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Review

Supply of precursors for carotenoid biosynthesis in plants

Manuel Rodríguez-Concepción *

Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB, Jordi Girona 18, 08034 Barcelona, Spain

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ABSTRACT

Carotenoids are isoprenoids of industrial and nutritional interest produced by all photosynthetic organisms, including plants. Too often, the metabolic engineering of plant carotenogenesis has been obstructed by our limited knowledge on how the endogenous pathway interacts with other related metabolic pathways, particularly with those involved in the production of isoprenoid precursors. However, recent discoveries are providing new insights into this field. All isoprenoids derive from prenyl diphosphate precursors. In the case of carotenoids, these precursors are produced predominantly by the methylerythritol 4-phosphate (MEP) pathway in plants. This review focuses on the progress in our understanding of how manipulation of the MEP pathway impacts carotenoid biosynthesis and on the discoveries underlining the central importance of coordinating the supply of MEP-derived precursors with the biosynthesis of carotenoids and other derived isoprenoids.

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Introduction

Carotenoids are a major group of plant pigments responsible for the yellow color of corn and bananas, the orange color of carrots, pumpkin, and oranges, and the red color of tomato and watermelon. In these organs, carotenoids accumulate in specialized plastids, the chromoplasts. High carotenoid levels are also found in the chloroplasts of photosynthetic tissues, where their distinctive yellow to red colors are masked by chlorophylls. They however become visible as the autumn colors of many leaves when chlorophylls are degraded. The characteristic yellow color of the cotyledons of dark-grown (etiolated) seedlings is also due to the presence of carotenoids in etioplasts. But the functions of plant carotenoids go beyond their role as pigments. Etioplast carotenoids appear to facilitate the transition to photosynthetic development upon illumination [1,2]. In chloroplasts, carotenoids act as membrane stabilizers and accessory light-harvesting pigments, and they are essential to channel excess energy away from chlorophylls for protection against photooxidative damage [3]. Furthermore, in these and other plastid types such as leucoplasts carotenoids serve as precursors of important growth regulators including abscisic acid and strigolactones [4,5].

Carotenoids are extensively used as natural pigments in the food, feed, and cosmetic industries, and their presence in the human diet provides health benefits as an important source of retinoids (including vitamin A) and antioxidants [6–8]. Mainly because of these health benefits, it is expected that the future demand for carotenoids of interest will exceed the levels that can be currently achieved by chemical synthesis or extraction from natural sources. A solution could be the biotechnological overproduction of carotenoids in plants. Metabolic engineering strategies based on overexpression of biosynthetic enzymes have actually been shown to be useful to increase carotenoid levels in transgenic plants [6,7,9]. However, there are still problems to be solved for an efficient biotechnological production of these pigments. Among them, the availability of metabolic precursors has been shown to limit the production of carotenoids in different plant systems [10–15]. Here I will review the current knowledge on how these precursors are synthesized and how their supply is coordinated with the production of carotenoids in plant cells.

Pathways involved in the production of carotenoid precursors

Like all isoprenoids, carotenoids derive from the five-carbon prenyl diphosphates isopentenyl diphosphate (IPP) and its double-bond isomer dimethylallyl diphosphate (DMAPP).[1] Until relatively recent times, it was believed that IPP was synthesized from acetyl-CoA via mevalonic acid (MVA) and then isomerized to DMAPP in all living organisms [16,17]. In the mid 1990s, however, compelling evidence for a completely novel pathway for the production of IPP and DMAPP was obtained (reviewed in [18–20]). This new pathway is currently known as the methylerythritol 4-phosphate (MEP) pathway [21,22]. It is now well established that the MEP pathway

* Fax: +34 932045904.
E-mail address: mrcgmp@cid.csic.es

[1] *Abbreviations used:* DMAPP, dimethylallyl diphosphate; DXP, deoxyxylulose 5-phosphate; DXR, DXP reductoisomerase; DXS, DXP synthase; GGPP, geranylgeranyl diphosphate; HDR, hydroxymethylbutenyl diphosphate synthase; IPP, isopentenyl diphosphate; MEP, methylerythritol 4-phosphate; PSY, phytoene synthase.

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is the only one present in most eubacteria and apicomplexan protozoa such as the malaria parasite, whereas archaebacteria, fungi and animals (including humans) only use the MVA pathway. By contrast, plants use both the MVA and the MEP pathways for the production of their universal isoprenoids precursors [22,23]. In plant cells, the MVA pathway synthesizes cytosolic IPP, which can be transported to mitochondria for the biosynthesis of mitochondrial isoprenoids [24]. In the plastid, IPP and DMAPP are synthesized by the MEP pathway (Fig. 1). Despite this compartmentation, there is ample evidence of a limited exchange of common precursors between the cytosol and plastids [23,25–32]. The mechanism regulating this exchange, however, remains unknown. The presence of putative transporters of prenyl diphosphates in the plastid envelope has been reported in several plant systems [25,33,34] but it is likely that regulating the contribution of the MVA and MEP pathways to the production of particular isoprenoids requires more than a simple shuttle of metabolites between the cytosol and the plastid [31]. The extent of the exchange can be manipulated by supplying intermediates of the MVA or the MEP pathways exogenously and is sensitive to metabolic, environmental, and developmental cues [27,28,31,32,35]. However, the exchange rate is not high enough to rescue the production of plastidial isoprenoids when the MEP pathway is pharmacologically or genetically blocked or of cytosolic and mitochondrial isoprenoids when the MVA pathway is inhibited [10,30,32,36–45].

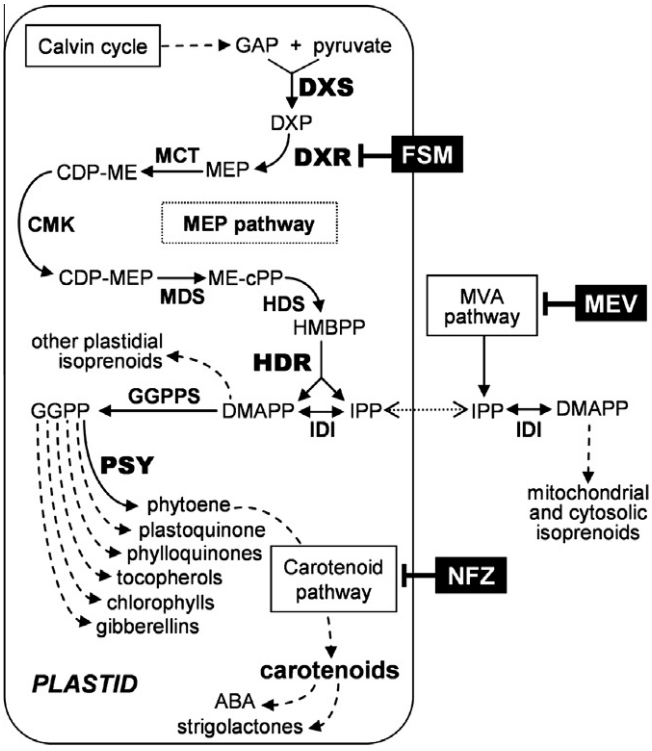

Fig. 1. Pathways involved in the production of carotenoids in plant cells. ABA, abscisic acid; CDP-ME, 4-diphosphocytidyl-methylerythritol; CDP-MEP, 4-diphosphocytidyl-methylerythritol 2-phosphate; DMAPP, dimethylallyl diphosphate; DXP, deoxyxylulose 5-phosphate; GAP, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl diphosphate; HBMPP, hydroxymethylbutenyl 4-diphosphate; IPP, isopentenyl diphosphate; ME-cPP, methylerythritol 2,4-cyclodiphosphate; MEP, methylerythritol 4-phosphate; MVA, mevalonic acid. Enzymes are indicated in bold: CMK, CDP-ME kinase (EC 2.7.1.148); DXR, DXP reductoisomerase (EC 1.1.1.267); DXS, DXP synthase (EC 4.1.3.37); GGPPS, GGPP synthase (EC 2.5.1.29); HDR, HMBPP reductase (EC 1.17.1.2); HDS, HMBPP synthase (EC 1.17.4.3); IDI, IPP isomerase (EC 5.3.3.2); MCT, MEP cytidylyltransferase (EC 2.7.7.60); MDS, ME-cPP synthase (EC 4.6.1.12); PSY, phytoene synthase (EC 2.5.1.32). The steps and pathways blocked by the specific inhibitors fosmidomycin (FSM), mevinolin (MEV) and norflurazon (NFZ) are indicated. Dashed arrows indicate multiple steps.

As a consequence of the limited exchange of isoprenoid precursors among cell compartments, sterols, brassinosteroids, sesquiterpenes, and polyprenols are mainly (but not exclusively) formed from MVA-derived precursors, whereas the MEP pathway provides most (but not all) precursors for the production of gibberellins, monoterpenes, isoprene, and the side chain of chlorophylls, tocopherols, phylloquinones, and plastoquinone. Several lines of evidence indicate that carotenoids are formed predominantly, but not exclusively, from MEP-derived precursors. Mutant Arabidopsis seedlings with a complete block in the MEP pathway have been shown to still accumulate low levels of carotenoids, suggesting an import of cytosolic MVA-derived isoprenoid precursors to the plastids [32,41,46,47]. In agreement, feeding with labeled MVA resulted in labeling of carotenoids in seedlings [28,30]. However, overproduction of the MEP pathway enzymes deoxyxylulose 5-phosphate (DXP) synthase (DXS), DXP reductoisomerase (DXR), or hydroxymethylbutenyl diphosphate (HMBPP) synthase (HDR) in transgenic Arabidopsis plants and tomato fruit led to increased carotenoid levels, whereas no changes were observed when flux through the MVA pathway was up-regulated [10,12,13,36]. Additionally, block of the MEP pathway with the specific inhibitor fosmidomycin generates a phenotype in seedlings germinating in the light and in tomato fruit very similar to that observed when the production of carotenoids is specifically inhibited with norflurazon, whereas no impact in carotenoid accumulation is observed when the MVA pathway is blocked with mevinolin (Fig. 2). Together, the MEP pathway appears to be the main pathway provid-

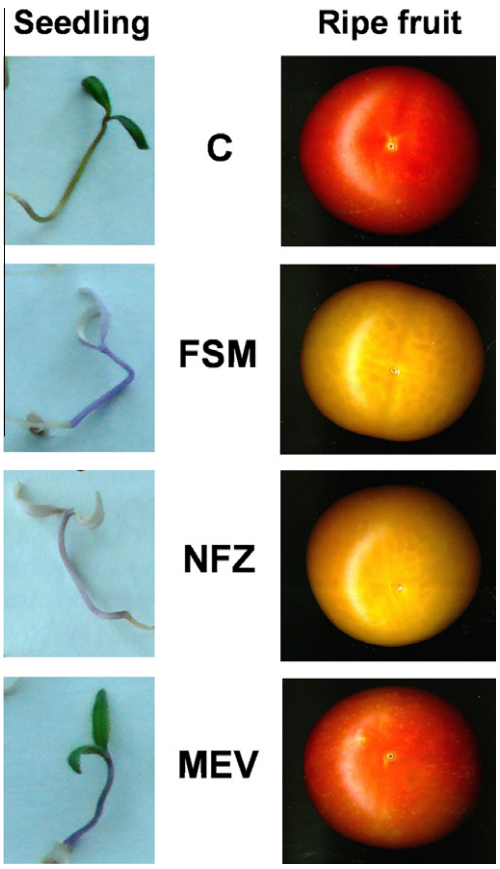

Fig. 2. Phenotypes of tomato seedlings and fruit after treatment with inhibitors. Panels on the left show representative tomato seedlings germinated and grown in the light for one week in the absence (C) or presence of the indicated inhibitor (see Fig. 1). Panels on the right show representative fruit injected at the mature green stage with the inhibitors or a mock solution (C) and collected two weeks later, at the ripe stage.

ing the metabolic precursors for plant carotenoids under normal conditions.

The MEP pathway and beyond

The enzymes of the MEP pathway (Fig. 1) are encoded by nuclear genes and targeted to plastids [22,23]. Rules for the nomenclature of the plant MEP pathway genes, enzymes, and mutants have been recently proposed [21]. The initial reaction of the MEP pathway, catalyzed by DXS, is the condensation of (hydroxyethyl)thiamin derived from pyruvate with the C1 aldehyde group of glyceraldehyde 3-phosphate (GAP) to produce DXP. In the second step, DXR synthesizes MEP by an intramolecular rearrangement and reduction of DXP. MEP then is converted into HMBPP in four enzymatic steps (Fig. 1). HMBPP is finally converted to a mixture of IPP and DMAPP by HDR. The progress in the analysis of these and other MEP pathway enzymes, their role in the control of MEP pathway flux, and their regulation at the transcriptional and post-transcriptional levels have been recently covered elsewhere [21,48–50].

DXS and, to a lower extent, DXR and HDR have rate-determining roles in the control of the MEP pathway flux in plants [10,12–15,36,51–53]. In most plants, DXS is encoded by a small gene family with members of at least two classes [54,55]. Class 1 DXS isozymes proposedly participate in the biosynthesis of essential or housekeeping isoprenoids, whereas class 2 DXS enzymes appear to be associated to the production of secondary isoprenoids. Exceptions include the presence of exclusively class 1 sequences in Arabidopsis and soybean [21,56] and the existence of a third class of DXS sequences in maize, sorghum and rice [14,57]. All three classes of DXS enzymes participate in carotenoid biosynthesis in different plant systems. Thus, carotenoid accumulation correlates with the expression of genes encoding class 1 isoforms in photosynthetic tissues [54] and ripening fruit [11,58], class 2 isoforms in mycorrhizal fungi-colonized roots [54,59,60] and flower petals [61,62], and class 3 isoforms in seed endosperm tissues [14]. The rest of the MEP pathway enzymes are encoded by a single gene in many plants (including those with a fully sequenced genome, such as Arabidopsis and rice). However, there are reports of plants harboring more than one gene encoding the flux-controlling enzymes DXR [63] and HDR [64,65]. No information is available yet regarding the involvement of particular isoforms in carotenoid biosynthesis.

IPP and DMAPP can be interconverted by the activity of the enzyme IPP/DMAPP isomerase (IDI). In plant cells, IDI is required for the production of DMAPP in the cytosol and likely the mitochondria but not in plastids, where the MEP pathway produces both IPP and DMAPP [66]. IDI is encoded by small gene families in plants, with two genes present in Arabidopsis [67–71]. A plastidial localization has been found for the Arabidopsis IDI1 isoform but mutants specifically defective in this enzyme do not show changes in carotenoid levels, consistent with the dispensable role of this enzyme in plastids [67,69]. Addition of three IPP units to one DMAPP molecule results in the production of geranylgeranyl diphosphate (GGPP) in a reaction catalyzed by GGPP synthase (GGPPS) enzymes. GGPP is converted to phytoene by phytoene synthase (PSY) in the first committed reaction of the carotenoid pathway (Fig. 1). But GGPP is also the precursor of other plastidial isoprenoids such as gibberellins and the side chain of chlorophylls and tocopherols [23]. The presence of several genes encoding putative GGPPS enzymes in most plants (more than 10 in Arabidopsis [71]) suggests that different isozymes might be involved in the production of specific groups of isoprenoids (including carotenoids). In agreement with this hypothesis, when transcript levels of three maize GGPPS-encoding genes were compared with kernel carotenoid content in a germplasm collection, only one of them (*GGPPS1*) was found to be positively correlated with endosperm carotenoid biosynthesis [14]. Also consistent with a role for specific GGPPS isoforms in channeling precursors to the carotenoid pathway, multienzyme complexes harboring GGPPS and PSY activities to directly convert IPP into phytoene have been isolated from chromoplasts [72–74].

Cross-talk between MEP and carotenoid pathways

Increasing evidence demonstrates that an active cross-talk exists between the MEP pathway and the carotenoid pathway [9,50,75]. It is likely that such communication contributes to ensure that the prenyl diphosphate precursors required for carotenoid biosynthesis will be supplied when necessary, as deduced from the phenotypes resulting when flux through only one of the two pathways is up-regulated in transgenic plants. Thus, overexpression of flux-controlling enzymes of the MEP pathway such as DXS or HDR in Arabidopsis led to increased carotenoid levels in photosynthetic tissues (where the activity of PSY, the enzyme channeling MEP-derived precursors to the carotenoid pathway, is not limiting) but not in etiolated seedlings (where PSY activity instead of precursor supply limits the production of carotenoids) [2,12]. In potato tubers, overexpression of DXS was found to cause a number of developmental phenotypes (probably as a result of an increased production of MEP-derived cytokinins) and to result in higher phytoene levels, a hint that phytoene desaturation rather than PSY activity is limiting in this tissue [15]. On the other hand, overexpression of *PSY* in photosynthetic tissues resulted in severe phenotypic effects including dwarfism and reduced pigmentation likely because an increased amount of the limited GGPP pool available was used for the production of carotenoids and diverted away from the pathways leading to the biosynthesis of other plastidial isoprenoids such as gibberellins and chlorophylls [76–78].

To prevent these deleterious effects, a significant degree of coordination exists in wild type plants between the MEP pathway and the production of carotenoids at the gene expression level. The expression of MEP pathway genes has been shown to either precede or parallel the production of carotenoids in pepper and tomato fruit and apocarotenoids in monocot roots [11,12,59,79,80]. In Arabidopsis, the analysis of global gene expression profiles has shown a close regulatory connection between genes of the MEP pathway and those involved in the biosynthesis of carotenoids [81]. In many cases, the coordination is exerted at the level of key flux-controlling steps of both pathways. For example, the induction of the MEP pathway *DXS* and *HDR* genes appears to be coordinated with that of *PSY* when carotenoid biosynthesis is up-regulated during early light-triggered deetiolation in Arabidopsis [12,82]. Although the mechanisms behind this coordination are unknown, a common *cis* motif (ATCTA) has been found in the promoters of the genes encoding DXS, HDR, PSY and other enzymes involved in photosynthesis-related biosynthetic pathways [12,50,83]. However, the only *trans*-acting factor found to bind to this motif does not appear to be instrumental for the control of carotenoid gene expression or biosynthesis [84], suggesting that the identified factor might be just one component of a complex network of regulatory proteins required for the transcriptional control of genes involved in carotenogenesis. Work in our lab has shown that phytochrome-interacting factors (PIFs), basic helix-loop-helix (bHLH) transcription factors with a central role in a variety of light-mediated responses [85], also modulate the production of carotenoids by regulating the expression of Arabidopsis genes from both the MEP pathway and the carotenoid pathway [86]. In particular, PIF1 can directly bind to a G-box motif (CACGTG) in the Arabidopsis *PSY* promoter to repress gene expression [86]. PIFs

also repress the expression of *DXS* in dark-grown seedlings [86,87]. But because the *DXS* promoter lacks G-box motifs, it is likely that the repression of *DXS* expression by these transcription factors occurs by an indirect mechanism. Light-triggered degradation of PIFs upon illumination of etiolated seedlings therefore results in a rapid derepression of *PSY* and *DXS* gene expression and a burst in the production of carotenoids in coordination with chlorophyll biosynthesis and chloroplast development for an optimal transition to photosynthetic metabolism during deetiolation [86,87]. Additionally, up-regulation of PSY activity in deetiolating seedlings has been recently shown to initiate a feedback mechanism that eventually results in the post-transcriptional accumulation of higher DXS protein levels [2,44]. The signals and metabolites involved in the feedback communication between the carotenoid pathway and the MEP pathway in Arabidopsis seedlings are a matter of speculation [2,29,44]. Together, it is likely that the up-regulation of DXS both transcriptionally (in response to light signals and mediated by PIFs) and post-transcriptionally (in response to metabolic feedback triggered by PSY up-regulation) ensures an appropriate supply of MEP-derived precursors for the biosynthesis of carotenoids that protect the emerging photosynthetic apparatus from photooxidative damage during the critical stages of early seedling development in the light.

A coordinated induction of genes encoding DXS and PSY has also been observed when carotenoid production is boosted during fruit ripening [11,12,88]. Furthermore, a clear cross-talk exists between the MEP pathway and the carotenoid pathway in tomato fruit at the level of these two steps. Thus, *DXS* expression increases in mutant fruits defective in the fruit-specific PSY1 isoform and decreases in transgenic fruits overexpressing PSY1 [11,78]. Changes in protein levels, however, might follow a different pattern. Thus, a feedback mechanism acting at a post-transcriptional level similar to that described for deetiolating Arabidopsis seedlings might explain the increase in DXS activity observed in PSY1-overexpressing fruits with lower *DXS* transcripts [78]. On the other hand, *PSY1* expression and, to a lower extent, PSY activity are increased in tomato fruit when the levels of DXP (the product of DXS activity) are up-regulated [11,13,89]. A very strong induction in *PSY* expression was also observed in tubers of DXS-overexpressing potato lines [15], whereas a down-regulated accumulation of transcripts encoding PSY has been reported when the MEP pathway is pharmacologically or genetically blocked in Arabidopsis seedlings [29]. Additional evidence that an active communication exists between the MEP and carotenoid pathways in plant cells is available in other recent reviews [9,48,75].

Concluding remarks

In the last few years it has been a huge progress in our understanding of how carotenoid precursors are produced in plant cells. Following the discovery of the MEP pathway, work of several groups contributed to identify rate-determining steps in a relatively short time, and we are now achieving an impressive progress in the identification of mechanisms that regulate the flux through this pathway and the production of carotenoid precursors. Recent results have also provided hints of the central importance of coordinating the supply of these MEP-derived precursors with the biosynthesis of carotenoids and other plastidial isoprenoids. Coordination at the gene expression level and feedback mechanisms have been found to regulate the cross-talk between these pathways, but future work will be necessary to unveil the specific components involved. It is expected than these and future discoveries in model and crop plants will contribute to develop more rational approaches for the generation of new plant varieties enriched in carotenoids of industrial or nutritional interest.

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