004) The biosynthesis and nutritional uses of carotenoids. *Prog. Lipid Res.* 43, 228–265
3 Dellapenna, D. and Pogson, B.J. (2006) Vitamin synthesis in plants: tocopherols and carotenoids. *Annu. Rev. Plant Biol.* 57, 711–738
4 Li, F. *et al.* (2007) Maize Y9 encodes a product essential for 15-cis-{zeta}-carotene isomerization. *Plant Physiol.* 144, 1181–1189
5 Fiore, A. *et al.* (2006) Elucidation of the beta-carotene hydroxylation pathway in *Arabidopsis thaliana*. *FEBS Lett.* 580, 4718–4722
6 Kim, J. and DellaPenna, D. (2006) Defining the primary route for lutein synthesis in plants: the role of *Arabidopsis* carotenoid beta-ring hydroxylase CYP97A3. *Proc. Natl. Acad. Sci. U. S. A.* 103, 3474–3479
7 Quinlan, R.F. *et al.* (2007) *Escherichia coli* as a platform for functional expression of plant P450 carotene hydroxylases. *Arch. Biochem. Biophys.* 458, 146–157
8 Galpaz, N. *et al.* (2006) A chromoplast-specific carotenoid biosynthesis pathway is revealed by cloning of the tomato white-flower locus. *Plant Cell* 18, 1947–1960
9 Tian, L. *et al.* (2004) The *Arabidopsis* LUT1 locus encodes a member of the cytochrome p450 family that is required for carotenoid epsilon-ring hydroxylation activity. *Proc. Natl. Acad. Sci. U. S. A.* 101, 402–407
10 Al-Babili, S. *et al.* (2000) Identification of a novel gene coding for neoxanthin synthase from *Solanum tuberosum*. *FEBS Lett.* 485, 168–172
11 Bouvier, F. *et al.* (2000) Identification of neoxanthin synthase as a carotenoid cyclase paralog. *Eur. J. Biochem.* 267, 6346–6352
12 Ronen, G. *et al.* (2000) An alternative pathway to beta-carotene formation in plant chromoplasts discovered by map-based cloning of beta and old-gold color mutations in tomato. *Proc. Natl. Acad. Sci. U. S. A.* 97, 11102–11107
13 North, H.M. *et al.* (2007) The *Arabidopsis* ABA-deficient mutant aba4 demonstrates that the major route for stress-induced ABA accumulation is via neoxanthin isomers. *Plant J.* 50, 810–824
14 Matsubara, S. *et al.* (2007) Short- and long-term operation of the lutein-epoxide cycle in light-harvesting antenna complexes. *Plant Physiol.* 144, 926–941
15 Welsch, R. *et al.* (2007) Transcription factor RAP2.2 and its interacting partner SINAT2: stable elements in the carotenogenesis of *Arabidopsis* leaves. *Plant Physiol.* 145, 1073–1085
16 Ohmiya, A. *et al.* (2006) Carotenoid cleavage dioxygenase (CmCCD4a) contributes to white color formation in chrysanthemum petals. *Plant Physiol.* 142, 1193–1201
17 Galpaz, N. *et al.* (2008) Abscisic acid deficiency in the tomato mutant high-pigment 3 (hp3) leading to increased plastid number and higher fruit lycopene. *Plant J.*. DOI: 10.1111/j.1365-313X.2007.03362.x (http://www.blackwell-synergy.com/loi/tpj)
18 von Lintig, J. and Vogt, K. (2004) Vitamin A formation in animals: molecular identification and functional characterization of carotene cleaving enzymes. *J. Nutr.* 134, 251S–256S
19 Shewmaker, C.K. *et al.* (1999) Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J.* 20, 401–412
20 Ravanello, M.P. *et al.* (2003) Coordinate expression of multiple bacterial carotenoid genes in canola leading to altered carotenoid production. *Metab. Eng.* 5, 255–263
21 Ducreux, L.J. *et al.* (2005) Metabolic engineering of high carotenoid potato tubers containing enhanced levels of beta-carotene and lutein. *J. Exp. Bot.* 56, 81–89
22 Diretto, G. *et al.* (2007) Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. *PLoS ONE* 2, e350
23 Morris, W.L. *et al.* (2006) Overexpression of a bacterial 1-deoxy-D-xylulose 5-phosphate synthase gene in potato tubers perturbs the isoprenoid metabolic network: implications for the control of the tuber life cycle. *J. Exp. Bot.* 57, 3007–3018
24 Romer, S. *et al.* (2002) Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. *Metab. Eng.* 4, 263–272
25 Diretto, G. *et al.* (2006) Metabolic engineering of potato tuber carotenoids through tuber-specific silencing of lycopene epsilon cyclase. *BMC Plant Biol.* 6, 13
26 van Eck, J. *et al.* (2007) Enhancing beta-carotene content in potato by RNAi-mediated silencing of the beta-carotene hydroxylase gene. *Am. J. Potato Res.* 84, 331–342
27 Burkhardt, P.K. *et al.* (1997) Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. *Plant J.* 11, 1071–1078
28 Ye, X. *et al.* (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287, 303–305
29 Schaub, P. *et al.* (2005) Why is golden rice golden (yellow) instead of red? *Plant Physiol.* 138, 441–450
30 Paine, J.A. *et al.* (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotechnol.* 23, 482–487
31 Fray, R.G. *et al.* (1995) Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. *Plant J.* 8, 693–701
32 Gann, P.H. *et al.* (1999) Lower prostate cancer risk in men with elevated plasma lycopene levels: results of a prospective analysis. *Cancer Res.* 59, 1225–1230
33 Fraser, P.D. *et al.* (2002) Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. *Proc. Natl. Acad. Sci. U. S. A.* 99, 1092–1097
34 Enfissi, E.M. *et al.* (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotechnol. J.* 3, 17–27
35 Rosati, C. *et al.* (2000) Metabolic engineering of beta-carotene and lycopene content in tomato fruit. *Plant J.* 24, 413–420

36 Simkin, A.J. *et al.* (2007) Fibrillin influence on plastid ultrastructure and pigment content in tomato fruit. *Phytochemistry* 68, 1545–1556
37 Romer, S. *et al.* (2000) Elevation of the provitamin A content of transgenic tomato plants. *Nat. Biotechnol.* 18, 666–669
38 D'Ambrosio, C. *et al.* (2004) Virtually complete conversion of lycopene into β-carotene in fruits of tomato plants transformed with the tomato lycopene β-cyclase (tLCY-b) cDNA. *Plant Sci.* 166, 207–214
39 Wurbs, D. *et al.* (2007) Contained metabolic engineering in tomatoes by expression of carotenoid biosynthesis genes from the plastid genome. *Plant J.* 49, 276–288
40 Stegemann, S. and Bock, R. (2006) Experimental reconstruction of functional gene transfer from the tobacco plastid genome to the nucleus. *Plant Cell* 18, 2869–2878
41 Azhagiri, A.K. and Maliga, P. (2007) Exceptional paternal inheritance of plastids in *Arabidopsis* suggests that low-frequency leakage of plastids via pollen might be universal in plants. *Plant J.* 52, 817–823
42 Marz, U. (2005) *The Global Market for Carotenoids.* Business Communication, Inc
43 Mann, V. *et al.* (2000) Metabolic engineering of astaxanthin production in tobacco flowers. *Nat. Biotechnol.* 18, 888–892
44 Ralley, L. *et al.* (2004) Metabolic engineering of ketocarotenoid formation in higher plants. *Plant J.* 39, 477–486
45 Dharmapuri, S. *et al.* (2002) Metabolic engineering of xanthophyll content in tomato fruits. *FEBS Lett.* 519, 30–34
46 Gerjets, T. and Sandmann, G. (2006) Ketocarotenoid formation in transgenic potato. *J. Exp. Bot.* 57, 3639–3645
47 Morris, W.L. *et al.* (2006) Engineering ketocarotenoid biosynthesis in potato tubers. *Metab. Eng.* 8, 253–263
48 Jayaraj, J. *et al.* (2007) Metabolic engineering of novel ketocarotenoid production in carrot plants. *Transgenic Res*. DOI: 10.1007/s11248-007-9120-0 (http://www.springerlink.com/content/100225/)
49 Morosinotto, T. *et al.* (2002) Dynamics of chromophore binding to Lhc proteins *in vivo* and *in vitro* during operation of the xanthophyll cycle. *J. Biol. Chem.* 277, 36913–36920
50 Demmig-Adams, B. *et al.* (1996) Carotenoids 3: *in vivo* function of carotenoids in higher plants. *FASEB J.* 10, 403–412
51 Havaux, M. and Niyogi, K.K. (1999) The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. *Proc. Natl. Acad. Sci. U. S. A.* 96, 8762–8767
52 Havaux, M. *et al.* (2004) The effect of zeaxanthin as the only xanthophyll on the structure and function of the photosynthetic apparatus in *Arabidopsis thaliana*. *J. Biol. Chem.* 279, 13878–13888
53 Pogson, B.J. and Rissler, H.M. (2000) Genetic manipulation of carotenoid biosynthesis and photoprotection. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 355, 1395–1403
54 Gotz, T. *et al.* (2002) Expression of a bacterial carotene hydroxylase gene (crtZ) enhances UV tolerance in tobacco. *Plant Mol. Biol.* 50, 129–142
55 Davison, P.A. *et al.* (2002) Overexpression of beta-carotene hydroxylase enhances stress tolerance in *Arabidopsis*. *Nature* 418, 203–206
56 Johnson, M.P. *et al.* (2007) Elevated zeaxanthin bound to oligomeric LHCII enhances the resistance of *Arabidopsis* to photooxidative stress by a lipid-protective, antioxidant mechanism. *J. Biol. Chem.* 282, 22605–22618
57 Dall'Osto, L. *et al.* (2007) Different roles of alpha- and beta-branch xanthophylls in photosystem assembly and photoprotection. *J. Biol. Chem.* 282, 35056–35068
58 Botella-Pavia, P. *et al.* (2004) Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors. *Plant J.* 40, 188–199
59 von Lintig, J. *et al.* (1997) Light-dependent regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression and is mediated by phytochrome in *Sinapis alba* and *Arabidopsis thaliana* seedlings. *Plant J.* 12, 625–634
60 Alba, R. *et al.* (2000) Fruit-localized phytochromes regulate lycopene accumulation independently of ethylene production in tomato. *Plant Physiol.* 123, 363–370
61 Giliberto, L. *et al.* (2005) Manipulation of the blue light photoreceptor cryptochrome 2 in tomato affects vegetative development, flowering time, and fruit antioxidant content. *Plant Physiol.* 137, 199–208
62 Liu, Y. *et al.* (2004) Manipulation of light signal transduction as a means of modifying fruit nutritional quality in tomato. *Proc. Natl. Acad. Sci. U. S. A.* 101, 9897–9902
63 Davuluri, G.R. *et al.* (2004) Manipulation of DET1 expression in tomato results in photomorphogenic phenotypes caused by post-transcriptional gene silencing. *Plant J.* 40, 344–354
64 Davuluri, G.R. *et al.* (2005) Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nat. Biotechnol.* 23, 890–895
65 Vishnevetsky, M. *et al.* (1999) Carotenoid sequestration in plants: the role of carotenoid-associated proteins. *Trends Plant Sci.* 4, 232–235
66 Vishnevetsky, M. *et al.* (1999) Molecular mechanisms underlying carotenogenesis in the chromoplast: multilevel regulation of carotenoid-associated genes. *Plant J.* 20, 423–431
67 Leitner-Dagan, Y. *et al.* (2006) CHRD, a plant member of the evolutionarily conserved YjgF family, influences photosynthesis and chromoplastogenesis. *Planta* 225, 89–102
68 Lu, S. *et al.* (2006) The cauliflower Or gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high-levels of {beta}-carotene accumulation. *Plant Cell* 18, 3594–3605
69 Giuliano, G. and Diretto, G. (2007) Of chromoplasts and chaperones. *Trends Plant Sci.* 12, 529–531
70 Li, L. and Van Eck, J. (2007) Metabolic engineering of carotenoid accumulation by creating a metabolic sink. *Transgenic Res.* 16, 581–585
71 Vishnevetsky, M. *et al.* (1996) Molecular cloning of a carotenoid-associated protein from *Cucumis sativus* corollas: Homologous genes involved in carotenoid sequestration in chromoplasts. *Plant J.* 10, 1111–1118
72 Zhu, C. *et al.* (2003) cDNAs for the synthesis of cyclic carotenoids in petals of *Gentiana lutea* and their regulation during flower development. *Biochim. Biophys. Acta* 1625, 305–308
73 Suzuki, S. *et al.* (2007) Flower color alteration in *Lotus japonicus* by modification of the carotenoid biosynthetic pathway. *Plant Cell Rep.* 26, 951–959
74 Zhu, C. *et al.* (2007) Nicotiana glauca engineered for the production of ketocarotenoids in flowers and leaves by expressing the cyanobacterial crtO ketolase gene. *Transgenic Res.* 16, 813–821
75 Wu, S. *et al.* (2006) Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. *Nat. Biotechnol.* 24, 1441–1447
76 Fraser, P.D. *et al.* (2007) Manipulation of phytoene levels in tomato fruit: effects on isoprenoids, plastids, and intermediary metabolism. *Plant Cell* 19, 3194–3211
77 Davidovich-Rikanati, R. *et al.* (2007) Enrichment of tomato flavor by diversion of the early plastidial terpenoid pathway. *Nat. Biotechnol.* 25, 899–901
78 Howe, J.A. and Tanumihardjo, S.A. (2006) Carotenoid-biofortified maize maintains adequate vitamin a status in Mongolian gerbils. *J. Nutr.* 136, 2562–2567
79 West, K.P., Jr *et al.* (1999) Double blind, cluster randomised trial of low dose supplementation with vitamin A or beta carotene on mortality related to pregnancy in Nepal. The NNIPS-2 Study Group. *BMJ* 318, 570–575
80 Diretto, G. *et al.* (2007) Silencing of beta-carotene hydroxylase increases total carotenoid and beta-carotene levels in potato tubers. *BMC Plant Biol.* 7, 11
81 Gerjets, T. *et al.* (2007) Metabolic engineering of ketocarotenoid biosynthesis in leaves and flowers of tobacco species. *Biotechnol. J.* 2, 1263–1269
82 Kumagai, M.H. *et al.* (1998) Functional integration of non-native carotenoids into chloroplasts by viral-derived expression of capsanthin-capsorubin synthase in *Nicotiana benthamiana*. *Plant J.* 14, 305–315
83 Stalberg, K. *et al.* (2003) Synthesis of ketocarotenoids in the seed of *Arabidopsis thaliana*. *Plant J.* 36, 771–779