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SCIENCE DIRECT
Metabolic Engineering 8 (2006) 291–302
METABOLIC ENGINEERING
www.elsevier.com/locate/ymben

Minireview

Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants

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Received 1 August 2005; received in revised form 26 January 2006; accepted 31 January 2006
Available online 18 April 2006

Abstract

As a proof of concept, the qualitative and quantitative engineering of carotenoid formation has been achieved in crop plants. Successful reports in tomato, potato, rice, and canola all describe the enhancement of carotenoid with nutritional value, while in model systems such as tobacco and *Arabidopsis* the engineering of carotenoid to confer abiotic stress has been described. For all the successful applications there have been many examples of unintended/unpredicted phenotypes and results. Typically this has resided from our lack of understanding of carotenoid formation and its regulation. In the present article, we will review advances in carotenoid formation and its regulation to illustrate how metabolic engineering experiments have shed light on regulatory mechanisms.

Keywords: Carotenoid; Metabolic engineering; Crop plants; And health-promoting phytochemicals

1. Introduction

Carotenoids accumulate in leaves, flowers and fruit of plants. Their major function in green tissue is to protect the photosynthetic apparatus from excess light. In this respect, they are essential for functional photosynthesis. In addition to their high antioxidative potential (Stahl and Sies, 2003), carotenoids are also important components of the human diet. Besides pro-vitamin A activity (Mayne, 1996) there is now strong evidence showing that diets rich in carotenoids prevent the onset of certain chronic disease states such as age-related macular degeneration (Landrum and Bone, 2001) and certain cancers (Giovannucci, 2002; Sharoni et al., 2003). The health promoting properties of carotenoids have intensified interest in carotenoid biosynthesis in plants and its commercial exploitation.

The genetic manipulation of the carotenoid pathway is of substantial interest in order to generate plants that are (i) resistant to environmental stresses (e.g., high light and UV-B), (ii) possess improved nutritional content and (iii) cell factories for the production of high-value industrial carotenoids. Several attempts have been made to genetically manipulate carotenoid biosynthesis in crop plants, thus increasing the nutritional value. Many of these aspects have been reviewed comprehensively in the following articles (Hirschberg, 2001; DellaPenna, 2001; Tucker, 2003; Fraser and Bramley, 2004). A broad diverse array of carotenogenic genes from pro- and eukaryotic organisms are available for the enhancement of individual reactions or the establishment of new branches in the pathway together with other genetic tools are available (Shewmaker et al., 1999; Mann et al., 2000). In addition, genetic modification of carotenogenesis relies on our basic biochemical knowledge of carotenoid biosynthesis and the regulation of the pathway. Although the biochemistry of carotenogenesis has been well established over the past decades, a major limitation is our poor knowledge of how endogenous carotenogenic gene expression is regulated in higher plants (Sandmann, 2001). Furthermore, it is still puzzling that in several cases endogenous carotenogenic genes are newly expressed or over-expressed upon the introduction of foreign genes. The present review provides an update of carotenoid biosynthesis and its regulation in

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1096-7176/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2006.01.005

different organs (plastid types) and highlights the achievements made in the genetic engineering of carotenogenesis as well as illustrating how these studies have elucidated regulatory mechanisms that can be exploited or will hinder future genetic engineering.

2. The pathway

For the genetic manipulation of the carotenoid biosynthetic pathway, fundamental knowledge on the reactions and enzymes involved in carotenoid formation as well as those pathways supplying prenyl pyrophosphates precursors and catabolising carotenoids is decisive. Isotope labelling and functional genomics have demonstrated that the prenyl pyrophosphates utilised in the formation of carotenoids is derived from the plastid localised desoxyxylulose 5-phosphate pathway and not from the mevalonate pathway operating in the cytoplasm (reviewed in Rodriguez-Concepcion and Boronat, 2002). However, it has been demonstrated that radioactivity from ^{14}C-mevalonate fed to corn leaves can be recovered in carotenoids and that an in vitro exchange of isopentenyl pyrophosphate (IPP) across the plastid envelope occurs (Albrecht and Sandmann, 1994; Bick and Lange, 2003). Furthermore, in vivo incorporation of isotope labelled intermediates of both pathways indicats that prenyl pyrophosphates can be exchanged between both the plastidic and the cytosolic terpenoid pathways (Kasahara et al., 2002). It should be pointed out, that prenyl pyrophosphates and especially geranylgeranyl pyrophosphate (GGPP) are not only the precursor for carotenoids but also participate in the synthesis of many other terpenoid compounds synthesised in plants. This pool of GGPP represents the metabolic link between the biosynthesis of carotenoids and other terpenoids and is responsible for inter pathway regulation via competition for GGPP.

The first carotene to be formed in the specific pathway is phytoene, resulting from the condensation of two GGPP molecules (see review by Cunningham and Gantt (1998) and Sandmann (2001) for older references on reactions, enzymes and genes of the pathway). The product of the phytoene synthase-catalysed reaction is a 15-*cis* isomer (Fig. 1A). Then, four double bonds are introduced into the phytoene molecule, first at C-11,12 and C-11′,12′ by phytoene desaturase (Pds) and then at C-7,8 and C-7′,8′ by ζ-carotene desaturase (Zds). NMR studies of the pigments found in the tangerine tomato mutant, early in vitro studies with daffodil chromoplasts and complementation experiment of desaturases in *Escherichia coli* all suggest the presence of a *cis* carotenoid desaturation pathway in plants to *tetracis* prolycopene. Involvement of individual isomers was elucidated by in vitro reactions of the corresponding enzyme. Determination of substrate and product specificity of phytoene and Zds (Breitenbach and Sandmann, 2004) revealed that 15-*cis* phytoene is converted to 9,15,9′-*tricis* ζ-carotene with 15,9′-*dicis* phytofluene as intermediate by the first desaturase. Prior to a

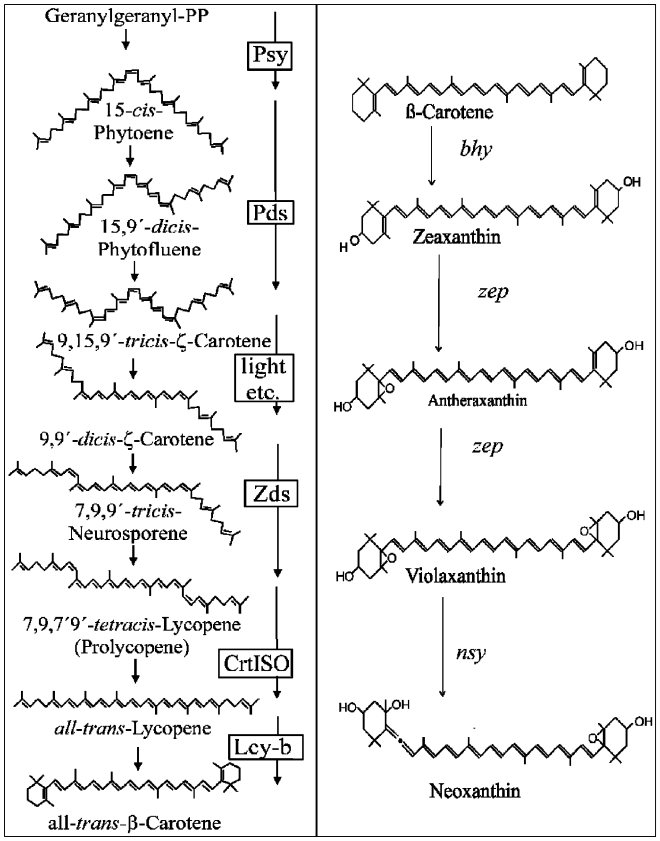

Fig. 1. Isomeric intermediates in the carotenogenic pathway from 15-*cis* phytoene to all-*trans* β-carotene (A) and reaction sequence to neoxanthin (B).

subsequent conversion by Zds, the 15-*cis* double bond of 9,15,9′-*tricis* ζ-carotene has to be isomerised to all-*trans*. Then, the resulting 9,9′-*dicis* ζ-carotene is utilised by Zds via 7,9,9′-*tricis* neurosporene to 7,9,7′,9′-*tetracis* prolycopene. This product of the desaturation reactions must finally be isomerised by a specific isomerase to all-*trans* lycopene. This is a prerequisite for cyclisation to β-carotene. The desaturation sequence via the individual geometric isomers is provided in Fig. 1A.

The plant phytoene and ζ-carotene desaturases Pds and Zds are highly homologous and follow the same reaction mechanism as a dehydrogenase with plastoquinone as the hydrogen acceptor (Fig. 2A). Via plastoquinone, carotene desaturation is connected to the photosynthetic electron transport chain. Immunogold localisation demonstrated that Pds is integrated into the thylakoid membrane of chloroplasts (Linden et al., 1993). Recently, the genes for a carotene isomerase *crtISO* have been isolated from *Arabidopsis* (Park et al., 2002) and tomato (Isaacson et al., 2002). The accumulation of precursors in the *Arabidopsis* mutant and tangerine tomato mutants, along with in vitro studies with the tomato and cyanobacterial carotene isomerase (Isaacson et al., 2004; Breitenbach et al., 2001) indicate that the products of these genes catalyse the *cis* to *trans* conversion of *tetracis* prolycopene to all–*trans* lycopene. This isomerase is evolutionary related

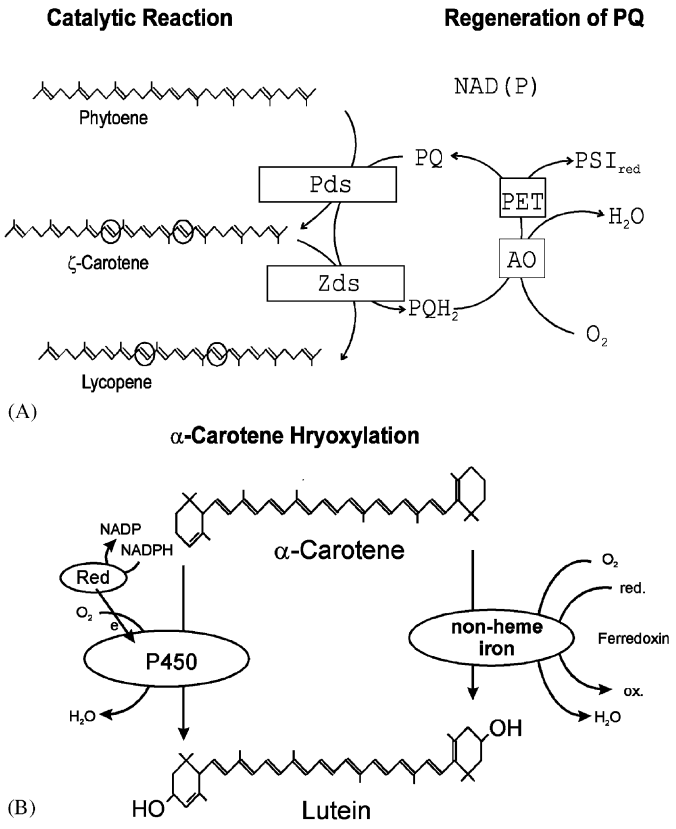

Fig. 2. Mechanism of phytoene and ζ-carotene desaturation and co-factor regeneration in chloroplast thylakoid membranes (A) and of α-carotene hydroxylations to lutein catalysed by two different enzymes (B). Abbreviations: Red, P450 reductase; PQ(H_2), plasoquinone (hydroquinone); PET, photosynthetic electron transport; AO, plastidic alternative oxidase; Pds, phytoene desaturase; Zds, ζ-carotene desaturase.

to bacterial phytoene desaturase. The cyclisation reactions of all-*trans* lycopene introduce ε- and β-ionone end groups. The reactions are catalysed by Lcy-e and Lcy-b, respectively, yielding α- and β-carotene.

In plant leaves and many chromoplasts, α-carotene is typically modified to lutein by hydroxylation at positions 3 and 3′ (Fig. 2B). Two hydroxylase are involved. The modification of the β-ionone ring is catalysed by a ferredoxin-dependent di-iron monooxygenase termed Bhy. However, for the ε-ring a completely unrelated cytochrome P450-type monooxygenase is responsible. The gene *lut1* encoding this enzyme has very recently been cloned and the functional properties of its product analysed (Tian et al., 2004). β-Carotene with two β-ionone end groups is hydroxylated by Bhy at C-3 and C-3′ to zeaxanthin (Fig. 1B). Then, two epoxy groups are inserted at positions C-5,6 and C-5′,6′ by zeaxanthin epoxidase (Zep) yielding violaxanthin. The reaction involves oxygen and ferredoxin as reductant. Especially under high light conditions, violaxanthin can be de-epoxidated back to zeaxanthin by violaxanthin de-epoxidase (Vde). The gene has been cloned from lettuce (Burgos et al., 1998). Neoxanthin is formed from violaxanthin by the action of neoxanthin synthase (Nsy). The cDNA encoding this enzyme has been isolated and functionally characterised from potato and tomato.

3. Pathway regulation

Regulatory processes in connection with plastid development have a direct impact on plant carotenogenesis. In addition, light has a strong influence on carotenoid content. In photosynthetic tissue, the dominant function of carotenoids is the quenching of the triplet chlorophyll as a mechanism to protect the photosynthetic apparatus (Siefermann-Harms, 1985). Although the triplet state of chlorophyll is transferred to carotenoids and the energy dissipated as heat, simultaneous oxidation of carotenoids occurs to some extent (Grumbach and Lichtenthaler, 1982). Under high light conditions carotenoid degradation in leaves may exceed the capacity of biosynthesis (Simkin et al., 2003a). Consequently, biosynthesis is regulated to compensate this loss ensuring the maintenance of a basal level of photoprotective carotenoids. Other regulatory processes are linked to plastid development and transformation. For example, chloroplast to chromoplast transition in flowers and fruit is accompanied by a massive increase of the carotenoid content. In some cases also a qualitative change of the carotenoid composition is observed. A deeper understanding of how the carotenogenic pathway is regulated may led to new strategies to engineer enhanced carotenoid production apart from overexpression of limiting enzymes.

3.1. Light-dependent carotenoid synthesis in leaves

Direct evidence of light-enhanced biosynthesis of leaf carotenoids comes from early radio-labelling studies (Grumbach and Lichtenthaler, 1982). Furthermore, it was recently demonstrated that under prolonged darkness, carotenoid biosynthesis is completely stalled (Simkin et al., 2003a). This control of carotenogenesis by light is predominately regulated at the transcriptional level. In dark-adapted leaves only trace amounts of the messenger levels for phytoene synthase Psy and Pds compared to illuminated leaves were detectable in pepper. Later carotenogenic genes like Zds or lycopene cyclase were down regulated to a much lesser extent. Obviously, the down-regulation of Psy is an influential step in regulating carotenoid biosynthesis. For Pds, a correlation between the amounts of mRNA and the enzyme level after prolonged dark period could be established in tomato (Simkin et al., 2003b). Regulatory elements have been functionally identified in the promoter region of the Psy gene from *Arabidopsis thaliana* (Welsch et al., 2003). Enhanced levels of Psy mRNA in the light have been reported for tomato leaves (Giuliano et al., 1993), *Sinapis alba* and *A. thaliana* (von Lintig et al., 1997). Little is know about the sensing of the light signal for up-regulation of carotenogenesis in chloroplast. The most likely options include phytochrome photoreceptors, redox control or even oxidation products

of carotenoids acting as signalling molecules. Some indications point to the influence of carotenoid content on gene expression (Simkin et al., 2003b). It has been further proposed that the induction of the *pds* promoter responds to end product regulation with β-carotene and/or xanthophylls being candidate compounds (Corona et al., 1996). Another regulatory aspect is the connection of carotene desaturation to photosynthetic electron transport since both processes share the thylakoid plastoquinone pool. Under photostress, the pool of plastoquinone is mainly oxidised which facilitates carotene desaturation. This metabolic redox control resembles a regulative adaptation to carotenoid demand in oxygenic photosynthesis. It was evolutionary inherited from the cyanobacteria, that have acquired the plant-type plastoquinone-dependent Pds in parallel with oxygenic photosynthesis.

Recently manipulation of the signalling pathways associated with light perception pathways have illustrated that elevated carotenoid content as well as other metabolites in tomato can arise as a pleiotropic effect (Liu et al., 2004). The exploitation of these regulators for enhancing health-promoting phytochemicals in plants has been elegantly shown (Davuluri et al., 2005; Giliberto et al., 2005). It has been speculated that increased total plastid area in both chloroplasts and chromoplasts is the reason for increased carotenoid levels in these transgenic and mutant tomato plants (Cookson et al., 2003).

3.2. Metabolite interaction between and within pathways

Carotenoid biosynthesis is part of the plastidic terpenoid metabolism. The engineering of any terpenoid pathway may have a direct effect on other branches of the pathway. In the case of carotenoid overproduction, limitations may be encountered especially for the synthesis of the phytohormone gibberellin (with a negative effect on growth) or the prenyl side chains of chlorophyll and redox active quinones (with impairment of photosynthesis). Some insight into control points of the pathway as well as regulation/de-regulation of carotenogenesis comes from plants genetically modified in the carotenoid biosynthetic pathway. Over-expression of Psy-1 under a constitutive promoter in tomato or tobacco elevated the carotenoid content indicate that phytoene synthase exerts the greatest control of precursor flux into the carotenoid pathway (Fray et al., 1995; Busch et al., 2002). However, the expression resulted in altered chlorophyll content and a dwarf plant phenotype. This dwarf phenotype was due to the depletion of the endogenous precursor pool of GGPP leading to a shortage in gibberellins. Contrastingly in Psy-1 antisense plants in tissues where carotenoids were reduced gibberellins were elevated (Fraser et al., 1995). Recently, seed-specific over-expression of the endogenous phytoene synthase in *Arabidopsis* caused a 43-fold increase in β-carotene levels, but also resulted in delayed germination due to increased ABA levels (Lindgren et al., 2003). These data clearly illustrate the metabolic interactions between the different isoprenoid branches and their common precursors (Hemmerlin et al., 2003; Laule et al., 2003). Transformation of *A. thaliana* with a 1-deoxy-D-xylulose-5-phosphate gene resulted in plants with different levels of this enzyme that correlated with the amounts of plastidic isoprenoids including carotenoids (Estevez et al., 2001). Obviously, the supply of prenyl pyrophosphates is a limiting factor in isoprenoid formation. This result implies that a certain competition exists for the different plastidic biosynthetic branches utilising prenyl pyrophosphates. However, in tomato where both 3 hydroxy-3 methylglutaryl CoA reductase (HMGR-CoA) and 1-deoxy-D-xylulose-5-phosphate synthase (DXS) steps were up-regulated in the cytosolic and plastid IPP forming pathways, respectively, metabolite profiling of the isoprenoids provided evidence for no metabolic interaction (Enfissi et al., 2005).

Channelling of metabolites within the carotenoid pathway is influenced by enzyme activities relative to each other. Canola transformed with a triple construct containing genes for phytoene synthase and *crtI* from a bacterial source as well as lycopene cyclases of either plant or bacterial origin synthesised an increased amount of carotenoids as expected (Ravanello et al., 2003). In addition, the enhanced expression of lycopene β-cyclase diverted xanthophyll formation away from α-carotene and lutein, shifting incorporation towards β-carotene formation. Studies on the enzymes involved in phytoene formation from IPP in tomato (Fraser et al., 2000), *Narcissus* (Lutzow and Beyer, 1988) and *Capsicum* (Dogbo and Camara, 1987) have also shown that channelling of precursors arises and a physical association of enzymes is likely. Further evidence has also been ascertained from the characterisation of transgenic tomato plants expressing a bacterial phytoene synthase (*crtB*) plants where it is suggested that the enzyme cannot contribute to the products (Fraser et al., 2002).

Unexpected up-regulations of carotenogenic genes have been observed in several transgenic plants with a manipulated carotenoid biosynthetic pathway. This was first observed when tobacco was transformed with a *crtI* gene from *Erwinia uredovora* (Misawa et al., 1994). The resulting plants formed larger amounts of β-carotene and its derivatives at the expense of lutein that originates from α-carotene. An enhancement of β-carotene content in tomato fruit was also achieved after transformation with the *crtI* gene. In both cases the elevated β-carotene formation was unexpected since the genetic modification did not directly altered cyclisation of lycopene to β-carotene. In the *crtI* tomato fruits a transcriptional up-regulation of the endogenous lycopene cyclases was detected (Römer et al., 2000) which could in part be responsible for the increase in the β-carotene content. Although, β-carotene content was increased in the *crtI* tomato transformants, total carotenoid content of these fruits was reduced. It is postulated that feedback regulation by β-carotene or one of its metabolites is responsible. In potato tubers using antisense inhibition of Zep and

co-suppression by the corresponding sense construct, zeaxanthin concentration could be increased up to 130-fold (Römer et al., 2002). Besides the anticipated elevation in zeaxanthin, the amount of total carotenoids present in the potato tubers was also increased significantly due to the observed transcriptional up-regulation of phytoene synthase in the transgenics lines. In rice endosperm, formation of β-carotene resulted from the expression of the *Narcissus* phytoene synthase and the *Erwinia crtI* gene but without the expression of a lycopene cyclase gene (Ye et al., 2000). However, this phenomenon has been shown not to be due to up-regulation of endogenous cyclase activity (Schaub et al., 2005).

3.3. Carotenoid formation during maturation of proplastids into chloroplasts

In etiolated leaves, etioplasts are formed instead of chloroplasts, to which they are transformed upon illumination, also referred to as de-etiolation. During this transition, the carotenoid content is strongly increased (Frosch and Mohr, 1980). In corn seedlings the increase of carotenoid content is very rapid and is paralleled by enhanced IPP isomerase activity (Albrecht and Sandmann, 1994). Up-regulation of several carotenoid biosynthetic genes in the process of light-dependent etioplast to chloroplast transition has been found in various plant species. During de-etiolation of tomato and mustard seedlings the expression level of phytoene synthase was increased, whereas the amounts of GGPP synthase and Pds transcripts in mustard were relatively constant (Giuliano et al., 1993; von Lintig et al., 1997). Phytoene synthase gene expression was shown to be under phytochrome control and to influence prolamellar body formation (Welsch et al., 2000). Recently, the analysis of the phytoene synthase promoter of *A. thaliana* allowed the identification of promoter regions responsible for the modulation to different light qualities (Welsch et al., 2003). In accordance, an involvement of phytochrome in the regulation of various xanthophylls biosynthetic genes during de-etiolation was postulated by Woitsch and Römer (2003) after exposure of tobacco seedlings to different light qualities. Moreover, different light quantities were shown to affect the transcript level of β-carotene hydroxylase. Evidence for a redox control of some of the xanthophylls biosynthetic genes by the plastoquinone pool was derived from studies using photosynthetic electron transport inhibitors (Woitsch and Römer, 2003).

Etioplast contain carotenoids to protect chlorophyll once it is synthesised. As pointed out above, plastoquinone acts as hydrogen acceptor for desaturations in carotenoid biosynthesis. Since etioplasts lack the photosynthetic electron transport chain for plastoquinone regeneration, another enzyme, a plastid terminal oxidase Pto, is essentially involved in carotenogenesis (Carol et al., 1999). After de-etiolation, Pto still acts as a cofactor in carotenoid biosynthesis but is not crucial any more.

3.4. Chloroplast to chromoplast transition

Chromoplasts are typically found in fruit and flowers. They originate by differentiation of chloroplasts (Marano et al., 1993). This process is accompanied by a modification of the internal ultrastructure, a decrease of chlorophyll content and an increase of carotenoids (Emter et al., 1990). Especially in fruit, the carotenoid pattern may also be qualitatively changed.

3.4.1. Regulation during flower development

Carotenogenesis during flower development is best studied in *Tagetes erecta* and *Gentiana lutea*. The carotenoid found in *T. erecta* petals is lutein and lutein fatty acid esters. Varieties are known with a low or an extremely high content varying in flower coloration from light yellow to dark orange flowers. Transcript analysis of most of the carotenogenic genes and of some of the 1-deoxy-D-xylulose-5-phosphate pathway showed a correlation between carotenoid content and the m-RNA levels of 1-deoxy-D-xylulose-5-phosphate synthase and phytoene synthase (Moehs et al., 2001). The greatest enhancements of transcript amounts in the last stage of flower development were found for the ε-cyclase and the β-carotene hydroxylase.

During the development of *G. lutea* flowers, an increase in petal carotenoids is observed which is accompanied by a shift from α-carotene derived lutein to xanthophyll originating from β-carotene (Zhu et al., 2003). In addition, chlorophyll content is strongly decreased. The entire regulation of carotenogenesis is under transcriptional control. Following floral development, the highest increases in messenger levels (6- to 7-fold) were observed for *Psy* and *Zds* (Zhu et al., 2002). In addition, transcript levels of all genes related to β-carotene formation and metabolism (lycopene β-cyclase, β-carotene hydroxylase) increased by a factor of about 2. In contrast, the lycopene ε-cyclase transcript declined to less than half. These antagonistic changes of transcripts modulate the relative abundance of carotenoids from the α- or β-biosynthetic branch. They are rather moderate compared to the up-regulation of phytoene synthase which determines the metabolite flow into the carotenoid pathway.

Although the GGPP synthase reaction provides the direct precursor for carotenoid biosynthesis, the transcript levels of *GGPPS* are decreasing over the different steps of flower development in *G. lutea* (Zhu et al., 2003). This may be explained by an almost exclusive availability of the GGPP pool for carotenoid synthesis after chlorophyll formation is stalled and the phytol demand is negligible.

In tomato flower petals, the carotenoid contents increases during development by almost 10-fold (Giuliano et al., 1993). Concurrently, the mRNA levels of the initial enzyme Psy as well as of Pds, the second enzyme of the pathway, were strongly increased. In studies on the *pds* promoter fused to a reporter gene, high level protein expression was determined in flower petals and anthers

(Corona et al., 1996). Here, a co-ordinated up-regulation of at least two genes at the transcriptional level was demonstrated. For daffodil flowers, evidence for post-transcriptional enzyme activation was found. The activation of phytoene synthase (Schledz et al., 1996) as well as Pds (Al-Babili et al., 1996) may be involved in regulation of carotenogenesis during chromoplast formation.

3.4.2. Regulation during fruit development

In most systems where fruit development and ripening occurs, carotenoid formation and regulation is distinct from that arising in vegetative tissue. At the cellular level, chloroplasts typically evolve into chromoplasts. These organelles are associated with high carotenoid accumulation. During tomato fruit ripening, a massive accumulation of lycopene occurs. In addition, phytoene, ζ-carotene and phytofluene accumulate while xanthophylls diminish (Fraser et al., 1994). Determination of metabolic flux coefficients indicate that phytoene synthase has the greatest influence over the pathway flux, while cyclisation is reduced (Fraser et al., 2002). This profile of events is mimicked at the gene expression level suggesting that the regulation of carotenoid formation in tomato fruit is predominantly controlled at the transcriptional level (Fraser et al., 1994). Expression of phytoene synthase-1 and desaturase are significantly increased at the onset of ripening, while the cyclases are down regulated (Ronen et al., 1999; Ronen et al., 2000). Thus lycopene accumulation in tomato fruit arises from an increase of flux through the initial stages of the pathway and a restriction to end-products that are typically found in vegetative tissues. Chromoplast development is also an important aspect of *Capsicum* ripening (reviewed in Camara et al., 1995). Similar to tomato, a quantitative and qualitative change in carotenoid composition arises as ripening proceeds. Expression of carotenoid biosynthetic genes are increased concurrently with the ripening process indicating that transcriptional regulation is an important aspect controlling carotenoid formation in this tissue (Römer et al., 1993). In addition, ripe *Capsicum* fruit synthesise capsanthin and capsorubin which are carotenoids not found in vegetative tissues. Esterification of carotenoids also arises in ripe *Capsicum* fruit (Camara et al., 1995).

4. Successful attempts to engineer carotenoid content and composition in edible crops

Despite the numerous examples of unintended/pleiotropic effects resulting from the manipulation of carotenoid formation in plants it has been shown that both nutritionally important and high-value carotenoids can be altered and generated in crop plants. Examples of these successful engineering of carotenoids in crop plants are provided in Table 1. Detrimental effects resulting from perturbations in related biochemical pathways have to a certain extent been overcome by the use of tissue specific promoters, for example the expression of a phytoene synthase under the control of a fruit ripening specific promoter and seed specific promoter in the case of tomato and canola respectively (Fraser et al., 2002; Shewmaker et al., 1999). This has lead to the expression of lycopene cyclases and β-carotene hydroxylases (Rosati et al., 2000; Dharmapuri et al., 2002). Overcoming genetic effects such as gene silencing/co-suppresion has also been achieved by the use of non-homologous target genes. Nevertheless, high β-carotene formation has been achieved by over-expression of an endogenous lycopene β-cyclase gene in tomato under a constitutive promoter (d'Ambrosio et al., 2004). The *Erwinia uredovora* gene cluster have proven very important in this respect, with the generations of potato, tomato and canola varieties expressing *Erwinia* derived biosynthetic genes (Ducreux et al., 2005; Römer et al., 2000; Fraser et al., 2002; Shewmaker et al., 1999; Ravanello et al., 2003). It is also importamt to note that the phenotypes are stable over numerous generations with these non-homologous genes. The production of high zeaxanthin potato tuber has also been successfully achieved by down-regulating metabolism of zeaxanthin (Römer et al., 2002). The most publicised example of genetic manipulation of the carotenoid pathway is the so-called "Golden Rice" (Ye et al., 2000). This strategy is now beyond proof of concept and entered the develop stage. Golden rice II has now been reported and the levels now reached will provide the recommended daily allowance (RDA) of β-carotene in one average daily portion of rice (300 g), although this does not take into account the bioavailablity from starchy material. To achieve this, a systematic approach was used to evaluate different phytoene synthases helped to overcome the rate limiting step in the formation of carotenoids in golden rice (Paine et al., 2005).

Through this review, the principle focus has been on the manipulation of specific steps in the pathway. Recent publications relating to the manipulation of light signal transduction pathway components (Liu et al., 2004; Davuluri et al., 2005) and photoreceptors (Giliberto et al., 2005) have illustrated the power of these global effects on pathways. For example the fruit specific silencing of *DET*-1 (*DE-ETIOLATED*-1) gene in tomato has lead to significant increases in carotenoids and other health-promoting phytochemicals such as phenolics and flavonoids (Davuluri et al., 2005). Similar findings have been reported following the overexpression of the tomato cryptochrome 2 (*cry-2*) gene product in tomato (Giliberto et al., 2005).

Besides improving nutrition properties and protection against abiotic stresses, several carotenoids are high-value fine chemicals. For example ketocarotenoids such as canthaxanthin and astaxanthin are used as feed supplements. In the case of aquaculture the cost of astaxanthin addition to feed can represent 15% of the total costs farming costs (Lorenz and Cysewski, 2000). In several attempts, bacteria have been engineered as biological systems for the production of various carotenoids (see reviews by Sandmann, 2002; Mijts and Schmidt-Dannert,

Table 1
Genetic modification for increase of carotenoids in edible crops

Carotenoid	Modification	Promoter	Estimated content in μg/g Dw (increase versus WT)	Ref.
β-Carotene				
Canola	te *crtB*	ss	1180[a] (314-fold)	(1)
Rice	te *psy, crtI*	ss	89[b]	(2)
Tomato	te *crtI*	fs	520 (1.9-fold)	(3)
Tomato	ee, *lcy*-b	ub	2050[c] (31.7-fold)	(4)
Tomato	te *lcy*-b	fe	546[c] (7-fold)	(5)
Tomato	te *cry*-2	fe	101 (1.3-fold)	(6)
Tomato	as *det*-1	fs	130[c] (8-fold)	(7)
Lycopene				
Tomato	te *crtB*	fs	2000 (1.5-fold)	(8)
Tomato	as *lcy*-b	fe	970 (1.3-fold)	(4)
Tomato	te *cry*-2	fe	1353 (1.7-fold)	(5)
Tomato	as *det*-1	fs	1227[c] (2.3-fold)	(6)
Zeaxanthin				
Potato	as *zep*	ts	40 (137-fold)	(7)
Tomato	te *lcy-b* + *bhy*-b	fe	130[c]	(8)
Ketocarotenoids[d]				
Potato	te *bkt*-1	ts	14[b,e]	(9)
	te *crtO*	ub	4[b]	(10)

Abbreviations: as, antisense and sense inactivation; ee, endogenous gene overexpressed; fs, fruit specific; ss, seed specific; te, trans gene expression; ts, tuber specific; ub, ubiquitous; WT, wild type.
References (1) Shewmaker et al. (1999); (2) Paine et al. (2005); (3) Römer et al. (2000); (4) d'Ambrosio et al. (2004); (5) Rosati et al. (2000); (6) Giliberto et al. (2005); (7) Davaluri et al. (2005); (8) Dharmapuri et al. (2002); (9) Morris et al. (2005); (10) Gerjets and Sandmann (2005).
[a]Assuming a water content of 20%.
[b]Wild-type tissue devoid of this carotenoids.
[c]Assuming a water content of 90%.
[d]Wild-type tissue devoid of this carotenoids including 3′-hydroxyechinenone, 4-ketozeaxanthin, ketolutein, and astaxanthin.
[e]Wild-type tissue devoid of this carotenoids including 3′-hydroxyechinenone, 4-ketozeaxanthin, ketolutein, and astaxanthin in *Solanum phureja*.

2003). However, higher plants are a cost-efficient competitive source for secondary products (Giddings et al., 2000) and their yield of metabolites can also be improved by genetic enhancement of biosynthetic capacity (Lessard et al., 2002). Several examples have been reported where the gene products responsible for astaxanthin formation have been expressed in higher plants (Mann et al., 2000; Stalberg, et al., 2003; Ralley, et al., 2004; Morris et al., 2005; Gerjets and Sandmann, 2005). Interestingly, accumulating ketocarotenoids were typically localised to specific plant tissues and in most cases resided in esterified forms. Thus it would appear that the plant has endogenous mechanisms for sequestering excess or altered carotenoids.

5. Metabolisation and degradation

The amount of carotenoids found in a plant tissue represents a steady-state value determined by biosynthesis and metabolisation. Control of the degradation processes may assist in maintaining high carotenoid concentrations. In addition to non-enzymatic degradation especially by the invasive effect of light, defined enzymatic cleavage reactions are known. The products of these oxidative reactions have roles as phytohormones, defence compounds and volatile aromas. Furthermore, carotenoid cleavage products may act as signalling molecules associated with branching (Sorefan et al., 2003) and have antifungal properties (Fester et al., 1999). Recently, a number of genes encoding non-heme iron oxygenases involved in oxidative cleavage of carotenoids have been identified (for review see Giuliano et al., 2003) indicating that at least some of the carotenoid degradation products originate from enzymatic reactions. It is now possible to use these genes for the production of apocarotenoids such as the pigments bixin and crocetin as well as carotenoid-derived aroma compounds.

5.1. Enzymatic carotenoid cleavage

Cleavage enzymes and their respective genes have been classified according to their carotenoid substrate and the cleavage position within the carotenoid molecule.

9-cis-Epoxy hydroxy carotenoid dioxygenases (Nced) cleaving at C-11,12. The maize *viviparous* 14 (*vp*14) (see Schwartz et al., 2003 for review) and tomato *notabilis* (*not*, Burbidge et al., 1999) genes encode *Nceds*. Characterisation of respective mutants has revealed their involvement in the formation of abscisic acid. In vitro the Vp14 enzyme displays oxidative cleavage of 9-*cis* violaxanthin, 9-*cis* neoxanthin and 9-*cis* zeaxanthin at the 11,12 double bond

to form xanthoxin, a precursor of abscisic acid. The common structural elements in all substrate molecules mentioned above are the individual 3-HO-5,6-epoxy substituted β-ionone end groups of carotenoids which are recognised by the dioxygenase enzyme. Furthermore, there is a strict requirement for a 9-*cis* double bond (Fig. 3).

Carotenoid dioxygenase (Ccd) cleaving at C-9,10. An *Arabidopsis Ccd* has been functional characterised in *E. coli* by complementation and shown to have an extremely broad substrate specificity for acyclic, cyclic and hydroxylated carotenoids cleaving these carotenoids at the 9,10, (9′,10′) position (Schwartz et al., 2001). A cDNA for a similar *Ccd* has also been cloned from *Crocus sativus* together with a 7,8 (7′,8′) cleavage enzyme using zeaxanthin as a substrate designated *Zcd* (Bouvier et al., 2003a) (Fig. 3). Interestingly, immuno-localisation indicated that the *Crocus* Ccd protein was localised in the cytoplasm while the Zcd was plastid located (Bouvier et al., 2003a). The 9,10 (9′,10′) dioxygenase is responsible for the formation of saffron aroma compounds whereas the 7,8 (7′,8′) dioxygenase appear to be involved in the formation of crocetin. Zcd expression is restricted to the style tissue of *Crocus* and is enhanced by draught stress. In contrast, Ccd is constitutively expressed in flowers and leaves. In the tomato genome, two genes encoding 9,10 (9′,10′) cleaving Ccd-type dioxygenases have been identified (Simkin et al., 2004). One of which is highly expressed in ripening fruit. Their proteins are not plastid localised. Different cyclic and acyclic C_{40} carotenoids are converted as substrates yielding a C_{14} dialdehyde and two C_{13} cyclohexones. Antisense inhibition of the *ccd* genes resulted in a decrease of volatile compounds such as β-ionone from β-carotene and geranylacetone derived from lycopene (Fig. 3).

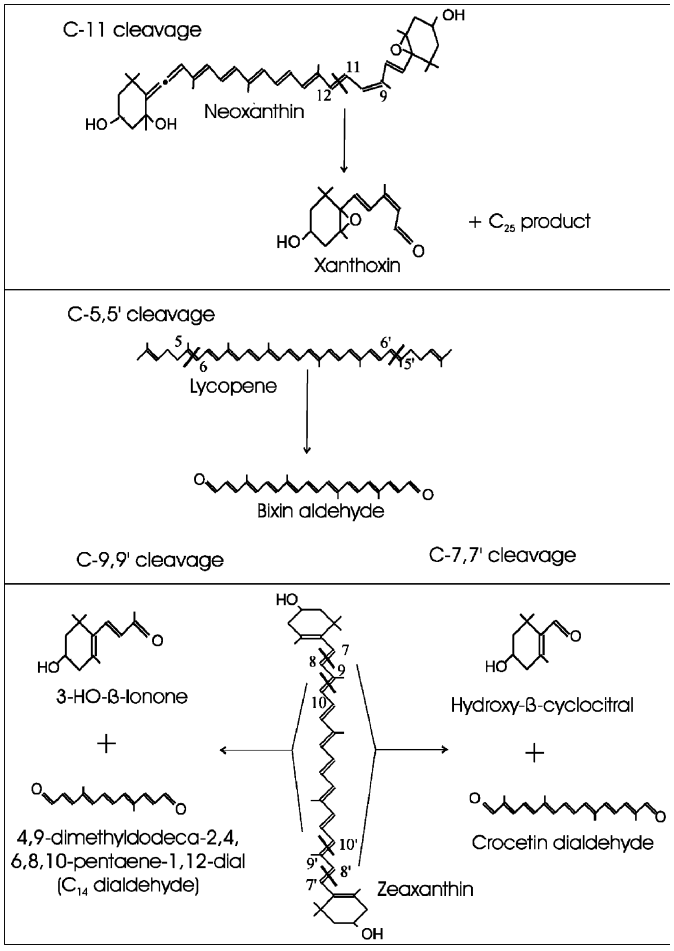

Fig. 3. Enzymatic cleavage of various carotenoid substrates: of 9-*cis*-neoxanthin at C-11, of lycopene at C-5 and C-5′ and of zeaxanthin at C-9 and C-9′ or C-7 and C-7′.

Zeaxanthin dioxygenase (Zcd) cleaving at C-7,8. The cDNA of this dioxygenase has been cloned from *Crocus sativus*. The enzyme specifically cleaves zeaxanthin to hydroxyl-β-cyclocitral and crocetin dialdehyde (Bouvier et al., 2003a). The latter is a precursor of crocetin glycosides.

Lycopene dioxygenase (Lcd) cleaving at C-5,6. Recently, the cleavage of lycopene but not β-carotene or zeaxanthin at positions 5,6 and 5′,6′ has been demonstrated (Bouvier et al., 2003b) yielding bixin aldehyde (Fig. 3). The cDNA encoding the enzyme was isolated from seeds of the tropical tree *Bixia orellana*. The gene product is the first enzyme in the biosynthesis of bixin. The cloning of the *lcd* cDNA from developing *B. orellana* seeds was based on the similarity to other carotenoid cleavage enzymes by using degenerate oligonucleotides designed for conserved regions of carotenoid oxygenases.

5.2. Non-enzymatic oxidation

Physical quenching of excited molecules by carotenoids is well established. However, chemical quenching resulting in irreversible carotenoid modification is an additional process. The dominant degradations of carotenoids are by photooxidation in green tissue. The degradation reactions are determined by the chemistry of the individual carotenoid molecule. Obviously, β-carotene is the carotenoid most susceptible to chlorophyll photosensitise as well as radical reactions. This may be due to its presence and exposure in both photosystems. The only detectable oxidation product of β-carotene in leaves and chloroplasts is β-carotene-5,6-epoxide in addition to lutein-5,6-epoxide. These compounds are optical inactive indicating their non-enzymatic origin (Young et al., 1989). The extent of the formation of β-carotene-5,6-epoxide in leaves correlates with the degree of light exposure (Ashikawa et al., 1987). The amount of carotenoid photooxidative degradation in green pepper leaves under high light (intensity below a level causing photoinhibition) was estimated to 1 mg per day (Simkin et al., 2003a). This loss exceeds the degree of permanent biosynthesis resulting in lower steady state levels.

The fate of β-carotene-5,6-epoxide and other oxidation products in the leaves, e.g., by exhaustive oxidation is very poorly understood. Analysis of β-carotene oxidation in in vitro model systems by singlet oxygen indicate that the 5,6-epoxide and the 5,8-endoperoxide are the major reaction

products (Yamauchi et al., 1998). Other oxidation products are the 5,8,5′,8′-diepoxide and related compounds with 4 and 6 oxygen atoms (Fiedor et al., 2001). Further oxidation leads to the break down of the C_{40} skeleton to shorter degradation products which are difficult to analyse. In vitro β-carotene modification to apo-carotenals of variable chain length from β-apo-14′-carotenal to β-apo-13-carotenone could be demonstrated (Henry et al., 2000). Also acyclic carotenes are susceptible to photooxidation. Phytoene accumulated in the presence of an inhibitor of the biosynthetic pathway was photooxidised to a 1,2-epoxide and to hydroxy derivatives (Albrecht et al., 1991) by attacking preferentially the isolated double bonds (Albrecht and Sandmann, 1995). Also during radical-mediated oxidation of carotenoids, the double bonds of the polyene chain are highly susceptible to radical addition reactions. For example, C-11 and C-15 formyl β-carotene together with cyclic ethers are formed during radical-mediated co-oxidation with methyl lineolate (Yamauchi et al., 1998). In a system where linoleoyl peroxide radicals were generated by lipoxygenase, a broad variety of β-carotene degradation products were detected (Wu and Robinson, 1999). They include 5,6- and 5,8-epoxy apocarotenals cleaved at the alkene chain at C-8′ C-10′, C-12′ and C-14′. In addition, C_{12} and C_{17} dicarbonyl compounds were identified as secondary oxidation product of the cyclohexane ring.

6. Conclusion

Plant tissues like flower petals and fruit are used as conventional production systems for carotenoids. The yield of certain carotenoids for feed and human nutrition can be increased by genetic optimisation of crop and production plants. The feasibility of this metabolic engineering approach has been demonstrated by several examples: For example, the amount of pre-existing β-carotene has been increased in rape seed and β-carotene biosynthesis has been engineered into rice endosperm, a tissue devoid of carotenoids (Table 1). The carotenoid pathway of tomato fruit and potato has been shifted to end products of higher nutritional value. In all cases of genetic engineering of the carotenoid pathway unintended effects have arisen. These include the co-ordinated expression of the *trans* gene with the other pathway components, the metabolic channelling within the pathway hindering end-product formation, interference with pathway regulation and the sequestering of the carotenoid products. The later problem may be overcome by choosing appropriate plant tissues with high storage capacity for carotenoid overproduction.

Progress has been made towards a better understanding of the regulation of the carotenoid pathway. Most knowledge results from transcript analysis in transgenic and non-transgenic plants. Several control steps of the carotenogenic pathway have been revealed (Fig. 4). In most cases, these are the initial reactions like formation of 1-deoxy-D-xylulose-5-phosphate synthase by Dxs in the general terpenoid pathway to prenyl pyrophosphates or the

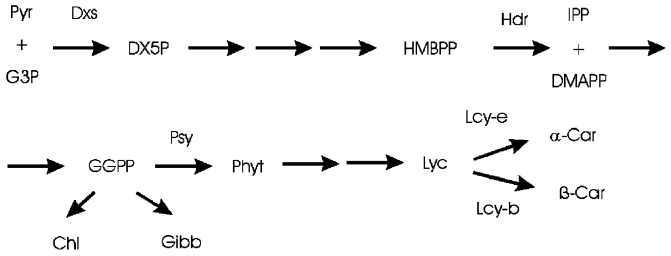

Fig. 4. Metabolic steps controlling prenyl pyrophosphates synthesis and their metabolisation to carotenoids. Pyr, pyruvate; G3P, glyceraldehyde 3-phosphate; DX5P, 1-deoxy-D-xylulose-5-phosphate; HMBPP, hydroxymethylbutenyl pyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; Phy, phytoene; Lyc, lycopene; α-Car, α-carotene; β-Car, β-carotene; Dxs, DX5P synthase; Hpr, HMBPP reductase; Psy, Phy synthase; Lcy-e, lycopene ε-cyclase; Lcy-b, lycopene β-cyclase.

phytoene synthesis, the first committed step to carotenoids catalysed by Psy. Also at the branch point to α- and β-carotene the cyclisation of lycopene, the relative activities of lycopene cyclases Lcy-e and Lcy-b determine the product composition. One should also account for competitive effects on common substrates used in biosynthesis of other terpenoids. This is the case for GGPP which is not only a direct precursor for carotenoids but also for the synthesis of gibberellins and the chlorophyll prenyl side chain. Optimisation of carotenoid composition can now be attempted by a co-ordinated approach to overexpress the genes catalysing these reactions or by additional introduction of foreign carotenogenic genes responsible for the formation of new carotenoids. With the current knowledge on carotenoid biosynthesis, the prospects are good for the metabolic engineering of food crops enriched with provitamin A and other carotenoids like zeaxanthin and lutein which are essential functional components of the human eye. Furthermore, plants have a potential as a factory for carotenoids with a pharmaceutical potential. This may be the case for some apocarotenoids for which several genes have been recently cloned. Following the initial proof of concept approach, Golden Rice has been developed further (e.g., Golden Rice II) so that the levels are now at those required to supply enough provitamin A, in order to overcome vitamin A deficiency (Paine et al., 2005). Finally the manipulation of light signal transduction pathways has shown the potential of altering gene products with the potential of altering multiple steps or pathways. This work also utilises endogenous plant genes and therefore the application of technologies such as Tilling offers the opportunity to breed these high carotenoid traits into crop plants without the use of GM technology (Slade and Knauf, 2005).

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