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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-*

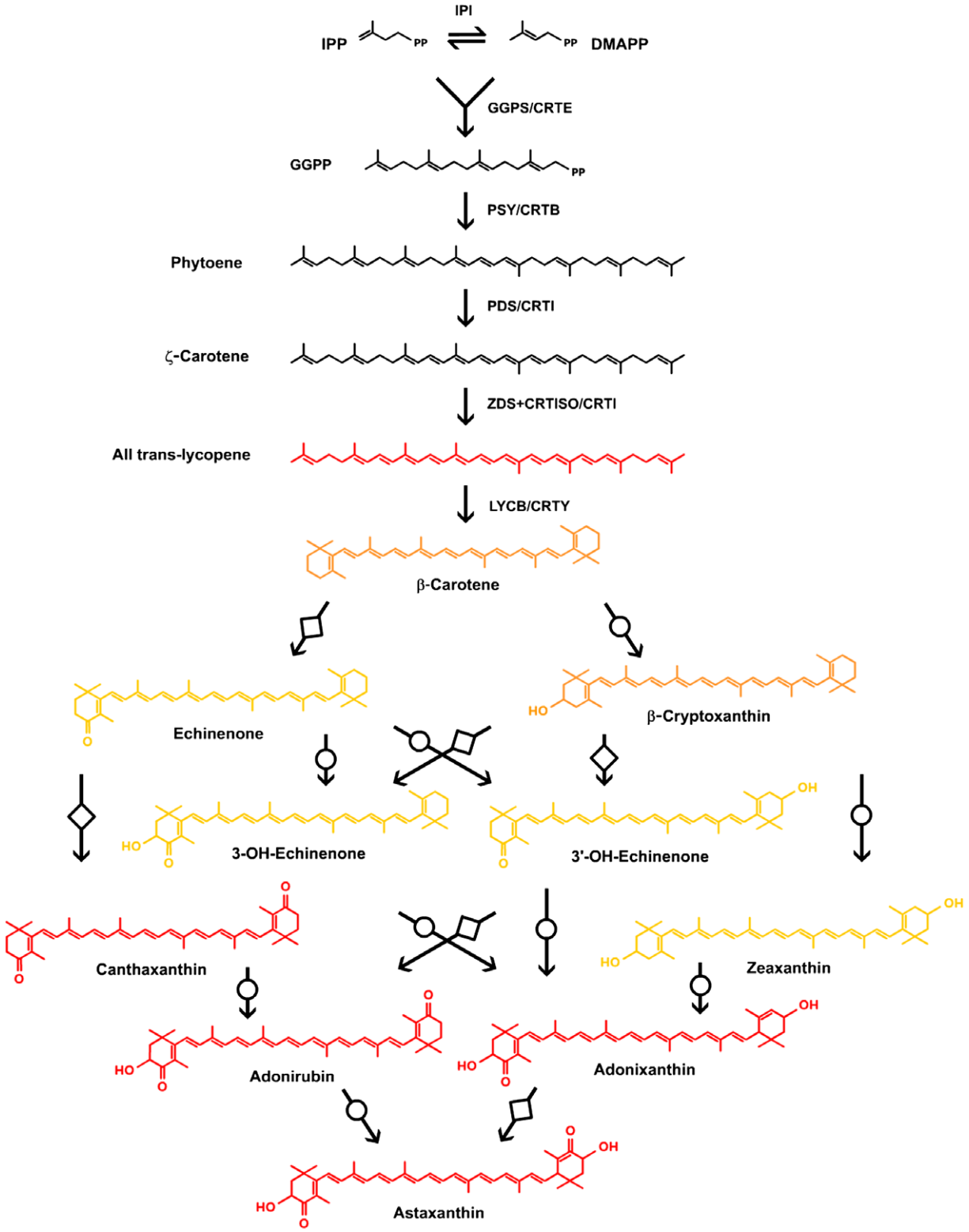

Fig. 1. The carotenoid biosynthetic pathway. The pathway shows the primary steps in algae and bacteria. IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; IPI, isopentenyl pyrophosphate isomerase; GGPP, geranylgeranyl pyrophosphate; GGPS, geranylgeranyl pyrophosphate synthase; CRTE, bacterial geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; CRTB, bacterial phytoene synthase; PDS, phytoene desaturase; CRTI, bacterial phytoene desaturase/isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β-cyclase; CRTY, bacterial lycopene β-cyclase. Arrow with square in the middle, β-carotene ketolase (BKT, CRTW or CRTO). Arrow with circle in the middle, β-carotene hydroxylase (BCH or CRTZ). In Cyanobacteria CrtP is used for PDS, CrtQ for ZDS and CrtH for CRTISO.

vialis, the reader is referred to Jin et al. [45]. In *H. pluvialis*, astaxanthin accumulation occurs in extra-plastidic lipid globules as a secondary carotenoid [22]. Although astaxanthin accumulates in the cytoplasm, the PDS enzyme of the general carotenoid pathway

Table 1
β-Carotene ketolase genes whose function has been confirmed in *in vitro* expression systems.

Genes	Accession number/Gene	Species (source)	Enzymatic characteristics	References
bkt-type	X86782	*Haematococcus pluvialis* 34/7	Diketolation at position 4 and 4′ to canthaxanthin; unable to convert zeaxanthin to astaxanthin	[33]
	D45881	*Haematococcus pluvialis* Flotow NIES-144	Diketolation at position 4 and 4′ to canthaxanthin; unable to convert zeaxanthin to astaxanthin	[28,34]
	AY603347	*Haematococcus pluvialis* Flotow NIES-144	Diketolation at position 4 and 4′ to canthaxanthin; unable to convert zeaxanthin to astaxanthin	[27]
	AY772714	*Chlorella zofingiensis*	Diketolation at position 4 and 4′ to canthaxanthin	[35]
crtW-type	D58420	*Paracoccus* sp. N81106 (former *Agrobacterium aurantiacum*)	Diketolation at position 4 and 4′ to canthaxanthin	[46]
	D58422	*Paracoccus* sp. PC-1 (former *Alcaligenes* sp. PC-1)	Diketolation at position 4 and 4′ to canthaxanthin	[31]
	Y15112	*Paracoccus marcusii*	Diketolation at position 4 and 4′ to canthaxanthin	[30]
	AY957386	*Paracoccus haeundaensis*	Diketolation at position 4 and 4′ to canthaxanthin	[54,55]
	AF218415	*Bradyrhizobium* sp. ORS278	Diketolation at position 4 and 4′ to canthaxanthin	[29]
	NpF4798	*Nostoc punctiforme* PCC 73102	Diketolation at position 4 and 4′ to canthaxanthin	[56]
	NpF5919	*Nostoc punctiforme* PCC 73102	Monoketolation at position 4 to echinenone	[56]
	alr3189	*Anabaena* (also known as *Nostoc*) sp. PCC 7120	Conversion of myxol to ketomyxol	[59]
	AB181388	*Brevundimonas* sp. SD212	Diketolation at position 4 and 4′ to canthaxanthin	[50]
	DQ309446	*Brevundimonas vesicularis* DC263	Diketolation at position 4 and 4′ to canthaxanthin	[53]
	gll1728	*Gloeobacter violaceus* PCC 7421	Diketolation at position 4 and 4′ to canthaxanthin	[32,91]
	DQ400932	*Sphingomonas* sp. DC18	Diketolation at position 4 and 4′ to canthaxanthin	[92]
crtO-type	slr0088	*Synechocystis* sp. PCC 6803	Monoketolation at position 4 to echinenone; unable to convert zeaxanthin to astaxanthin	[57]
	all3744	*Anabaena* sp. PCC 7120	Monoketolation at position 4 to echinenone	[59]
	AY705709	*Rhodococcus erythropolis* AN12	Diketolation at position 4 and 4′ to canthaxanthin	[58]
		Rhodococcus erythropolis PR4	Diketolation at position 4 and 4′ to canthaxanthin; unable to convert zeaxanthin to astaxanthin	[61]
	DR0093	*Deinococcus radiodurans* R1	Diketolation at position 4 and 4′ to canthaxanthin	[58]
	gll0394	*Gloeobacter violaceus* PCC 7421	Monoketolation at position 4 to echinenone	[32]
asy	AX034665	*Xanthophyllomyces dendrorhous*	Diketolation and dihydroxylation to astaxanthin	[63,64]
keto 1 and *keto* 2	AY644757 and AY644758	*Adonis aestivalis*	3,4-desaturation subsequent to 4-hydroxylation of the β-ring leads to the formation of a 4-keto-β-ring carotenoid	[65]

appears to be localized in the chloroplast [22]. Generally expression of carotenogenic genes in *H. pluvialis* was shown to be upregulated at either the mRNA or protein levels, or both, in response to environmental stresses [22,39–41,44].

In microorganisms most β-carotene ketolases are CRTW-type enzymes that are homologus to *H. pluvialis* BKT. CRTW-type ketolase acts symmetrically to introduce two keto groups on each β-ionone ring of β-carotene to produce canthaxanthin via the mono-keto intermediate, echinenone (Table 1). Genes encoding CRTW-type ketolase have been cloned and characterized in bacteria such as *Paracoccus* [46]. The astaxanthin biosynthetic pathway in bacteria was elucidated at the gene and enzyme levels by *in vivo* [46] and *in vitro* [47,48] experiments. In *Paracoccus* β-carotene hydroxylase encoded by *crtZ* catalyzes the hydroxylation of the β-ionone rings of β-carotene to produce [3*R*,3*R*′]-zeaxanthin (β,β-carotene-3,3′-diol or 3,3′-dihydroxy-β,β-carotene) via β-cryptoxanthin (3-hydroxy-β,β-carotene) [46] similarly to *Erwinia uredovora* (reclassified as *Pantoea ananatis*) hydroxylation reactions [49]. The *crtW* gene which encodes β-carotene ketolase in *Paracoccus* converts β-carotene into canthaxanthin via echinenone. In the first step, β-carotene ketolase and β-carotene hydroxylase can carry out reactions independently, but further events are dependent on which reaction occurs first [48]. *Paracoccus* β-carotene ketolase (CRTW) has a strong preference for carotenoids with at least one non-hydroxylated β-ionone ring, e.g. β-carotene, β-cryptoxanthin, echinenone and 3-hydroxy-echinenone. In contrast, 3-hydroxylated β-ionone rings such as those in zeaxanthin, 3′-hydroxy-echinenone and adonixanthin (3,3′-dihydroxy-β,β-caroten-4-one; 4-ketozeaxanthin; one keto-group) are poor substrates for this enzyme [48]. Accumulation of six different ketocarotenoids in *in vivo* studies demonstrated the versatility of these two enzymes [46]. *In vitro* experiments have shown that for *Paracoccus* CRTW and *H. pluvialis* BKT the conversion of adonixanthin to astaxanthin appears to be an important limiting step [47]. A *crtW* gene has been isolated from the astaxanthin-producing marine bacterium *Brevundimonas* sp. SD212 [50]. The CRTW protein from *Brevundimonas* exhibited 45% identity to that of *Paracoccus* [50]. Choi et al. characterized a variety of bacterial CRTW and CRTZ enzymes by complementation analysis in *Escherichia coli*. They demonstrated that their astaxanthin-accumulating efficiencies were different, and that *Brevundimonas* CRTW and CRTZ possessed the most efficient catalytic mechanism for astaxanthin synthesis [50,51]. Recently, mutational analysis of *crtW* has been carried out to obtain a mutated *crtW* gene favorable to the production of astaxanthin [52,53]. CRTZ and CRTW have also been identified and characterized in *Paracoccus haeundaensis* [54,55] and a non-marine *Brevendimonas* DC263 [53].

Two open reading frames ORF148 (NpF4798) and ORF38 (NpF5919) from *Nostoc punctiforme* PCC 73102 exhibits sequence similarities to CRTW-type ketolases found in *Paracoccus*. They were also shown to contain conserved iron binding motifs. Functional complementation experiments in *E. coli* revealed that both CRTW148 (ORF148) and CRTW38 (ORF38) can successfully convert β-carotene to canthaxanthin via the mono-keto intermediate, echinenone. When provided with zeaxanthin, only CRTW148 could convert it to astaxanthin. Consequently ORF148 encodes a ketolase which is able to function in concert with the bacterial CRTZ type hydroxylase resulting in astaxanthin synthesis. While CRTW38 is similar to CRTW type ketolase found in *Paracoccus* and BKT type ketolase in *H. pluvialis*, it converts zeaxanthin to astaxanthin inefficiently [56].

CRTO is a third type of ketolase that has been reported in some bacteria such as *Synechocystis* sp, *Anabaena*, *Gloeobacter*, *Rhodococcus* and *Deinococcus* (Table 1). CRTO-type ketolase exhibits homol-

ogy to bacterial phytoene desaturase but has no such activity [57,58]. CRTO-type β-carotene ketolases from these species are homologous but *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 CRTO add a keto group to only one of the two rings of β-carotene to generate echinenone [57,59]. Although, in *Synechocystis* the β-carotene ketolase (CRTO) mediates the formation of the monoketo product echinenone, it was demonstrated that higher oxygen supply and higher levels of the protein favor the formation of diketo products [60]. On the other hand *Rhodococcus* and *Deinococcus* CRTO can add two keto groups on β-carotene to produce canthaxanthin [58]. CRTO enzymes from *Synechosystis* sp. PCC 6803 or *R. erythropolis* PR4 are unable to utilize zeaxanthin to produce astaxanthin [61]. It is very likely that CRTO cannot accept a 3-hydroxylated β-ionone ring as substrate. CRTW-type ketolases contain four conserved regions with three iron-binding motifs [34,58]. CRTO-type ketolases have six different conserved regions including the FAD-binding motif which is also present in phytoene desaturases [58]. Choi et al. [61] compared the catalytic function of a CRTO ketolase from the marine bacterium *Rhodococus erthropolis* PR4 to that of a CRTO derived from the cyanobacterium *Synechosistis* sp. PCC6803, and CRTW derived from *Brevundimonas* sp. SD212, by complementation analysis in *E. coli* expressing the known *crt* genes. Results strongly suggested that a CRTO-type ketolase was unable to synthesize astaxanthin from zeaxanthin, i.e. only a CRTW-type ketolase could accept a 3-hydroxy-β-ionone ring as substrate [61]. CRTO enzymes (>500 amino acids) are almost twice the size of CRTW enzymes and do not share significant sequence homology. They do, however, exhibit significant homology to bacterial phytoene desaturases, CRTI [57,58]. These differences probably translate to different catalytic functions for CRTO-type and CRTW-type ketolases [61].

A fourth unique and species specific ketolase, related to cytochrome P450 enzymes, has been identified in the yeast *X. dendrorhous* [62,63]. The enzyme is known as astaxanthin synthase (ASY) and converts β-carotene to astaxanthin, carrying out both hydroxylation and ketolation reactions [64]. These authors demonstrated that ASY is a cytochrome P450 enzyme which obtains the electron for β-carotene oxygenation from a cytochrome P450 reductase. It is postulated that C4 is hydroxylated twice, followed by the spontaneous elimination of water, to form a keto group as proposed in *Adonis aestivialis* [65]. In the case of *X. dendrorhous*, the reaction starts with ketolation of β-carotene to echinenone and can branch depending on subsequent 4′-ketolation or 3-hydroxylation reactions. The resulting canthaxanthin is hydroxylated or 3-hydroxyechineone is ketolated to produce phoenicoxanthin (adonirubin, 3-hydroxy-β,β-carotene-4,4′-dione; two keto-groups), which is the direct precursor of astaxanthin. The side reaction that produces 3-hydroxy-4-ketotorulene from torulene involves torulene as an intermediate. The carotenoid biosynthetic pathway of *X. dendrorhous* is also well understood to the stage of β-carotene formation. Only two genes participate in this section of the pathway. Both genes have been cloned and their functions established. One is a fusion CRTYB gene encoding a phytoene synthase (CRTB) and a lycopene cyclase (CRTY) [66]. The other is the CRTI gene encoding a phytoene desaturase [67]. Its product catalyzes the introduction of four double bonds into phytoene, yielding all-trans lycopene. Thus, in *X. dendrorhous* astaxanthin is derived from GGPP through the action of CRTYB, CRTI and ASY.

Enzymes identified in *A. aestivalis* are proposed to be a fifth type of ketolases that add a carbonyl functional group to number 4 carbon of β-rings via an indirect route that involves keto-enol tautomerization. The cDNAs *Adketo 1* and *Adketo 2* identified in *A. aestivialis* that encode β-carotene 4-ketolases are homologous to plant type β-carotene 3-hydroxylase. However, these two enzymes exhibited neither 4-ketolase nor 3-hydroxylase activity when presented with β-carotene as the substrate in *E. coli* [65]. Instead, the products of the *Adonis* cDNAs were found to modify β-rings in two distinctly different ways: desaturation at the 3,4 position and hydroxylation of the number 4 carbon. It was proposed that a 3,4-desaturation subsequent to 4-hydroxylation of the β-ring leads to the formation of a 4-keto-β-ring via keto-enol tautomerization [65]. In the case of *Adonis* a 4-hydroxy-β-ring is an intermediate in the synthesis of the 4-keto-β-ring. Thus a further oxidation of 4-hydroxy-β-ring yields a 4-keto group [65]. *Adketo 1* and *Adketo 2* encode specific ketolases that are only found in *A. aestivalis*.

Metabolic engineering of ketocarotenoid biosynthesis in plants

Since the creation of the first transgenic plants in the early 80s that expressed single transgene together with a selectable/screenable marker gene, we have witnessed a plethora of examples in many different plant species whereby one or a relatively few transgenes were introduced either concomitantly or sequentially into plants. In the context of creating plants containing what are commonly referred to as "output or value-added traits", there is a serious bottleneck that needs to be overcome, i.e. general and effective ways to introduce into plants multiple transgenes that need to be expressed sequentially and coordinately in a manner that will mimic/enhance endogenous or reconstitute exogenous multi-step pathways [19,68,69]. This bottleneck is perhaps not widely appreciated, but is amply evident by the huge body of literature describing the manipulation or expression of single gene in plants and the comparative paucity of publications dealing with the manipulation of multiple genes [17]. Until very recently multi-gene engineering constituted a significant hurdle in complex pathway analysis due to the diminishing rate of return as more transgenes are introduced simultaneously. As discussed subsequently in this review, one effective way to overcome this bottleneck is combinatorial nuclear genetic transformation as applied to carotenoid and ketocarotenoid manipulation in maize [18]. Thus the entire carotenoid pathway was engineered in white maize, creating a rainbow of kernel colors visually equivalent to Indian corn depending on the integrated transgene complement. This system has an added advantage from a commercial perspective in that these methods circumvent problems with traditional approaches that not only limit the amount of sequences transferred but may disrupt native genes or lead to poor expression of the transgene, thus reducing both the numbers of transgenic plants that must be screened and the subsequent breeding and introgression steps required to select a suitable commercial candidate [70].

As discussed earlier, 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. Even though higher plants synthesize different hydroxylated carotenoids, they are not able to form ketocarotenoids, other than very few species such as *Adonis* [71] which accumulates ketocarotenoids in flowers. Transgenic expression of *H. pluvialis bkt* encoding β-carotene ketolase in the cyanobacterium *Synechococcus* PCC7942, which normally accumulates β-carotene and zeaxanthin, generated significant levels of astaxanthin and other ketocarotenoid intermediates [72]. These results provided the first evidence of genetic modification of a plant-type carotenoid biosynthesis pathway towards the production of novel carotenoids. The overexpression of *H. pluvilis bkt* under the control of the chromoplast-associated tomato *pds* promoter resulted in the accumulation of high levels of astaxanthin and other ketocarotenoids in the nectary, a flower tissue rich in xanthophyll-containing chromoplasts [16]. This finding demonstrated that ketocarotenoid accumulation in higher plants can be genetically engineered by overexpression of a β-carotene ketolase gene. The

substrate for ketocarotenoid formation, β-carotene, is present in substantial quantities in all photosynthetic plant tissues and most non-photosynthetic tissue including flowers, fruits and carrot roots. Moreover, *H. pluvialis* and *Paracoccus* orthologous ketolase genes of the *bkt*/*crtW* family [33,34,46], *Synechocystis* sp. PCC 6803 *crtO* [57] or *X. dendrohous* P450 monooxygenase *asy* [64] are available for the genetic engineering of ketocarotenoids (Table 1). All heterologous ketolases act on β-carotene yielding 4-keto derivatives followed by hydroxylation at position 3 by hydroxylases. This leads to a diverse array of potential products. Genetic modification for enhanced ketocarotenoid accumulation has been accomplished in bacteria [73], yeast [74], and with mixed success in model plants and several crops including tomato, potato, carrot, rapeseed and maize (Table 2).

Model plant systems

Constitutive expression of the *H. pluvialis* β-carotene ketolase (*bkt*) in tobacco resulted in the production of astaxanthin, canthanxanthin, adonixanthin and 3′-hydroxy-echinenone in nectary tissues [16]. The same gene was also expressed in a seed-specific manner in *Arabidopsis thaliana* [75]. In transgenic *Arabidopsis* seeds the major ketocarotenoids were 4-ketolutein, adonirubin (3-hydroxy-β,β-carotene-4,4′-dione, phoenicoxanthin) and canthaxanthin. In order to enhance the levels of ketocarotenoids, crosses were made with transgenic *Arabidopsis* over-expressing an endogenous phytoene synthase gene [76]. Resulting plants exhibited an increase in ketocarotenoid content of up to 13-fold over that measured in the single β-carotene ketolase-expressing plants [75]. Ralley et al. [77] reported that simultaneously constitutive expression of *Paracoccus crtW* and *crtZ* genes in *N. tabacum* resulted in a 9-fold increase of ketocarotenoid levels in nectaries. These levels were greater than previously reported in plants only expressing the algal ketolase [16,75]. Echinenone was the principal ketocarotenoid found in tobacco leaves in the *crtZ/crtW*-expressing transgenic plants; the compound accumulated typically between 600 and 900 μg/g DW.

Constitutive expression of a *Synechocystis* sp. PCC 6803 ketolase gene (*crtO*) [57] shifted the pathway towards the formation of 3′-hydroxyechinenone, 3-hydroxyechinenone, 4-ketozeaxanthin (adonixanthin) esters, 4-ketolutein and 4′-ketolutein esters in tobacco nectary tissue [78]. Some of these ketocarotenoids e.g. echinenone (derived from β-carotene) and 4-ketolutein (derived from lutein) also accumulated in leaves. Obviously such ketocarotenoids are formed in leaves by mono ketolation of carotenoids with the highest pools. As in tomato [77] and potato [60], echinenone was one of the major ketocarotenoids in tobacco leaves [78]. 4-Ketolutein had not been detected in leaves and nectaries earlier. Previously, its accumulation could be demonstrated in *A. thaliana* seeds [75] and in potato tubers [60]. In the nectaries of transgenic tobacco plants more than 50% of carotenoids were keto-derivatives. Levels of ketocarotenoid esters were much lower and a higher percentage of free ketocarotenoids accumulated. These results provide new opportunities for metabolic engineering of keto-hydroxy carotenoid production in carotenogenic flowers [78].

Nicotiana glauca flowers have carotenoid-pigmented petals, sepals, pistil, ovary and nectary tissues. This tobacco species was genetically engineered for ketocarotenoid biosynthesis by constitutive expression of either a *Nostoc punctiforme* PCC 73102 *crtW* gene (ORF148) or a cyanobacterial *Synechocystis* sp. PCC 6803 *crtO* gene. Plants transformed with the *crtW* ketolase accumulated 4-ketozeaxanthin (adonixanthin) as the only detectable ketocarotenoid [78]. In contrast the *crtO* transformants exhibited a wider spectrum of ketocarotenoids comprising 4′-ketolutein, echinenone, 3′-hydroxyechinenone and 4-ketozeaxanthin [79]. The total ketocarotenoid content in petals was over 3-fold higher in the later plants. Furthermore *crtO*-transformants exhibited an unexpected up-regulation of total carotenoid biosynthesis in leaves and more so in flower petals [79].

Transgenic *L. japonicus* plants expressing constitutively a *Paracoccus crtW* gene targeted to the plastids accumulated astaxanthin,

Table 2
Ketocarotenoid biosynthesis in higher plants through metabolic engineering.

Species	Genes (origin)	Promoters	Major ketocarotenoids/site of accumulation	References
Nicotiana tabacum cv. NN	*Bkt* (*Haematococcus pluvialis* 34/7)	Tomato phytoene synthase gene	Astaxanthin and canthaxanthin/nectaries	[16]
N. tabacum cv. Samsum	*CrtW* and *CrtZ* (*Paracoccus* sp. N81106)	Cauliflower mosaic virus (CaMV) 35S	Echinenone, astaxanthin, adonirubin and adonixanthin/leaves and nectaries	[77]
N. tabacum	*CrtO* (*Synechocystis* sp. PCC 6803); *CrtZ* (*Pantoea ananatis*)	CaMV 35S	Echinenone and 4-keto-lutein accumulation in leaves; adonixanthin, echinenone and 4-keto-lutein/nectaries	[78]
N. glauca	*CrtW*148 (*Nostoc* 73102)	CaMV 35S	Adonixanthin/flower petals	[78]
N. glauca	*CrtO* (*Synechocystis* sp. PCC 6803)	CaMV 35S	Echinenone, 4-keto-lutein, adonixanthin and 3́-hydroxyechinenone/leaves and flower petals	[79]
N. tabacum	*CrtW* and *CrtZ* (*Brevundimonas* sp. SD212)	Tobacco *rrn*	Astaxanthin and 4-ketoantherxanthin/leaves	[83]
Arabidopsis thaliana (Wassilewskija)	*Bkt* (*H. pluvialis*)	2S seed storage protein napA (oilseed rape)	4-Keto-lutein, canthaxanthin and adonirubin/seeds	[75]
A. thaliana (Columbia)	*Bch* (*A. thaliana*)	CaMV 35S	Astaxanthin/leaves	[81]
Lotus japonicus	*CrtW* (*Paracoccus* sp. N81106)	CaMV 35S	Astaxanthin, adonixanthin, canthaxanthin and echinenone/flower petals	[80]
Lycopersicon esculentum Mill. cv. Ailsa Craig	*CrtW* and *CrtZ* (*Paracoccus* sp. N81106)	CaMV 35S	Echinenone/leaves and fruits	[77]
Solanum tuberosum cv. Baltica	*CrtO* (*Synechocystis* sp. PCC 6803)	CaMV 35S	Echinenone, 3′-hydroxyechinenone and adonixanthin/leaves; astaxanthin, 3′-hydroxyechinenone and adonixanthin/tubers	[60]
S. tuberosum cv. Desiree	*Bkt* (*H. pluvialis* 34/7)	Patatin (potato)	4-Ketolutein and astaxanthin/tubers	[86]
S. phureja cv. Mayan Gold	*Bkt* (*H. pluvialis* 34/7)	Patatin (potato)	4-Ketolutein and astaxanthin/tubers	[86]
Daucus carota L. cv. HCM	*Bkt* (*H. pluvialis*)	Double CaMV 35S promoter	Astaxanthin, adonirubin, canxanthin, echinenone and adonixanthin/carrot roots	[87]
Brassica napus L.	*CrtW* and *CrtZ* (*Brevundimonas* sp. SD212)	Napin seed-specific (*B. napus*)	Echinenone, canthaxanthin, astaxanthin and adonixanthin/seeds	[88,89]
Zea mays L. cv. M37W	*CrtW* (*Paracoccus* sp. N81106); *Bch* (*Gentiana lutea*)	γ-zein (maize); glutelin-1 (rice)	Astaxanthin, adonixanthin, 3′-hydroxyechinenone and echinenone/endosperm	[18]

adonixanthin, canthaxanthin and echinenone in flower petals and leaves [80]. The total amount of carotenoids was increased 1.5-fold compared to wild type plants, and the newly formed ketocarotenoids constituted a quarter of the total carotenoid content in the transgenic plants.

To investigate the effect of β-carotene hydroxylase (*bch*) on oxycarotenoid biosynthesis, transgenic *A. thaliana* plants constitutively expressing a homologous *bch* gene were generated [81]. Astaxanthin accumulated in leaves at levels of up to 19.1 nmol/g DW. As astaxanthin is formed from β-carotene by the action of two different enzymes, β-carotene ketolase and β-carotene hydroxylase this report establishes the presence of a β-carotene ketolase in *Arabidopsis*. Astaxanthin accumulated most likely as a result of activation of β-carotene ketolase by the accumulation of metabolites in the transgenic plants expressing the introduced *bch*. An alternative explanation is that BCH might also exhibit ketolase activity. This, however, has not as yet been reported in the literature for the *Arabidopsis* enzyme.

Because carotenogenic enzymes are plastid-localized it is possible to engineer the pathway directly in plastids, at least in the few systems that are tractable in terms of plastid transformation [82]. Very recently, Hasunuma et al. [83] have introduced codon-optimized *crtW* and *crtZ* genes from *Brevundimonas* SD212 into tobacco (*N. tabacum*) chloroplasts. Transplastomic tobacco produced more than 0.5% (dry-weight) astaxanthin (more than 70% of total carotenoids) in leaves, turning their green color into reddish-brown.

Crop plants

Tomato (Lycopersicon esculentum)

A high β-carotene tomato (*Lycopersicon esculentum* cv. Ailsa Craig) was used as template to accumulate ketocarotenoids through constitutive co-expression of *Parococcus* β-carotene ketolase (CRTW) and β-carotene hydroxylase (CRTZ). The levels of ketocarotenoid (echinenone) in transgenic tomato leaves were approximately 50-fold less compared to transgenic tobacco plants expressing the same two transgenes [77]. Despite accumulation of mRNA for both introduced transgenes, negligible amounts (0.01 μg/mg DW) of echinenone were found in tomato fruits [77]. This finding suggests that β-carotene accumulated in high β-carotene tomato fruits is not accessible to ketolation or hydroxylation. In contrast, simultaneous expression of lycopene β-cyclase (LYCB) and β-carotene hydroxylase (BCH) in tomato fruits leads to the formation of zeaxanthin [84], indicating possible metabolic channeling between LYCB and BCH. The exact set of CRTW and CRTZ or BCH enzymes leading to astaxanthin production in tomato fruits awaits elucidation.

Potato (Solanum sp)

Potato has been a useful platform to investigate ketocarotenoid biosynthesis. A transgenic line accumulating zeaxanthin following inactivation of zeaxanthin epoxidase (ZEP) [85] was re-transformed with the *Synechocystis* sp. PCC 6803 *crtO* (β-carotene ketolase) gene [60]. Transgenic plants expressing *crtO* constitutively accumulated echinenone, 3′-hydroxyechinenone, and 4-ketozeaxanthin together with astaxanthin in tubers. The newly formed ketocarotenoids comprised approximately 10–12% of total carotenoids in leaves and tubers.

Solanum phureja cultivar Mayan Gold, a wild diploid strain naturally accumulating high levels of violaxanthin and lutein in tubers, and *Solanum tuberosum* cultivar Desiree, a low carotenoid-accumulating cultivar, were engineered with the *Haematococcus bkt* gene [86]. Transgenic plants from both species accumulated ketolutein and astaxanthin. Interestingly no ketocarotenoid intermediates in the pathway between β-carotene and astaxanthin were detected in transgenic tubers. The ketocarotenoid levels in tubers of *S. tuberosum* Desiree co-expressing *crtB*, encoding phytoene synthase, and *bkt*, were not enhanced in contrast to lines expressing *bkt* alone [86]. As some *bkt*/*crtB* transgenic tubers accumulated β-carotene, this substarte was not utilized efficiently by the ketolase enzyme in the transgenic tuber. The authors postulated that β-carotene is stored in a form inaccessible to the ketolase or that the ketolase does not readily use β-carotene as a substrate in tubers [86].

Carrot (Daucus carota L.)

Carrots naturally accumulate high levels of α- and β-carotene as well as lutein in roots. Astaxanthin and other ketocarotenoids constituted the bulk of carotenoids in transgenic carrot roots expressing a *Haematococcus bkt* gene [87]. Because 4-ketolutein was not detected in transgenic roots, the authors inferred that over-expression of a single ketolase results in efficient re-direction of the vast majority of the carotenoid pool towards the formation of β-ketocarotenoids. Endogenous expression of carrot β-carotene hydroxylases was up-regulated in transgenic leaves and roots, and up to 70% of total carotenoids were converted to novel ketocarotenoids, with accumulation of up to 2400 μg/g root dry weight. Astaxanthin, adonirubin, and canthaxanthin were most prevalent, followed by echinenone, adonixanthin and β-cryptoxanthin [87].

Rapeseed (Brassica napus L.)

In an effort to increase total carotenoid levels and also to accumulate astaxanthin, seven genes were co-introduced into rapeseed using a co-integrative vector encompassing all seven genes via *Agrobacterium*-mediated gene transfer [88,89]. These genes were: the *Paracocus* sp. N81106 isopentenyl pyrophosphate isomerase gene (*ipi*) and the *P. ananatis* genes encoding GGPP synthase (*crtE*), phytoene synthase (*crtB*), phytoene desaturase (*crtI*), and lycopene β-cyclase (*crtY*) to increase metabolic flux to β-carotene; in addition, the marine bacterium *Brevundimonas* SD212 *crtW* and *crtZ* genes were also incorporated in the same transformation vector to divert flux towards desired ketocarotenoids, including astaxanthin. All seven expression cassettes included a plastidial targeting signal and transgene expression was controlled by different promoters as follows: the *B. napus* napin seed-specific promoter was used to drive expression of *ipi*, *crtW and crtZ*; the *Arabidopsis* fatty acid elongation enzyme 1 (FAE1) seed-specific promoter was used to control expression of *crtB*; and the constitutive CaMV 35S promoter for *crtE*, *crtI* and *crtY*. Total carotenoid levels in the seeds of a highly expressing line were 559 μg/g FW. This enhancement corresponded to 18.6-fold increase over wild type [88,89]. Ketocarotenoids including echinenone, canthaxanthin, astaxanthin and adonixanthin, not normally produced in wild type rapeseed accumulated in the seeds of transgenic plants. The maximum accumulation of astaxanthin in transgenic rapeseed reached 2.2 μg/g FW [88,89].

Corn (Zea mays L.)

A novel combinatorial nuclear transformation method enabling the simultaneous integration of multiple transgenes into one genetic locus in recipient transgenic plants was used to generate a maize mutant series with diverse carotenoid and ketocarotenoid profiles [18]. Direct DNA transfer [90] was used to co-transform a white maize variety (*Z. mays* L., cv. M37W) deficient for endosperm carotenoid synthesis with five carotenogenic genes: *Zmpsy1* (*Z. mays* phytoene synthase 1), *PacrtI* (*P. ananatis* phytoene desaturase), *Gllycb* (*Gentiana lutea* lycopene β-cyclase), *Glbch* (*G. lutea*

β-carotene hydroxylase) and *ParacrtW* (*Paracoccus* β-carotene ketolase), all under the control of endosperm-specific promoters. In addition to phenotypes accumulating different combinations of carotenoids, three ketocarotenoid-accumulating phenotypes were recovered [18]. One phenotype expressing *Zmpsy1*, *PacrtI*, *Glbch* and *ParacrtW* accumulated adonixanthin alone. A second phenotype expressing *Zmpsy1*, *PacrtI*, *Gllycb* and *ParacrtW* produced adonixanthin, echinenone and 3-hydroxyechinenone together with astaxanthin. The third phenotype expressing all five transgenes accumulated adonixanthin, echinenone and 3-hydroxyechinenone. Furthermore, the authors demonstrated that β-carotene hydroxylase and bacterial β-carotene ketolase compete for the unsubstituted β-ionone rings as substrates in four sequential steps of the extended pathway, so that plants expressing all five transgenes accumulated complex mixtures of hydroxy- and ketocarotenoids in addition to other carotenes [18].

Conclusions

Despite reported successes in generating transgenic plants with altered ketocarotenoid composition, knowledge of the regulation of the pathway is relatively limited, and the subject is currently an area of active research. Efficient conversion of zeaxanthin to astaxanthin via adonixanthin (4-ketozeaxanthin) appears to be important for the accumulation of astaxanthin at high levels in transgenic plants (Table 2). A number of studies demonstrated that bacterial β-carotene ketolases convert β-carotene to astaxanthin inefficiently. An exception is expression of *Brevundimonas crtW* and *crtZ* in transplastomic tobacco leaves [83]. Indeed *Brevundimonas* CRTW exhibited the most efficient catalytic function for astaxanthin synthesis in *E. coli* complementation experiments [50,51]. In contrast, cyanobacterial CRTO and *Paracoccus* CRTW enzymes were shown to catalyze poorly the conversion of zeaxanthin to astaxanthin via adonixanthin. This is because it is difficult for cyanobacterial CRTO and *Paracoccus* CRTW to accept molecules with a 3-hydroxy β-ionone ring as substrates [45–48,50,61,91]. Similarly, *H. pluvialis* BKT has low conversion efficiencies for adonixanthin to astaxanthin [50,57]. For many plants transformed with a ketolase encoded by the *Paracoccus crtW* or the *Synechocystis crtO* genes, conversion of adonixanthin to astaxanthin appears to be an important limiting step for astaxanthin biosynthesis [18,60,79]. It appears, therefore, that in order to accumulate astaxanthin in transgenic plants, adonixanthin formation needs to be minimized or preferably blocked. One strategy that may help to overcome this constraint is the use of *Brevundimonas* sp *crtW* genes which appear to be more efficient in ketolating 3-hydroxy-β-ionone end groups [50,51,53]. A different strategy to achieve the same objective might be to create a mutated *crtW* gene conducive to the production of astaxanthin from adonixanthin [52,53]. It might also be possible to utilize the *X. dendrohous* astaxanthin synthase (ASY) which is a unique multifunctional enzyme able to catalyze all steps from β-carotene to astaxanthin through oxygenation of carbons 3 and 4 [64]. The reaction involves 4-ketolation of β-carotene followed by 3-hydroxylation without the accumulation of 3-hydroxy intermediates. As more in depth knowledge of the functional properties of β-carotene ketolases is generated, better informed decisions on a "best mode of action" for modulating the ketocarotenoid pathway in plants will emerge.

Combinatorial genetic transformation as applied to the engineering of maize with multiple genes involved in carotenoid biosynthesis provides a unique and straightforward strategy for metabolic pathway analysis and multi-gene engineering in plants [18]. It involves the introduction and coordinated expression of multiple transgenes followed by the selection of stable lines expressing the specific combination of transgenes required for particular metabolic outputs. By examining the entire diverse population of plants, it becomes possible to dissect the pathway and subsequently reconstruct it either in its original form or with modifications, thus providing a basis for understanding and subsequently engineering the synthesis of novel metabolites. This novel methodology allows facile recovery of combinatorial populations of plants expressing diverse carotenoid profiles. It opens the way for the combination of novel innovations in transformation technology, particularly allowing the efficient co-transformation of clusters of genes involved in a given metabolic pathway. This, in combination with advances in the elucidation of function of novel enzymes will allow the development of plants as "cell factories" for producing specialty ketocarotenoids for industrial and health applications.

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