

Pathway engineering of plants toward astaxanthin production

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Abstract Astaxanthin responsible for the red color of red fish and crustacean has beneficial effects on human health as well as its utility as a pigmentation source in aquaculture. Astaxanthin biosynthetic pathway from β -carotene needs two enzymes, a carotenoid 4,4'-ketolase (oxygenase) and a carotenoid 3,3'-hydroxylase. β -Carotene is usually one of dominant carotenoids in higher plants, which lack the former enzyme. This mini-review elucidates in the earlier section the catalytic functions of a series of the ketolase and hydroxylase enzymes that have been isolated up to now. For the last ten years, pathway engineering approaches of plants including staple crops toward astaxanthin production have been undertaken by introducing and expressing in the plants a ketolase gene sometimes along with a hydroxylase gene. Several successful results have been achieved to produce large amounts of astaxanthin together with other ketocarotenoids in plant tissues such as carrot roots and tobacco leaves.

Key words: Astaxanthin, carotenoid, pathway engineering, metabolic engineering.

“Red hero” astaxanthin

Carotenoids responsible for color ranging from light yellow through orange to deep red are biosynthesized in all of the photosynthetic organisms containing cyanobacteria, algae and higher plants, and also in some of non-photosynthetic bacteria, yeasts and fungi. Several carotenoids including astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) have attracted great attention due to their beneficial effects on human health as well as their utility as food colorant and/or feed additive. Astaxanthin is responsible for the red color of red fish, crab and prawn, and chemically synthesized one has been utilized as a pigmentation source in the aquaculture of salmon, rainbow trout and red sea bream, whose global market was US\$ 240 millions in 2007 according to BCC research report (code: FOD025C). This pigment has also been shown to have strong antioxidant and singlet oxygen-quenching activity (Miki 1991; Tatsuzawa et al. 2000), protect human low-density lipoprotein from oxidation (Iwamoto et al. 2000), have preventative effects on diabetic nephropathy in diabetic db/db mice (Naito et al. 2004) and on bladder carcinogenesis in mice (Tanaka et al. 1994), have an inhibitory effect on mammary tumor growth in mice (Chew et al. 1999), and have the possibility of enhancing immune responses (Jyonouchi

et al. 1995; Chew and Park 2004). Due to such beneficial effects, the global market of astaxanthin that is extracted from an unicellular green alga *Haematococcus pluvialis* producing this pigment (ester forms) has been extended as a food supplement or nutraceutical, whose size is now estimated to be US\$ 20 millions/year (communicated from Dr. Eiji Yamasihita, Fuji Chemical Industry Co).

Genes mediating biosynthesis of astaxanthin from β -carotene

The biosynthesis of astaxanthin from β -carotene (β,β -carotene) involves the introduction of keto and hydroxyl moieties at the 4,4' and 3,3' positions of the β -ionone rings by a carotenoid 4,4'-ketolase (4,4'-oxygenase; β -carotene ketolase) and a carotenoid 3,3'-hydroxylase (β -carotene hydroxylase), respectively (Misawa et al. 1995b; Fraser et al. 1997; 1998). The eight intermediates of ketolated and/or hydroxylated carotenoids can be generated as shown in Figure 1. Higher plants ordinarily do not synthesize astaxanthin, since they lack a β -carotene ketolase. β -Carotene is usually one of dominant carotenoids in plants, and can be hydroxylated to form zeaxanthin (β,β -carotene-3,3'-diol) via β -cryptoxanthin by an endogenous β -carotene hydroxylase (Bhy) (Sun et al. 1996), which has substantial sequence homology

Abbreviations: Bkt, green-algal β -carotene (carotenoid) ketolase; CrtW, bacterial β -carotene (carotenoid) ketolase; CrtO, bacterial β -carotene ketolase; CrtZ, bacterial β -carotene (carotenoid) hydroxylase; CrtR, cyanobacterial β -carotene (carotenoid) hydroxylase; CYP175A1, bacterial β -carotene hydroxylase. The abbreviations of plant carotenogenic enzymes are represented in the Figure 2 legend.

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(31–37% identity) to a bacterial corresponding enzyme CrtZ (Misawa et al. 1990). Genes encoding β -carotene ketolase CrtW and hydroxylase CrtZ have been isolated from astaxanthin-producing non-photosynthetic bacteria (α -Proteobacteria) such as *Paracoccus* sp. and *Brevundimonas* sp. (Lee and Kim 2006; Misawa et al. 1995a; 1995b; Nishida et al. 2005; Tao et al. 2006a). Their homologous genes have also been isolated from *H. pluvialis* (Kajiwara et al. 1995; Linden 1999; Lotan and Hirschberg 1995). This alga was shown to possess three different β -carotene ketolase genes, which were renamed *bkt1*, *bkt2* and *bkt3* (Huang et al. 2006). The *bkt1* and *bkt2* genes corresponded to *crtO* (Lotan and Hirschberg 1995) and *bkt* (Kajiwara et al. 1995) previously isolated from *H. pluvialis* and designated. Sequences homologous to *crtW* have been found not only in the above-mentioned α -Proteobacteria but also in cyanobacterial strains such as *Anabaena* sp. and *Nostoc* sp., which produce not astaxanthin but 4-ketomyxol 2'-fucoside that includes the 4-ketolated β -ionone ring in the molecule (Mochimaru et al. 2005; Steiger and Sandmann 2004). Genes, named *crtO*, encoding a new type of β -carotene ketolases, which show no significant sequence homology to CrtW-type ketolases, have been isolated from cyanobacterium *Synechocystis* sp. strain PCC 6803 (Fernandez-Gonzalez et al. 1997) and non-photosynthetic bacteria *Rhodococcus erythropolis* and *Deinococcus radiodurans* (Tao and Cheng 2004). This *crtO* gene is structurally different from “*crtO*” from *H. pluvialis* (Giuliano et al. 2008; Lotan and Hirschberg 1995). Thus, I recommend you to call the latter gene not *crtO* but *bkt1*, as renamed by Huang et al. (2006). *Adonis aestivalis* is an unusual higher plant that synthesizes astaxanthin in the petals. Two genes involved in the ketolation were isolated from this plant, and the gene products were found to have sequence homology not to CrtW or CrtO but to β -carotene hydroxylase (Bhy; CHY) and mediate 4- and 4'-ketolation reactions by ways different from the other types of ketolases (Cunningham and Gantt 2005).

Sequences homologous to *crtZ* have been found not only in the above-mentioned α -Proteobacteria but also in γ -Proteobacteria *Pantoea* sp. (formerly called *Erwinia* sp.) that produce zeaxanthin and its glucosides (Misawa et al. 1990; Hundle et al. 1991), whereas the sequences have not been found in cyanobacteria. As genes coding for new types of β -carotene hydroxylases that are structurally different from CrtZ-type hydroxylases, two genes, *crtR* and *CYP175A1*, have been isolated from cyanobacteria such as *Synechocystis* sp. PCC 6803 (Masamoto et al. 1998) and *Anabaena* sp. (Makino et al, 2008; Mochimaru et al. 2008), and a thermophilic bacterium *Thermus thermophilus* (Blasco et al. 2004), respectively.

Catalytic functions of β -carotene ketolases

We initially confirmed the catalytic functions of the CrtW enzymes derived from marine bacteria, *Paracoccus* sp. N81106 (NBRC101723; formerly called *Agrobacterium aurantiacum*) and *Paracoccus* sp. PC1 (NBRC101025; formerly *Alcaligenes* PC-1), and the *H. pluvialis* Bkt2 enzyme, by *in vitro* experiments using crude enzyme extracts from recombinant *E. coli* cells that expressed the corresponding genes (Fraser et al. 1997; 1998). A result showed that the ketolation enzymes are very likely to be α -ketoglutarate (2-oxoglutarate)-dependent dioxygenases (Fraser et al. 1997). Next, we compared the catalytic functions of various CrtWs, which originated from marine bacteria (α -Proteobacteria) *Paracoccus* sp. N81106, *Paracoccus* sp. PC1 and *Brevundimonas* sp. SD212 (NBRC101024), and cyanobacteria *Anabaena* (also known as *Nostoc*) sp. PCC 7120 and *Nostoc punctiforme* PCC 73102 (CrtW148), by complementation analysis using recombinant *E. coli* cells (Choi et al. 2005; Makino et al. 2008). The individual *crtW* genes were expressed in *E. coli* synthesizing β -carotene and zeaxanthin due to the presence of plasmids pACCAR16 Δ crtX and pACCAR25 Δ crtX, respectively (Misawa et al. 1995b). These results revealed that all of these CrtW (and Bkt2) enzymes accept zeaxanthin as well as β -carotene as the substrates, i.e., they introduce a keto group not only into the β -ionone rings but also into the 3(3')-hydroxylated β -ionone rings at the 4 (4') positions (Figure 1). Specifically, the *Brevundimonas* sp. SD212 CrtW protein converted adonixanthin, which was difficult to convert with the *Paracoccus* CrtW (and Bkt2) enzymes, into astaxanthin efficiently (Choi et al. 2005). On the other hand, the cyanobacterial CrtWs, whose phylogenetic positions are independent from the groups of α -Proteobacteria, were found to convert zeaxanthin into astaxanthin poorly (Makino et al. 2008). Mutational analysis of *crtW* proteins including the *Paracoccus* sp.

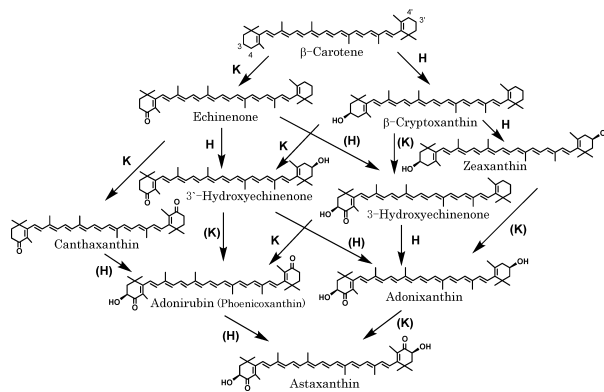


Figure 1. Pathway for biosynthesizing astaxanthin from β -carotene. K, carotenoid 4,4'-ketolase; H, 3,3'-hydroxylase. Parentheses (K) and (H) in Figure represent the steps that are postulated to be catalyzed only by CrtW (and Bkt1 and Bkt2) and CrtZ, respectively.

N81106 one was also performed to improve the conversion efficiency of the hydroxylated intermediates such as adonixanthin toward the production of astaxanthin (Ye et al. 2006; Tao et al. 2006b).

The catalytic function of CrtOs was also examined by the same method (Choi et al. 2007). It was very likely that CrtO enzymes from *Synechocystis* sp. PCC 6803 and *Rhodococcus erythropolis* PR4 (NBRC 100887) are unable to accept zeaxanthin, i.e., the 3(3′)-hydroxylated β -ionone rings as the substrates (Figure 1).

Catalytic functions of β -carotene hydroxylases

We also compared the catalytic functions of the CrtZ enzymes from soil bacterium (γ -*Proteobacteria*) *Pantoea ananatis* and from *Paracoccus* sp. N81106, *Paracoccus* sp. PC1 and *Brevundimonas* sp. SD212, by complementation analysis using recombinant *E. coli* cells (Choi et al. 2006). The individual *crtZ* genes were expressed in *E. coli* synthesizing β -carotene and canthaxanthin due to the presence of plasmids pACCAR16 Δ crtX and pAC-Cantha (Nishida et al. 2005), respectively. It was consequently shown that all of these CrtZ enzymes accept canthaxanthin as well as β -carotene as the substrates, i.e., they introduce a hydroxyl group not only into the β -ionone rings but also into the 4(4′)-ketolated β -ionone rings at the 3 (3′) positions (Figure 1). Specifically, the most efficient conversion from β -carotene to astaxanthin was performed by the *Brevundimonas* sp. SD212 CrtZ.

The catalytic functions of the CrtR enzymes derived from *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120 and *Anabaena variabilis* ATCC 29413, and cytochrome P450 (*CYP175A1*) from *T. thermophilus* HB27, whose structures are different from that of CrtZ, were also examined by the same method (Makino et al. 2008; Choi et al. 2006). Consequently, it was very likely that CrtR and CYP175A1 enzymes are not able to accept canthaxanthin, i.e., the 4(4′)-ketolated β -ionone rings, as the substrates (Figure 1). Moreover, the CrtR enzymes from *Anabaena* sp. PCC 7120 and *A. variabilis* ATCC 29413 were substantially unable to accept even β -carotene as the substrate (Makino et al. 2008), although the *Synechocystis* sp. PCC 6803 CrtR converted β -carotene into zeaxanthin efficiently (Makino et al. 2008; Masamoto et al. 1998).

Expression of a β -carotene ketolase gene in plants

Since higher plants do not possess a β -carotene ketolase gene, at least its introduction into plants and subsequent expression are necessary to confer the ability to produce ketocarotenoids such as astaxanthin. A β -carotene

ketolase gene is initially introduced into model plants tobacco (*Nicotiana tabacum*) and Arabidopsis (*Arabidopsis thaliana*), and ketocarotenoid formation was shown to be feasible in plant tissues: Mann et al. (2000) introduced the *H. pluvialis bkt1* gene into tobacco plants under the control of the tomato *pds* promoter. As a result, a transgenic tobacco produced ketocarotenoids (84% of total carotenoids) including 84 $\mu\text{g g}^{-1}$ fresh weight of astaxanthin in the nectary tissue (Table 1), although this transgenic plant did not accumulate them in the leaf tissue. Rally et al. (2004) introduced both the *crtW* and *crtZ* genes from *Paracoccus* sp. N81106 in tobacco plants under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and found that low levels of ketocarotenoids (2.5–3.2% of total carotenoids) including astaxanthin were formed in resultant transgenic leaves (Table 1; Figure 2). Stalberg et al. (2003) introduced the *H. pluvialis bkt2* gene into Arabidopsis plants under the regulation of the *Brassica napus napA* promoter, and found the formation of ketocarotenoids such as canthaxanthin, adonirubin and frittshiellaxanthin (4-ketolutein) (23% of total pigments) in the seeds. The β -carotene ketolase genes, *crtW* from *Paracoccus* sp. N81106 and *crtO* from *Synechocystis* sp. PCC 6803, were also expressed in the yellow petals of *Lotus japonicus* and *Nicotiana glauca*, respectively (Suzuki et al. 2007; Zhu et al. 2007). Resultant transgenic plants generated orange petals with ketocarotenoid formation (Table 1). Contents of the ketocarotenoids were similar in the two transgenic plants (23% and 29% of total carotenoids). On the other hand, astaxanthin was detected only in the *L. japonicus* flower, suggesting that the *crtW* gene is superior to the *crtO* to mediate the synthesis of astaxanthin, since the two genes were directed with the same CaMV 35S promoter. This finding coincides with our results of the complementation experiments using *E. coli* transformants. As for practical plants, several ketolase genes were tried to be expressed in several crops, i.e., tomato, potato and carrot plants (Rally et al. 2004; Morris et al. 2006; Gerjets and Sandmann 2006; Jayaraj et al. 2008). The *bkt1* and *crtO* genes were individually introduced into potato plants, and resultant transgenic plants were found to produce ketocarotenoids including astaxanthin in the tuber tissues (Morris et al. 2006; Gerjets and Sandmann 2006), as shown in Table 1. Jayaraj et al. (2008) expressed the *bkt1* gene in carrot roots using the double CaMV 35S promoter, and found that the transgenic roots were able to accumulate large amounts of ketocarotenoids (236 $\mu\text{g g}^{-1}$ fresh weight; 68% of total carotenoids) containing 91.6 $\mu\text{g g}^{-1}$ fresh weight of astaxanthin, 57.0 $\mu\text{g g}^{-1}$ fresh weight of adonirubin and 50.1 $\mu\text{g g}^{-1}$ fresh weight of canthaxanthin (Table 1). Recently, Hasunuma et al. (2008) introduced the genes [*crtW* (SD212) and *crtZ* (SD212)] encoding the CrtW and CrtZ proteins of

Table 1. Production of astaxanthin and ketocarotenoids in transgenic plants.

Transgenic plant (cultivar)	Ketolase gene [additional gene]	Promoter	Tissue	Astaxanthin [$\mu\text{g g}^{-1}$ fresh weight (%)]	Total ketocarotenoids [$\mu\text{g g}^{-1}$ fresh weight (%)]	Total carotenoids [$\mu\text{g g}^{-1}$ fresh weight (%)]	Reference
<i>Nicotiana tabacum</i> (NN)	<i>bkt1</i>	<i>pds</i>	nectary	84 (24%)	300 (84%)	357	Mann et al. (2000)
<i>N. tabacum</i> (Samsun)	<i>crtW</i> (N81106) [<i>crtZ</i> (N81106)]	CaMV 35S	leaf	detected	800* (3.2%)	25,000*	Rally et al. (2004)
<i>Lotus japonicus</i> (Miyakojima)	<i>crtW</i> (N81106)	CaMV 35S	petal	detected	89.8 (23%)	387	Suzuki et al. (2007)
<i>Nicotiana glauca</i>	<i>crtO</i> (6803)	CaMV 35S	petal	not detected	190* (29%)	666*	Zhu et al. (2007)
<i>Solanum phureja</i> (Mayan Gold)	<i>bkt1</i>	patatin	tuber	13.9* (46%)	19.8* (65%)	30.4*	Morris et al. (2006)
<i>Solanum tuberosum</i> (Baltica)	<i>crtO</i> (6803)	CaMV 35S	tuber	1.8* (4.6%)	10.7* (27%)	39.2*	Gerjets and Sandmann (2006)
<i>Daucus carota</i>	<i>bkt1</i>	CaMV 35S (double)	root	91.6 (27%)	236 (68%)	346	Jayaraj et al. (2008)
<i>N. tabacum</i> (Xanthi)	<i>crtW</i> (SD212) [<i>crtZ</i> (SD212)]	<i>rrn</i> (in chloroplast)	leaf	34.7 (15%)	52.3 (23%)	226	Hasunuma et al. (2008)
<i>N. tabacum</i> (Xanthi)	<i>crtW</i> (SD212)	<i>rrn</i> (in chloroplast)	leaf	5,440* (74%)	7,290* (99%)	7,380*	Hasunuma et al. (2008)
			leaf	1,880* (49%)	3,830* (100%)	3,840*	Hasunuma et al. (2008)

Asterisks indicate data for dry weight of tissue.

bkt1, *bkt2* from *H. pluvialis*; *crtW* (N81106) and *crtZ* (N81106), *crtW* and *crtZ* from *Paracoccus* sp. N81106; *crtO* (6803), *crtO* from *Synechocystis* sp. PCC 6803; *crtW* (SD212) and *crtZ* (SD212), chemically synthesized genes with the codon usage of *Brassica napus* that encode the *CrtW* and *CrtZ* proteins from *Brevundimonas* sp. SD212. In order to express these genes in plants and target the gene products into the plastids, a transit peptide sequence such as that of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) small subunit from pea is needed to be fused with 5' upstream of each gene, as initially described by Misawa et al. (1993 & 1994).

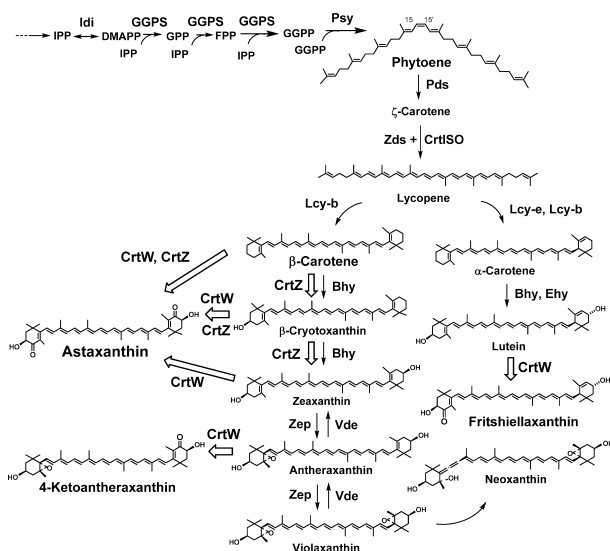


Figure 2. Carotenoid biosynthetic pathway in plants and the catalytic functions of *CrtW* and *CrtZ* introduced and expressed in tobacco leaves. IPP, isopentenyl diphosphate (pyrophosphate); DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; Idi, IPP isomerase; GGPS, GGPP synthase; Psy, phytoene synthase; Pds, phytoene desaturase; Zds, ζ -carotene desaturase; CrtISO, carotene isomerase; Lcy-b, lycopene β -cyclase; Lcy-e, lycopene ϵ -cyclase; Bhy, β -carotene hydroxylase; Zep, zeaxanthin epoxidase; Vde, violaxanthin de-epoxidase; Ehy, ϵ -carotene hydroxylase.

Brevundimonas sp. SD212, which are the combination of the most efficient enzymes for synthesizing astaxanthin from β -carotene, into the chloroplasts of tobacco plants by plastid transformation. It was consequently revealed that the transformed plants can produce the largest amount of astaxanthin as the predominant carotenoid in the leaves, without any significant damage to the plants: i.e., astaxanthin levels reached 5.44 mg g^{-1} dry weight (74% of total carotenoids) (Table 1), which could correspond to 0.54 mg g^{-1} fresh weight based on 90% moisture level in the leaves. Surprisingly, almost all of the carotenoids generated in the leaves were ketocarotenoids (99%), which contained the ketolated products of lutein and antheraxanthin, fritshiellaxanthin (4-ketolutein) and 4-ketoantheraxanthin, respectively, in addition to astaxanthin (Shindo et al. 2008). The pathway for synthesizing these ketocarotenoids is shown in Figure 2. Moreover, the total carotenoid amount in the transplastomic tobacco was 2.1-fold higher than that of wild-type tobacco (Hasunuma et al. 2008), suggesting up-regulation for carotenoid biosynthesis probably due to the stream from the plant-original carotenoids to the ketocarotenoid formation. Hasunuma et al. (2008) also found that the simultaneous high expression of *crtZ* (SD212) in addition to *crtW* (SD212) was necessary for the efficient synthesis of astaxanthin, i.e., a transplastomic tobacco plant expressing only the *crtW* (SD212) gene produced much lower level of astaxanthin (1.88 mg g^{-1}

dry weight) in the leaves (Table 1).

Outlook for commercial use of transgenic plants to engineer the astaxanthin biosynthetic pathway

As mentioned above, the high expression of the carotenoid 4,4'-ketolase gene *crtW*, *bkt1* or *bkt2* [specifically *crtW* (SD212)] in a desired plant tissue is needed for producing larger amounts of astaxanthin. The high expression of only the ketolase gene may seem enough for producing large amounts of astaxanthin, judging from the successful results with *bkt1* reported by Jayaraj et al. (2008). Whereas, the simultaneous high expression of the carotenoid 3,3'-hydroxylase gene *crtZ* (SD212) in addition to the ketolase gene was found to be useful for increasing astaxanthin levels (Hasunuma et al. 2008). In the transplastomic tobacco [the *crtW* (SD212) and *crtZ* gene (SD212)] producing the ketocarotenoids (99% of total carotenoids), the photosynthesis rate was shown to be similar to that of wild-type plants under ordinary light intensity (Hasunuma et al. 2008). Thus, we can utilize strong constitutive promoters such as CaMV 35S promoter as well as tissue-specific promoters for the high expression of the ketolase and hydroxylase genes. The step from GGPP to phytoene has been shown to be a very important key step to increase carotenoid levels, i.e., the expression of the phytoene synthase gene (*crtB*) from *P. ananatis* in a plant tissue has been found to elevate total carotenoid content (Shewmaker et al. 1999; Fraser et al. 2002; Morris et al. 2006; Fujisawa et al. 2008a). The *crtB* expression along with the high expression of the *crtW* (SD212) (or probably *bkt1*) and *crtZ* (SD212) genes in a desired plant tissue are very likely to be effective for producing great amounts of astaxanthin together with other commercially useful carotenoids. Based on such viewpoints, multiple carotenoid biosynthesis genes including *crtB*, *crtW* and *crtZ* were introduced and expressed in staple crops, rapeseed (canola; *B. napus*) and maize (*Zea mays*) (Fujisawa et al. 2008b; Zhu et al. 2008), awaiting their detailed reports. Continuous efforts focused on engineering of the astaxanthin biosynthetic pathway in plants would enable us to reach the commercial use of an astaxanthin-producing transgenic crop in the near future.

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