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Review

Metabolic engineering of ketocarotenoid biosynthesis in higher plants

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ABSTRACT

Ketocarotenoids such as astaxanthin and canthaxanthin have important applications in the nutraceutical, cosmetic, food and feed industries. Astaxanthin is derived from β-carotene by 3-hydroxylation and 4-ketolation at both ionone end groups. These reactions are catalyzed by β-carotene hydroxylase and β-carotene ketolase, respectively. The hydroxylation reaction is widespread in higher plants, but ketolation is restricted to a few bacteria, fungi, and some unicellular green algae. The recent cloning and characterization of β-carotene ketolase genes in conjunction with the development of effective co-transformation strategies permitting facile co-integration of multiple transgenes in target plants provided essential resources and tools to produce ketocarotenoids *in planta* by genetic engineering. In this review, we discuss ketocarotenoid biosynthesis in general, and characteristics and functional properties of β-carotene ketolases in particular. We also describe examples of ketocarotenoid engineering in plants and we conclude by discussing strategies to efficiently convert β-carotene to astaxanthin in transgenic plants.

Carotenoids are naturally occurring fat-soluble pigments synthesized by all plants and many microorganisms. In plants they participate in light harvesting in photosynthetic membranes and also protect the photosynthetic apparatus from photo-oxidation [1]. Carotenoids act as precursors of the growth regulator abscisic acid [2]. In addition carotenoids function as attractants to pollinators and seed dispersal agents [1]. Ketocarotenoids constitute a group of carotenoids that contain at least one keto group, either in the linear chain or on the β-ionone ring(s). A bathochromic shift in absorbance to longer wavelength occurs when the keto group is conjugated with the double bonds of the backbone [3]. Among different ketocarotenoids found in higher plants, algae, fungi or bacteria, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) and canthaxanthin (β,β-carotene-4,4′-dione) are the most important from a biotechnological point of view [4] and are widely used in aquaculture [5].

Astaxanthin is a red pigment abundant in marine animals including salmon, trout, shrimp and lobster. It is also present in birds such as flamingos and quails. These animals cannot synthesize this pigment but they accumulate significant amounts through their diet. Ketocarotenoids are rarely found in flower petals of higher plants, but many microorganisms such as the marine bacteria *Agrobacterium aurantiacum* (reclassified as *Paracoccus* sp. N81106) and *Alcaligenes* sp. PC-1 (reclassified as *Paracoccus* sp. PC-1), fresh water algae such as *Haematococcus pluvialis*, and the yeast *Xanthophyllomyces dendrorhous* (*former Phaffia rhodozyma*) synthesize ketocarotenoid pigments. The presence of hydroxyl and keto functional groups in ketocarotenoids makes them excellent antioxidants compared to the other carotenoids. Astaxanthin is a strong antioxidant [6] and contributes to general eye and skin health [5,7,8]. It has anti-inflammatory properties and inhibits oxidation of low-density lipoprotein in humans [9]. It is also implicated in the prevention of diabetic nephropathy in diabetic db/db mice [10], exhibits anticancer activity [11,12], and enhances immune responses [13,14].

Currently, a large proportion of ketocarotenoids including astaxanthin and canthaxanthin is produced through chemical synthesis. However, synthetic astaxanthin contains the stereoisomer by-products 3*S*,3*R*′ and 3*R*,3*R*′ in addition to the naturally occurring 3*S*,3*S*′ stereoisomer. The presence of the by-products may have an inhibitory effect on the biological activity of astaxanthin. Additionally, chemically-synthesized astaxanthin may be contaminated with other reaction by-products or intermediates. Thus, its commercial use is restricted mostly to feed supplementation particularly in aquaculture. Astaxanthin can also be produced biologically since several microorganisms are able to accumulate the compound at relatively high levels. For example, the green alga *H. pluvialis*, which produces astaxanthin at levels representing 4–5% of its dry weight, is used for the commercial production of this pigment as a functional food supplement for human consumption. However, this organism requires high light-intensities that increase production costs and its slow growth rate in culture increases the risks of contamination, which hinder its broader

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utility [4,5]. The yeast *X. dendrohous* is another microorganism which can accumulate astaxanthin up to 0.5% of its dry weight. However, the pigment produced by this microorganism has the 3*R*,3*R*′ configuration [15]. Metabolic engineering in higher plants using cloned heterologous genes is potentially one of the most powerful tools to produce astaxanthin, since plants have the ability to accumulate carotenoids in the thylakoid membranes and in the lipid globules within the plastid at very high concentrations [16].

Until very recently multi-gene transfer constituted one of the most significant bottlenecks in the genetic engineering of complex metabolic pathways in plants due to the diminishing rate of return as more transgenes are introduced simultaneously [17]. As the number of introduced genes increases, the chances of failure for at least one of the transgenes also increases, so huge numbers of transgenic plants would need to be generated to ensure that complete, stable pathways are constructed if such pathways have many enzymic steps. Alternative approaches such as individual transformation followed by crossing to 'stack' transgenes are unworkable for large numbers of transgenes because of the time taken to stack all transgenes in one line and the risk of segregation in later generations (reflecting the existence of multiple transgenic loci on different chromosomes). We have addressed this challenge by developing a combinatorial nuclear transformation strategy, allowing us to generate a combinatorial metabolic library for the investigation of carotenoid biosynthesis and the synthesis of specific combinations of carotenoids [18]. This approach provides a unique and surprisingly straightforward strategy for metabolic pathway analysis and multi-gene metabolic engineering in plants. Direct DNA transfer with separate vectors usually results in transgene integration at a random single locus, in the form of a multi-gene array [19,20]. The array may contain any number of transgenes but the distribution within a transgenic population tends to describe a normal curve as would be expected from random sampling [21] which means that within a suitably large population of transgenic plants, individuals carrying all possible combinations of transgenes can be recovered. The genotypes and phenotypes are genetically stable through generations.

Biosynthesis of ketocarotenoids

The green alga *H. pluvialis* accumulates higher amounts of ketocarotenoids in cytoplasmic lipid vesicles [22]. *H. pluvialis* under unfavorable environmental conditions ceases motility and transforms into cyst cell aplanospores, which accumulate high levels of astaxanthin. The carotenoid biosynthetic pathway has been elucidated in *H. pluvialis* by inhibitor studies [23]. In higher plants and green algae the carotenoid precursor isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) are derived from two alternative pathways: the classical acetate/mevalonate (MVA) pathway or the 2-C-methyl-D-erythritol-phosphate (MEP) pathway [24]. The MVA pathway, in which IPP is synthesized from three molecules of acetyl-CoA with the intermediate mevalonic acid (MVA), is responsible for the synthesis of phytosterols, sesquiterpenes and triterpenoids. This pathway is localized in the cytosol/endoplasmic reticulum [25]. Plastidial isoprenoids, such as carotenoids, phytol, plastoquinones and tocopherols are formed via the MEP pathway, which is also known as the non-MVA pathway or 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway [24]. In the MEP pathway, the five-carbon building blocks of isoprenoid metabolism, IPP and DMAPP, are synthesized from glyceraldehyde-3-phosphate and pyruvate. 1-Deoxy-D-xylulose 5-phosphate (DOXP), the product of the initial reaction, is the precursor of IPP. The resulting IPP is then transformed in plants by the enzyme isopentenyl pyrophosphate isomerase (IPI) to its isomer DMAPP, the starting molecule of isoprenoid biosynthesis, to which more IPP molecules are added by head-to-tail condensation to yield eventually the end isoprenoid product. It has been proposed that unlike higher plants *H. pluvialis* synthesizes isoprenoids only through the MEP pathway [22].

Geranylgeranyl pyrophosphate (GGPP) is a C_{20} compound, which is synthesized from IPP and DMAPP via geranyl pyrophosphate (C_{10}) and farnesyl pyrophosphate (C_{15}). The condensation of two molecules of GGPP produces the first C_{40} carotenoid, phytoene (Fig. 1). The first committed step is catalyzed by phytoene synthase (PSY). Phytoene is further converted to lycopene via ζ-carotene. Bacterial phytoene desaturase (CRTI) can introduce three to four double bonds into phytoene to produce neurosporene and lycopene. However, in plants and cyanobacteria phytoene desaturase (PDS) can only introduce two double bonds to produce ζ-carotene. Therefore a second enzyme, ζ-carotene desaturase (ZDS) that converts ζ-carotene to lycopene is required. Each of these enzymes requires plastoquinone and a plastid terminal oxidase, which act as electron acceptors. In *H. pluvialis,* the desaturation carried out by ZDS and isomerization reactions have not been fully elucidated. Lycopene is a substrate of two cyclases, lycopene β-cyclase (LYCB) and lycopene ε-cyclase (LYCE). LYCB can introduce two β-rings, one at each end of lycopene to form γ-carotene (monocyclic) or β-carotene (β,β-carotene) (dicyclic), while LYCE can only introduce one ε-ring into one end of lycopene to give rise to σ-carotene (monocyclic) [26]. α-carotene (β,ε-carotene) is produced from lycopene by the addition of an ε-ring at one end and a β-ring at the other, reactions catalyzed by LYCE and LYCB, respectively.

In both prokaryotes and eukaryotes, β-carotene is converted into astaxanthin by the addition of keto groups at the 4 and 4′-position and hydroxyl groups at the 3 and 3′ positions of the β-ionone rings via several ketocarotenoid intermediates (Fig. 1). These reactions are catalyzed by β-carotene ketolase (4,4′-oxygenase; CRTW, BKT or CRTO) and β-carotene hydroxylase (3,3′-oxygenase; BCH or CRTZ), respectively. The presence of echinenone (β,β-carotene-4-one; one keto-group) and canthaxanthin (β,β-carotene-4,4′-dione; two keto-groups) in microorganisms shows that the ketolation of β-carotene takes place prior to hydroxylation at 3,3′ carbons (Fig. 1). Several ketolase and hydroxylase encoding genes have been identified in microorganisms and higher plants. There exist three highly homologous functional *bkt* genes in *H. pluvialis* [27] (Table 1) encoding putative amino acid sequences ranging from 86.1% to 99.4%. These are *bkt1* from *H. pluvialis* 34/7 and *bkt2* and *bkt3* from *H. pluvialis* Flotow NIES-144 [27,33,34]. Some authors misused *bkt1* from *H. pluvialis* 34/7 as *crtO* [33]. It has also been demonstrated that *bkt1* and *bkt2* coexist in a single strain of *H. pluvialis* while *bkt3* is a third ketolase identified that share 95% identity with *bkt2* [27].

The ketolation of β-carotene to canthaxanthin is catalyzed by a β-carotene ketolase BKT via the mono ketocarotenoid intermediate echinenone (Fig. 1). It has been demonstrated that subsequent hydroxylation steps are carried out by a hydroxylase to produce astaxanthin [33]. *Chlorella zofingiensis* ketolase, a BKT-type ketolase together with CRTZ convert β-carotene to adonixanthin and astaxanthin [35]. Studies carried out by Huang et al. [35] suggest that in *C. zofingiensis* astaxanthin is synthesized by ketolation of zeaxanthin via adonixanthin (4-ketozeaxanthin). The presence of cathanxanthin denotes the end product of β-carotene oxygenation.

Haematococcus pluvialis accumulates astaxanthin under adverse environmental conditions, such as high irradiance and salinity, acetate addition, nutrient deprivation, influence of reactive oxygen species (ROS), and increased C/N ratio in culture media [36,37]. As a result, *H. pluvialis* is a valuable model for understanding carotenogenesis regulation. Increases in astaxanthin accumulation have corresponded with increased transcript abundance of carotenogenic genes in *H. pluvialis* [38–44]. For a comprehensive review of the regulation of astaxanthin formation in response to stress in *H. plu-*