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RESEARCH REVIEW

Metabolic Engineering Strategies for the Production of Beneficial Carotenoids in Plants

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Abstract Adequate consumption of carotenoids including lycopene, β-carotene, lutein, zeaxanthin, and astaxanthin have many benefits for human health. In plants, carotenoids are derived from isoprenoid precursors from the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway located in plastids. The MEP pathway is also required for the biosynthesis of chlorophyll, terpenoids, plant hormones, and other metabolites. Despite its complexity and difficulty, various strategies have been successfully used to improve the carotenoid biosynthesis in plants through metabolic engineering. Here, these metabolic engineering strategies are reviewed. In addition, the development of gene stacking technologies for carotenoid biosynthesis is evaluated. These technologies will expedite our efforts to bring the health benefits of carotenoids and other nutritional compounds to our diet.

Keywords: carotenoid, β-carotene, provitamin A, zeaxanthin, astaxanthin

Introduction

Carotenoids including lycopene, β-carotene, zeaxanthin, lutein, and astaxanthin have a myriad of benefits for human health. β-Carotene, which can be cleaved to produce retinol by carotene dioxygenase in the intestine, is the major source of vitamin A from our diet (1). Vitamin A deficiency (VAD) has many clinical manifestations, ranging from xerophthalmia (practically pathognomonic) to disturbances in growth and susceptibility to severe infection (2). VAD is a very significant nutritional problem in many areas of the developing world, affecting millions of children (3). Zeaxanthin and lutein are also important for maintaining eye health (4). These two carotenoids are macular pigments that can prevent age-related macular degeneration. Carotenoids have antioxidant properties, serving as antioxidant scavengers as well as inhibitors of pro-inflammatory and pro-thrombotic factors. These properties may provide potential benefits in the prevention of cardiovascular and other diseases (5,6). As a result, increase in carotenoid content in plants through metabolic engineering can improve the nutritional value of our diet.

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Carotenoid Biosynthesis in Higher Plants

Carotenoid biosynthesis uses the building units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) derived from the isoprenoid pathways (Fig. 1). In plants there are two distinct isoprenoid pathways: the mevalonate pathway (MVA) present in the cytoplasm and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway in plastids (7-9). The 1st intermediate in the MEP pathway is the 1-deoxyxylulose-5-phosphate (DXP) formed from pyruvate and glyceraldehyde-3-phosphate by the action of DXP synthase. The 2nd intermediate of the pathway is MEP. This step is carried out by the enzyme 1-deoxyxylulose-5-phosphate reductoisomerase (DXR), which is the target of antibiotic fosmidomycin. The MEP pathway is required for the biosynthesis of carotenoids, tocopherols, certain sesquiterpenes, monoterpenes, diterpenes, abscisic acid, and the side chains of chlorophylls and plastoquinone. For the MVA pathway, the 1st and 2nd intermediates are

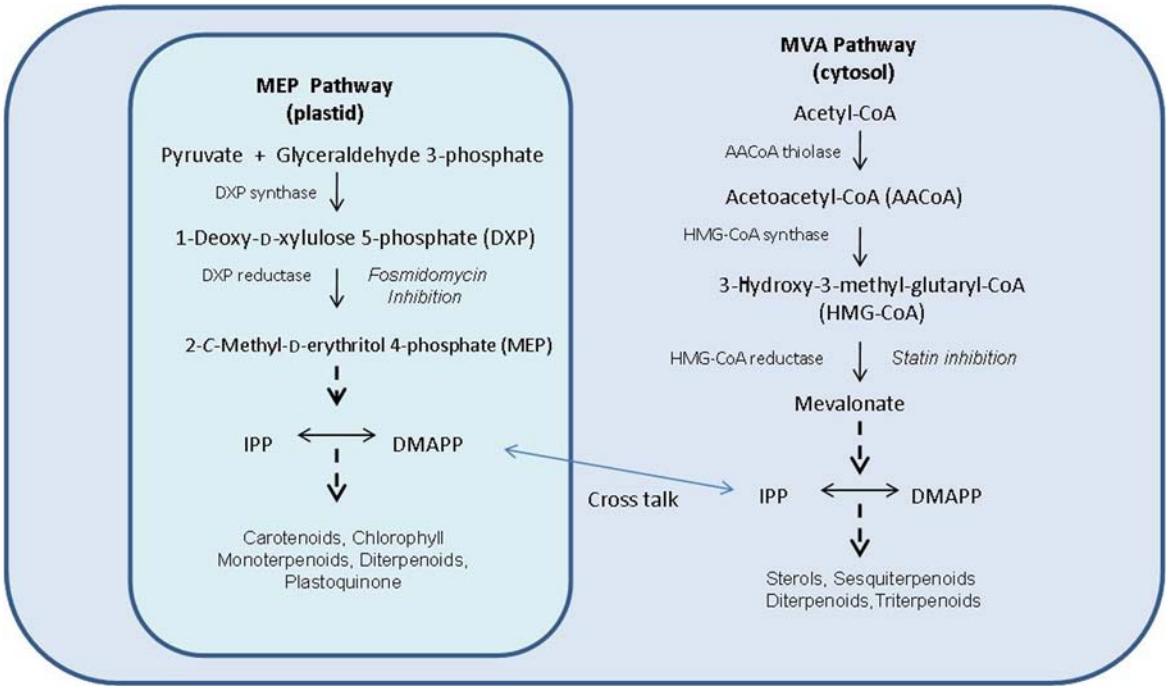

Fig. 1. Isoprenoid biosynthetic pathways in plants.

acetoacetyl-CoA and 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA), respectively. The key regulatory step in the MVA pathway is the conversion of HMG-CoA to mevalonate, which is carried out by HMG reductase (HMGR). This enzyme is the inhibition target of a class of drugs known as statins. The MVA pathway leads to the biosynthesis of sterols, brassinosteroids, certain sesquiterpenes, triterpenes, and ubiquinone side chain.

The dynamic of MEP and MVA pathways in plants are subjected to control by environmental and developmental factors such as light, plant pathogen interactions, and stages of growth (10). Even though enzymes involved in these two pathways are compartmentalized, cross talks between them is carried out by transportation of common metabolites (11,12). Precursors such as IPP and short prenyl diphosphates are believed to be the metabolites that connect these two networks.

For carotenoid biosynthesis the isoprene units IPP and DMAPP are condensed into the C_{10} molecule geranyl pyrophosphate (GPP), C_{15} compound farnesyl pyrophosphate (FPP), and the C_{20} geranylgeranyl pyrophosphate (GGPP) (Fig. 2). Formation of GGPP is catalyzed by the GGPP synthase (GGPPS). Phytoene synthase (PSY) catalyzes the formation of phytoene, the first C_{40} carotenoid from 2 GGPP molecules. Biosynthesis of all-*trans*-lycopene from phytoene involves desaturation and isomerization steps (13). Formation of β-carotene from lycopene is catalyzed by lycopene β-cyclase (LYC-b). On the other hand, lycopene ε-cyclase (LYC-e) converts lycopene to δ-carotene which is then converted to α-carotene by the LYC-b. Lutein production involves the hydroxylation of each ring on α-carotene at the C-3 positions (14). Hydroxylation the 3, 3' position of the β-ionone ring in β-carotene leads to the formation of zeaxanthin.

Biosynthesis of ketolated carotenoids such as canthaxanthin and astaxanthin is a result of the introduction of keto groups on the β-ionone ring structure at the 4,4' positions. In bacteria, 2 β-carotene ketolases, CRTW and CRTO have been characterized (15). The CRTW homolog found in algae is referred to as BKT. In the yeast *Xanthophyllomyces dendrorhous,* the astaxanthin synthase (Asy or CrtS) is found to catalyze the formation of astaxanthin from β-carotene (16,17). Asy is a cytochrome P450 monooxygenase. Higher plants generally lack ketolase activity. Only a few species in the genus *Adonis* are land plants that are known to produce the astaxanthin in significant amounts. In *Adonis* flowers, a novel pathway for astaxanthin biosynthesis was identified (18). This pathway requires carotenoid β-ring 4-dehydrogenase (CBFD) and 4-hydroxy-β-ring 4-dehydrogenase (HBFD). These two novel enzymes could be useful for engineering astaxanthin biosynthesis in plants.

Phytoene Synthase as a Key Step to Increase Carotenoid Biosynthesis in Plants

To engineer a high level of carotenoid production, an important strategy is to increase the overall carbon flux. Phytoene synthesis is an early step of carotenoid biosynthesis

(Fig. 2). Expression of the phytoene synthase gene in plants has been used to increase various carotenoid contents. In the oleaginous crop flaxseed, when the phytoene synthase gene (*crtB*) derived from a soil bacterium *Pantoea ananatis* strain 20D3 was introduced into *Linum usitatissimum* WARD cultivar, the transgenic plant formed orange seeds that contained α-carotene, β-carotene, and lutein (19). The total carotenoid content in seeds reached 156 μg/g fresh weight, a 18.6-fold increase as compared to controls. In potato plants, expression of the bacterial *crtB* gene also led to the production of potato tubers with a 4 to 6-fold increase in the amount of β-carotene and lutein, depending on the transgenic lines (20). In the knockout line of the Psy1 gene in tomato fruit there was no carotenoid accumulation in ripening fruit (21,22). Constitutive expression of an additional tomato Psy-1 gene product dramatically changed the carotenoid composition at the mature green stage (23). The total content of carotenoids was 6.7-fold greater in the Psy-1 fruit. The increases in the carotenoid content were mainly due to higher levels in β-carotene and lutein. Results from all these studies show that phytoene synthase is a rate-limiting step for carotenoid biosynthesis in plants. Proper regulation of this rate-limiting step during plant development is essential for metabolic engineering.

Engineering the β-Carotene Biosynthesis for the Production of "Golden" Staple Crops

One of the major driving forces to increase β-carotene content in plants is to combat vitamin A deficiency. The basic genetic engineering strategy to produce "Golden" staple crops is to combine the expression of phytoene synthase and the other early genes in the carotenoid to improve the β-carotene content. In rice, the combined expression of *PSY* gene from daffodils and *crtI* gene from the bacterium *Erwinia uredovora*v resulted in the generation of Golden Rice (24). The color was due to the production of carotenoids such as α-carotene, β-carotene, zeaxanthin, and lutein. The β-carotene content was, however, only 1.6 μg/g. Further optimization of the pathway by using maize and rice *PSY* genes improved the β-carotene content (25). This new generation of Golden Rice accumulated up to 31 μg/g β-carotene. In potato plants, tissue-specific expression of 3 early carotenoid pathway genes was also the best strategy to increase β-carotene. Over-expression of the mini-pathway containing *crtB*, *crtI*, and *crtY* genes led to the production of Golden Potato tubers (26,27). The content of β-carotene in these tubers reached 47 μg/g dry weight, the highest β-carotene level reported among 4 staple crops: maize, rice, wheat, and potato.

The daily recommended allowance of vitamin A is 300 μg for children and about half of that is enough for normal health. Given the ratio of β-carotene equivalency to vitamin A is 12:1, 100 to 200 g day of Golden Rice (dry weight) or 150 g/day (fresh weight) of Golden Potatoes meets the requirement as a dietary supplement for vitamin A deficiency in developing countries (28). In spite of technological success, one of the major hurdles for commercialization for genetically engineered crops is the legal requirements (29).

Improvement of Zeaxanthin Biosynthesis

Hydroxylation of β-carotene leads to the production of zeaxanthin. Similar to the production of β-carotene, an increase in carbon flux is necessary to increase the zeaxanthin biosynthesis. Plants, however, have both β-cyclase and ε-cyclase activities (Fig. 2). The carbon can be channeled to both β and ε branches of the pathway. In certain wild-type yellow endosperm corn variety, the ratio of endosperm β:ε activities favors the β-carotene/zeaxanthin branch. In transgenic maize that expressed the mini-pathway consisting of phytoene synthase, CrtI, and LYC-b, β-carotene was preferentially produced (30). Introgression of this transgenic strain into this wild-type yellow endosperm variety resulted in hybrids with the β:ε ratio elevated in an additive manner (31). The hybrids produced zeaxanthin at an unprecedented level, reaching 56 μg/g dry weight. The result demonstrates that the native metabolic capability can be explored to enhance the effectiveness of the heterologous pathways during pathway engineering in plants.

Other strategies to increase zeaxanthin production involve the re-direction of the carbon flux or elimination of branch pathways. LYC-e in the β-ε branch of the pathway can be blocked using gene silencing technology. In potato plants, when the LYC-e gene was silenced by introducing an antisense fragment, the level of carotenoids, including zeaxanthin, was increased by 2.5-fold in the transgenic potato tubers (32). In plants, zeaxanthin epoxidase catalyses the conversion of zeaxanthin to antheraxanthin and violaxanthin (Fig. 2). The conversion of zeaxanthin to violaxanthin can be inhibited. Two different potato (*Solanum tuberosum* L.) varieties were transformed with sense and antisense constructs encoding zeaxanthin epoxidase (33). Both antisense and co-suppression methods yielded potato tubers with zeaxanthin content elevated 4- to 130-fold, reaching 40 μg/g dry weight. The amount of violaxanthin was reduced drastically. Furthermore, most of the transgenic potato tubers with higher zeaxanthin levels also had higher carotenoid content. These studies show that inhibition of pathway branched points in the carotenoid pathway can result in the accumulation of higher amounts of desirable carotenoids in plants.

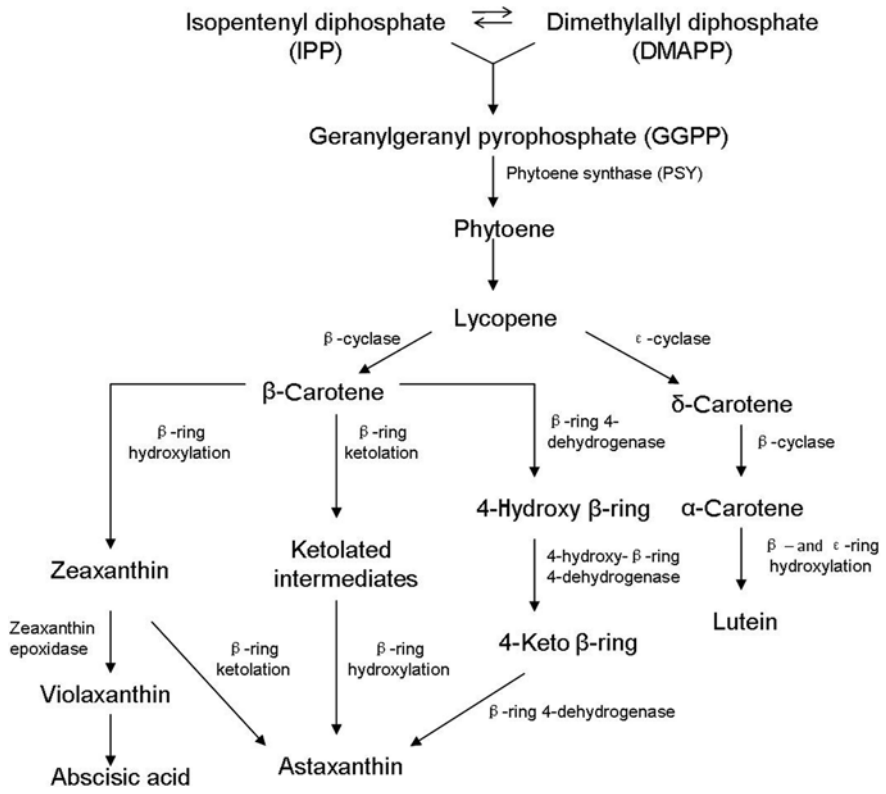

Fig. 2. Carotenoid biosynthetic pathways.

Engineering Biosynthetic Pathways for Astaxanthin Production in Plants

Astaxanthin is mainly used as a coloring agent for shrimp, trout, and salmon in the aquaculture industry. Currently, chemical synthesis is the major source of astaxanthin. Due to the low cost of production, engineering plants for astaxanthin biosynthesis is an attractive option. Astaxanthin biosynthesis requires both β-carotene ketolase and hydroxylase activities (Fig. 2). Most plants, however, do not have β-carotene ketolase activity, while they do exhibit a high level of carotenoid hydroxylase activity. Thus, the main strategy to engineering plants for astaxanthin production is the expression of efficient β-carotene ketolase in the appropriate host background. In *Arabidosis*, efficient ketolation of zeaxanthin is essential to obtain a high level of astaxanthin (34). One strategy employed was to screen for ketolases that have catalytic activity towards zeaxanthin. In this experiment, 3 putative β-carotene ketolases from microalgae (BKT) were initially expressed in *Escherichia coli* and analyzed for their ability to convert zeaxanthin to astaxanthin. These 3 genes were obtained from *Chlamydomonas reinhardtii* (*Cr*), *Chlorella zofingiensis* (*Cz*), and *Haematococcus pluvialis* (*Hp*). The CrBKT was identified to have the highest substrate specificity for zeaxanthin. Up to 85% of the total carotenoids were present as astaxanthin in the *E. coli* strain expressing CrBKT cDNA. When these CrBKT genes were transformed and expressed in *Arabidopsis*, the CrBKT was also more efficient for astaxanthin production than the CzBKT and HpBKT. The transgenic plant expressing the CrBKT produced up to 2 mg/g dry weight of astaxanthin in the leaves, which was about 43% of the total carotenoids. When different BKT enzymes were evaluated in tobacco plant, the CrBKT was again found to have a higher activity for astaxanthin production (35). The level of astaxanthin in the leaves was 1.6 mg/g dry weight, which was about 38% of the total carotenoids.

A higher percentage of the total carotenoids in the form of astaxanthin were achieved in engineered tobacco plant through the expression of the efficient CrtW and CrtZ from a marine bacterium *Brevundimonas* sp. strain SD212 in the chloroplasts (36). In the transgenic plants with these 2 genes, the astaxanthin made up about 70% of the total carotenoids, reaching 5.44 mg/g dry weight. In fact, the total carotenoid content in the transplastomic tobacco plants was 2.1-fold higher than that of wild-type tobacco plant.

The above studies emphasize the importance of expressing suitable bacterial or algal enzymes or enzyme combinations to engineer astaxanthin biosynthesis in plants. The newly identified 2-enzyme pathway required for biosynthesis in *Adonis* provides an intriguing alternative (Fig. 2). These 2 enzymes, CBFD and HBFD, have been expressed in *E.*

coli (18). The recombinant strain had a nearly complete conversion of β-carotene to astaxanthin, suggesting that both enzymes are portable and robust. Since these enzymes are already adapted to plant plastids, they should provide a new strategy for astaxanthin production in many crop plants.

Gene Stacking Strategies for Engineering Carotenoid Biosynthesis

The ability to simultaneously manipulate multiple genes is essential in metabolic engineering in both microbes and plants. Expression or suppression of multiple genes can overcome several carbon flux bottlenecks and enable the introduction of multiple pathway steps or even an entire pathway. In *E. coli*, the isoprene precursors IPP and DMAPP are derived from the MEP pathway. To eliminate the endogenous regulatory system and increase the overall carbon flux, a complete heterologous MVA pathway has been successfully expressed (37,38). A similar approach has also been accomplished in tobacco plants (39). As described earlier, the MVA pathway is located in the cytosol, while the MEP pathway occurs in the plastids (Fig. 1). Instead of using the gene by gene approach, the entire cytoplasmic MVA pathway with 6 enzymes was introduced into chloroplasts. Pathway function was demonstrated by unimpeded growth with fosmidomycin, a specific inhibitor for the MEP pathway. Expression of the cytoplasmic MVA pathway led to an increase in carotenoid contents. In another experiment, multiple gene constructs were designed to contain 7 key genes involved in ketocarotenoid biosynthesis (40). Each gene was constructed with an appropriate promoter and terminator and connected in a tandem manner. These large constructs were inserted into a binary vector and transformed into *Brassica napus*. Interestingly, 73-85% of the regenerated plants retained all the genes. The level of carotenoids in transgenic seeds was 412-657 μg/g fresh weight, which was about 19- to 30-fold higher than the level found in untransformed controls. Results from these two studies demonstrate the utility of the multigene strategy for pathway engineering in plants to redirect carbon flux for carotenoid biosynthesis.

Alternatively, multiple genes stacking in plants can be performed with polycistronic systems. The bicistronic systems using the 2A sequence from the foot-and-mouth disease virus and the internal ribosome entry site (IRES) sequence from the crucifer-infecting tobamovirus have been evaluated for the biosynthesis of carotenoids in rice endosperm (41). In this experiment, the bicistronic constructs consisted of phytoene synthase and carotene desaturase linked via either the synthetic 2A sequence or the IRES sequence under the control of the rice globulin promoter. The transgenic endosperm of rice with the 2A construct had a more intense golden color, suggesting that 2A was more efficient than IRES in coordinating gene expression. Protein quantification indicated that 2A was 9-fold more effective than IRES in driving translation. The endosperms with 2A construct accumulated an average of 1.3 μg/g of total carotenoids, which was 9-fold higher than the level obtained for endosperms with the IRES construct. Results from this experiment demonstrated that the 2A expression system can be an effective tool for expressing multiple proteins for carotenoid biosynthesis in plants.

A combinatorial genetic transformation for production of multiplex-transgenic plants has also been used to engineer pathways for carotenoid biosynthesis. Secondary metabolism in plants often consists of complex pathways with multiple branch points, multi-functional enzymes, and issues related to compartmentalization and tissue specificities. Besides, feedback inhibition can be complex, and multiple rate-limiting steps are common. One strategy to overcome this challenge in maize is the use of combinatorial nuclear transformation, which can generate a library of transgenic plants (30,42). The approach involves the introduction of multiple gene expression constructs and the subsequent selection of stable lines with a particular phenotype. The corresponding gene combination will be delineated, providing valuable information for the carbon flux and regulatory network of the pathway. Specifically, the experiment used different constructs consisting of the selection marker and expression cassettes for carotenoid biosynthesis. Each gene of interest was driven by a different endosperm-specific promoter. The transformation of corn and rice plants was carried out by particle bombardment. A population of regenerated plants with many different combinations of transgenes was obtained. Due to the color formation of carotenoids, a variety of distinct phenotypes was obtained based on visual inspection of the endosperm tissue. The combinatorial genetic transformation approach used here successfully identified a few bottleneck steps for carotenoid biosynthesis in maize. At the same time, the approach generated plants with extraordinary levels of â-carotene and other carotenoids.

Conclusion

Growing evidence for human health benefits of carotenoids has driven the metabolic engineering effort to produce these molecules in a sustainable and cost-effective manner. The overall biosynthetic pathway for carotenoid production is well-established (Fig. 1 and 2). The carbon flux through the isoprenoid pathway leading to the biosynthesis of IPP and DMAPP can be increased by overcoming rate limiting steps. The carotenoid biosynthetic pathway leads to the

formation of colorful carotenoids that can be visualized and analyzed. As a result, carotenoid biosynthesis provides a model system to develop and validate metabolic engineering strategies in plants. Development of new technologies will eventually expedite the effort to deliver the health benefits of carotenoids and other nutrients.

One of the major practical objectives to engineer pathways for carotenoid biosynthesis crops is to tackle vitamin A deficiency in developing countries and to deliver various health benefits of carotenoids to the general public through staple foods. Generation of staple foods rich in β-carotene represents significant progress over the past few decades (24,26,30,31). The future technological challenge will be the generation of crops rich in multiple nutrients, including carotenoids, with simultaneously reduced levels of undesirable products (43-45). This will require a metabolic balancing act and the ability to engineer multiple pathways at the same time, with the ultimate goal of improving nutrition and human health (46). With further advancement in metabolic engineering technologies and a better understanding of the plant carbon flux, metabolic cross talks, and regulatory networks, the future direction is the combination of nutritional value along with traits such as drought tolerance and insect and disease resistance. These types of crops would possess greater commercial appeal by addressing multiple challenges for sustainable agriculture in both developed and developing countries.

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