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ORIGINAL PAPER

Functional expression of the astaxanthin biosynthesis genes from a marine bacterium, *Paracoccus haeundaensis*

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Abstract The astaxanthin biosynthesis gene cluster in *Paracoccus haeundaensis* consists of six genes: *crtW*, *crtZ*, *crtY*, *crtI*, *crtB*, and *crtE* contain 726, 486, 1158, 1503, 912, and 879 base pairs, respectively. Individual carotenoid biosynthesis genes of *P. haeundaensis* have now been expressed in *E. coli* and each gene product has been purified to homogeneity. Their molecular characteristics, including enzymatic activities, are reported here.

Keywords Astaxanthin · Carotenoid · *crt* genes · Expression · Marine bacterium

Introduction

Carotenoids have recently attracted commercial interest not only as a source of pigmentation (Hencken 1992) but also for their beneficial effects on human health, including their function as antioxidants (Murtaugh et al. 2004), which includes a possible role in cancer prevention (Kurihara et al. 2002) and in enhancing immune responses (Amar et al. 2004).

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Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is an abundant carotenoid found in marine animals, including salmonids and crustaceans. In addition to its antioxidative activity, astaxanthin has numerous biological functions including being a vitamin A precursor, enhancing gap junction communications, modulating the immune system, and possessing antitumor activities (Amar et al. 2004). Astaxanthin has been isolated from various sources, including the yeast *Phaffia rhodozyma* (Johnson et al. 1978), the green alga *Haematococcus pluvialis* (Bubrick 1991), the Gram-positive bacterium *Brevibacterium linens* (Krubasik and Sandmann 2000), the Gram-negative bacterium *Agrobacterium aurantiacum* (Misawa et al. 1995), and the marine bacterium *Paracoccus haeundaensis* (Lee et al. 2004).

Many carotenoid biosynthesis genes have been cloned and characterized from the various organisms that produce astaxanthin, and the functions of the gene products have been extensively studied (Harker et al. 1998; Lee and Kim 2006). Early steps in the biosynthesis of carotenoids include the formation of geranyl geranyl pyrophosphate (GGPP) from farnesyl pyrophosphate (FPP) by GGPP synthase, an enzyme encoded by the *crtE* gene. The formation of phytoene from GGPP is catalyzed by a phytoene synthase encoded by the *crtB* gene. Phytoene is then dehydrated by a phytoene dehydrogenase, which is encoded by the *crtI* gene. In many

carotenogenic organisms, the process is completed with the cyclization of lycopene β-cyclase, which is encoded by the *crtY* gene, yielding a β-carotene. In other organisms, carotenoids are modified with oxygen-containing functional groups, thus creating xanthophylls. Both prokaryotes and eukaryotes can convert β-carotene into astaxanthin by using several ketocarotenoids as intermediates and only two enzymes: a β-carotene ketolase, which is encoded by the *crtW* gene, and a β-carotene hydroxylase, encoded by the *crtZ* gene. β-Carotene ketolase (*crtW* gene) initially converts β-carotene to canthaxanthin via echinenone, and β-carotene hydroxylase (*crtZ* gene) initially mediates the conversion of β-carotene to zeaxanthin via β-cryptoxanthin. Finally, through a few more intermediates, the *crtW* and *crtZ* gene products act in combination to produce astaxanthin (Lee and Kim 2006). A scheme of the astaxanthin biosynthesis pathways is shown in Fig. 1.

In bacteria that cannot naturally synthesize carotenoids, such as *E. coli*, *de novo* carotenoid biosynthesis has been introduced by transformation with the carotenogenic biosynthesis genes (Hundle et al. 1992). We have previously isolated and characterized a new marine bacterium, *Paracoccus haeundaensis*, which produces carotenoids, primarily astaxanthin (Lee et al. 2004). Furthermore, we have cloned and characterized a carotenoid biosynthesis gene cluster responsible for the production of astaxanthin from this organism (Lee and Kim 2006).

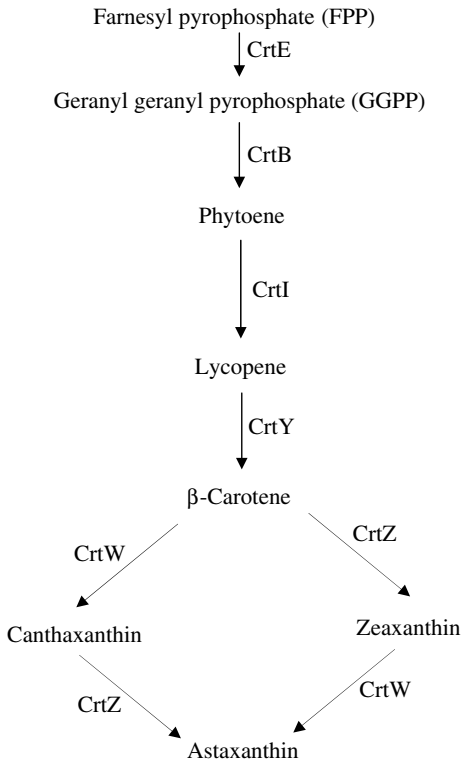

Fig. 1 Scheme of the astaxanthin biosynthesis pathways from the *Paracoccus haeundaensis*

Here, high-level expressions of carotenoid biosynthesis genes from *P. haeundaensis* were achieved by introducing these genes into *E. coli.* The recombinant proteins were produced in the cytoplasm of the transformed cells. We describe the purification and characterization of expressed proteins from the transformed cells with each gene encoding the carotenoid biosynthetic enzymes from *P. haeundaensis*. These results will provide a wider knowledge of the primary structures of the astaxanthin biosynthesis enzymes at the molecular level and facilitate the biotechnological applications of carotenoids.

Materials and methods

Expression of the carotenoid biosynthesis genes in *E. coli*

To express each gene product from the carotenoid biosynthesis enzymes of *Paracoccus haeundaensis*, the expression plasmid was constructed with the cluster's individual genes. Each gene was amplified by PCR using a pair of corresponding oligonucleotides specifically designed for cloning each individual gene, which were loaded onto the plasmid pCR-XL-TOPO-Crtfull as templates (Table 1). The resultant plasmid, pCR-XL-TOPO-Crtfrull, therefore contained the carotenoid biosynthesis gene cluster (Lee and Kim 2006). The amplified fragment was subcloned into the vector pGEM-T (Promega, Madison, WI, USA) and its nucleotide sequence was confirmed by DNA sequencing. The subcloned plasmid was digested with *Nde*I and *Xho*I restriction enzymes. The excised fragment was then ligated into the expression plasmid pET44-a(+) vector, and the resulting plasmids carrying an individual gene of

Table 1 Oligonucleotide primers used in this study

Name	Nucleotide sequence	Remarks
CrtW-1	5′-CCATATGAGCGCACATGCCCTG-3′	Primer for *crtW*, Forward (*Nde*I site)
CrtW-2	5′-CTCGAGTGCGGTGTCCCC-3′	Probe for *crtW*, Reverse (not included stop codon, *Xho*I site)
CrtZ-1	5′-GCATATGACCAATTTCCTGATC-3′	Probe for *crtZ*, Forward (*Nde*I site)
CrtZ-2	5′-CTCGAGGGGTCACGTGCGC-3′	Probe for *crtZ*, Reverse (not included stop codon, *Xho*I site)
CrtY-1	5′-GCATATGACCATGACGTGCTG-3′	Primer for *crtY*, Forward (*Nde*I site)
CrtY-2	5′-CTCGAGTCATGCGTTTTCCTTCAG-3′	Probe for *crtY*, Reverse (not included stop codon, *Xho*I site)
CrtI-1	5′-TCATATGAACGCCCATTCGC-3′	Primer for *crtI*, Forward (*Nde*I site)
CrtI-2	5′-CTCGAGCGACAGGACCAGATC-3′	Probe for *crtI*, Reverse (not included stop codon, *Xho*I site)
CrtB-1	5′-GCATATGAGCGATCTGGTCCTG-3′	Primer for *crtB*, Forward (*Nde*I site)
CrtB-2	5′-CTCGAGAGACGTGATGCGG-3′	Probe for *crtB*, Reverse (not included stop codon, *Xho*I site)
CrtE-1	5′-CCATATGAGACGAGACGTCAA-3′	Primer for *crtE*, Forward (*Nde*I site)
CrtE-2	5′-GCCGCCTCGCTAGGCGC-3′	Probe for *crtE*, Reverse (not included stop codon, *Xho*I site)

the carotenoid biosynthesis enzymes were designated pET-44a(+)-CrtW, pET-44a(+)-CrtZ, pET-44a(+)-CrtY, pET-44a(+)-CrtI, pET-44a(+)-CrtB, and pET-44a(+)-CrtE (Fig. 2). The plasmids containing inserts were sequenced using an ABI Prism DNA sequencing kit and an ABI 377 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

The plasmids containing the individual genes were transformed into the competent *E. coli* strain BL21(DE3) Codon plus. The cells harboring a plasmid containing each of the carotenoid biosynthesis genes were cultured overnight at 30°C in LB medium (containing 50 μg ampicillin/ml) and induced by adding IPTG to 1 mM at cell density of OD_{600} = 0.5. The production of carotenoids was analyzed after extracting the pigments produced from the transformed *E. coli* strains.

Purification of recombinant carotenoid biosynthesis proteins in transformed *E. coli*

Subcloning of the carotenoid biosynthesis genes into the pET-44a(+) vector produces a fusion protein tagged with histidine residues in the carboxyl terminus, resulting in a CRT-His fusion protein. Cultured cells expressing the individual carotenoid biosynthesis protein were harvested by centrifugation, and the cell pellets were re-suspended in extraction buffer (20 mM Tris/HCl, pH 8.0) then sonicated with an ultrasonicator (VC130; Sonics and Materials Inc., USA). The samples were centrifuged for 10 min at 12,000*g* and the precipitates were re-suspended in isolation buffer (20 mM Tris/HCl, 2 M urea, 0.5 M NaCl, 2% Triton X-100, pH 8.0), sonicated again, and harvested by centrifugation for 10 min at 12,000*g*. The pellets were then re-suspended with binding buffer (20 mM Tris/HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0) and filtrated with a 0.45 μm filter. Prepared samples were loaded onto an affinity column using a His-Trap Chelating HP column Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacture's instructions. The protein content was determined according to the Bradford method.

Western blot analysis of recombinant carotenoid biosynthesis proteins

Purified proteins, 10 μg, were electrophoresed on a 15% SDS-PAGE. Immunoblot analysis was conducted by standard procedures (Gershoni and Palade 1983). The blot was incubated for 1 h with 3% (w/v) gelatin in TTBS (20 mM Tris/HCl [pH 7.4], 500 mM NaCl, 0.05% Tween 20) and then rinsed with TTBS. Subsequently, a polyclonal antibody against goat anti-6-histidine (diluted 1:500) was added and incubated for 1 h at room temperature. After rinsing three times with TTBS, the membrane was incubated with alkaline phosphatase-conjugated anti-goat IgG (diluted 1:2000 in TTBS containing 1% gelatin; Sigma) as the secondary antibody at room temperature for 30 min. The membrane was then rinsed three times with TTBS and developed at room temperature.

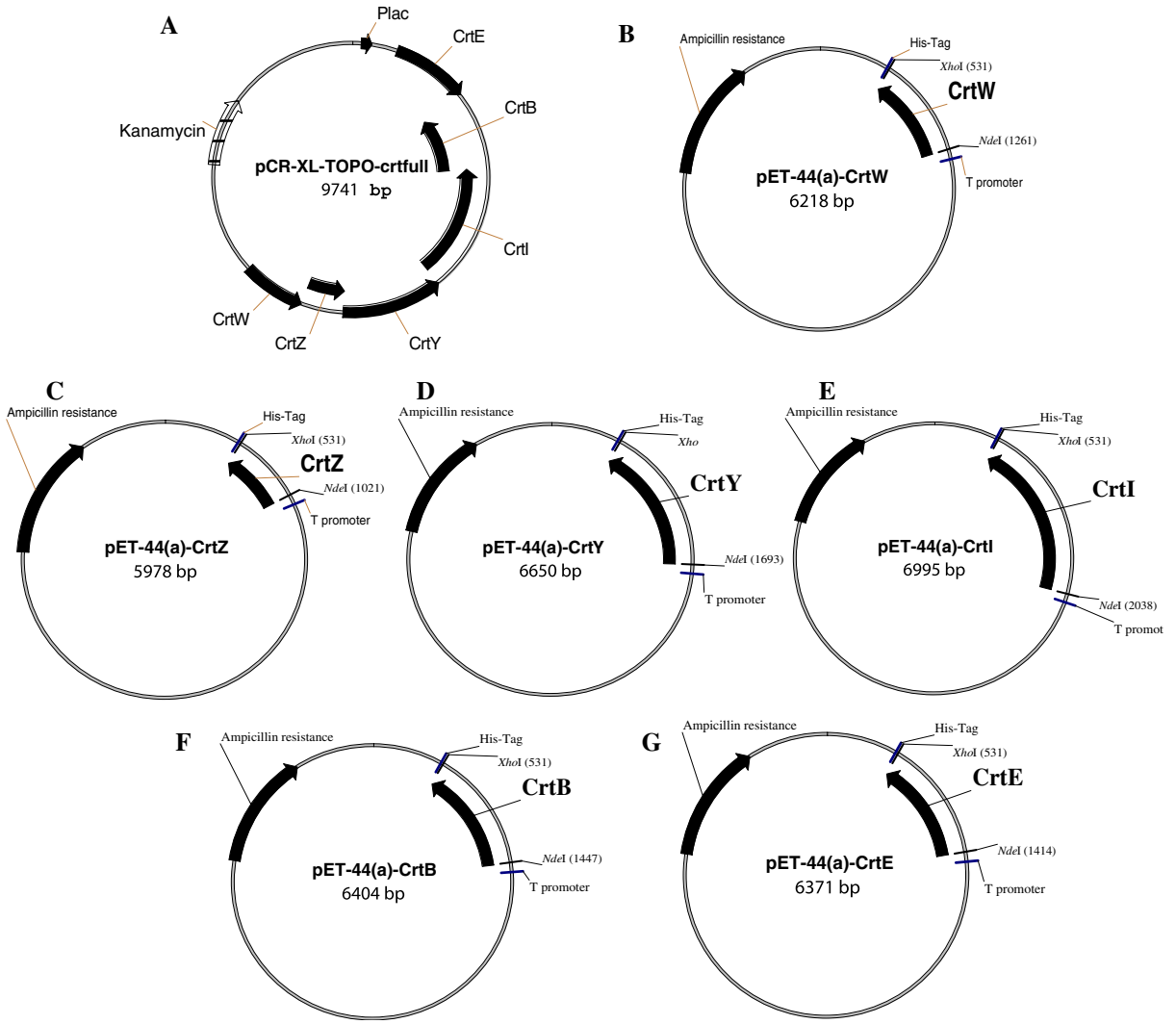

Fig. 2 Genetic map of the expression plasmid of the *Paracoccus haeundaensis* carotenoid biosynthesis enzymes. **A**: pCR-XL-TOPO-Crtfull, **B**: pET-44a(+)-CrtW, **C**: pET-44a(+)-CrtZ, **D**: pET-44a(+)-CrtY, **E**: pET-44a(+)-CrtI, **F**: pET-44a(+)-CrtB, and **G**: pET-44a(+)-CrtE

Enzyme assay

The biological activities of the carotenoid biosynthesis enzymes were measured according to previously described methods (Hannibal et al. 2000) with the following modifications: the incubation mixture (500 µl) for the enzyme assay was made with 0.1 M Tris/HCl buffer, pH 8, containing 10 mM ATP, 1 mM dithiothreitol, 0.5 mM $FeSO_4$, 0.5 mM 2-oxoglutarate, 6 mM MnCl, 5 mM ascorbic acid, 4 mM $MgCl_2$, and deoxycholate (0.1% mass/vol). Additions of 5 µg of each substrate (FPP for CrtE, GGPP for CrtB, phytoene for CrtI, lycopene for CrtY, β-carotene for CrtZ, and zeaxanthin for CrtW) and 300 ng protein of each purified carotenoid biosynthesis enzymes were performed. The assays were carried out at 37°C for 15 h under constant shaking and dark conditions. After termination of the enzyme reaction by the addition of 2.5 ml of methanol, the carotenoids were partitioned into 10% diethylether in petroleum ether, evaporated, redissolved in acetone, and then separated by HPLC analysis using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped

with a temperature-controlled autosampler and a diode array detector. The column was a YMC Carotenoid C_{30} column (250 mm × 4.6 mm; Waters Corp., Milford, MA, USA), and the guard column was a Pelliguard LC-18 cartridge (20 mm; SUPELCO, Bellefonte, PA, USA). The mobile phase was methanol/methyl tert-butyl ether (A/B) at 1 ml/min gradient with the following parameters (all percentages expressed as v/v): start, A/B (80:20, v/v) for 10 min, A/B (65:35, v/v) for 20 min, and A/B (10:90, v/v) for 10 min. The injection volume and column temperature were 10 μl and 15°C, respectively. The detection of GGPP and phytoene was measured at 283 nm with the other carotenoids detected at 470 nm. Astaxanthin, *β*-carotene, lycopene, geranyl geranyl pyrophosphate (GGPP), and farnesyl phyrophosphate (FPP) were purchased from Sigma, and phytoene and zeaxanthin were purchased from Roth Karlsruhe (Germany) as authentic samples. Protein concentration was estimated using the Bio-Rad protein assay dye reagent following the manufacturer's instructions. Enzyme activity was expressed as pmol formed per h/mg of protein in all cases.

Extraction and purification of carotenoids produced in transformed *E. coli*

Acetone was used to extract the carotenoid pigments from the transformants carrying the carotenoid biosynthesis genes. The acetone extracts were evaporated to dryness and were further extracted with chloroform/methanol (9:1, v/v). After being dissolved in a small volume of *n*-hexane, the carotenoids were analyzed by HPLC using a Nova-Pak HR 6 U C_{18} column (3.9 mm × 300 mm) with acetonitrile/methanol/2-propanol (90:6:4, by vol.) at 1 ml/min, and the elution profiles were recorded with a photodiode array detector.

The pigments were purified by TLC silica gel, developed with chloroform/methanol (50:1, v/v) and were further purified by Sephadex LH-20 (Amersham Pharmacia Biotech) column chromatography developed with chloroform/methanol (1:1, v/v). Synthetic FPP, GGPP, phytoene, lycopene, *β*-carotene, zeaxanthin, and astaxanthin were used as references.

Results and discussion

We have previously described the cloning and sequence analysis of genes encoding the astaxanthin biosynthetic enzymes from *P. haeundaensis* (Lee and Kim 2006). All six genes of the carotenoid biosynthesis gene cluster of *P. haeundaensis* (*crtW*, *crtZ*, *crtY*, *crtI*, *crtB*, and *crtE*) necessary for astaxanthin biosynthesis were cloned and characterized. When *E. coli* BL21(DE3) was transformed with the plasmid containing the entire gene cluster (pCR-XL-TOPO-Crt-full), carotenoids, mainly astaxanthin, were produced without a requirement for additional *P. haeundaensis*, suggesting that *P. haeundaensis* astaxanthin biosynthesis genes can be functionally expressed in *E. coli*. In addition, the expression occurred without using any *E. coli* genes (Lee and Kim 2006). Despite this, detailed studies of each enzyme from the astaxanthin biosynthetic enzymes have not yet been conducted. In an effort to elucidate the mechanism responsible for controlling the astaxanthin biosynthetic pathway and the intracellular carotenoid concentration, it would therefore be necessary to conduct a comparative analysis of the structure, expression, and function of the astaxanthin biosynthesis genes. We have further studied their expression and characterization to elucidate the organization and characteristics of the individual enzyme of the carotenoid biosynthesis genes.

To express an individual gene product of the carotenoid biosynthesis genes, plasmids containing an individual gene of *crtW*, *crtZ*, *crtY*, *crtI*, *crtB*, or *crtE* genes were constructed into the expression vector, pET44-a(+), under the control of T7 promoter, which allows expression of recombinant protein with *C*-terminal fusion His-tag. The resulting expression plasmid was designated as pET-CrtW, pET-CrtZ, pET-CrtY, pET-CrtI, pET-CrtB and pET-CrtE (Fig. 2). The *β*-carotene ketolase gene (*crtW*) isolated from *P. haeundaensis* consisted of 726 bp encoding a polypeptide of 242 amino acid residues; the ß-carotene hydroxylase gene (*crtZ*) contained 486 bp and encoded a 162-amino acid polypeptide; the lycopene cyclase gene (*crtY*) consisted of 1158 bp encoding a polypeptide of 386 amino acids; the phytoene dehydrogenase gene (*crtI*),

consisting of 1503 bp, encoded a 501-amino acid polypeptide; the phytoene synthase gene (*crtB*) consisted of 912 bp, which encoded a polypeptide of 304 amino acids; and the geranyl diphosphate synthase gene (*crtE*) was composed of 879 bp encoding a polypeptide of 293 amino acid residues. The plasmids harboring individual genes were transformed into *E. coli* BL21 (DE3) Codon Plus strain and the expression of each gene was accomplished by the addition of IPTG. When the transformants containing Crt genes were cultured at 37°C, the expressed recombinant protein was aggregated into inclusion bodies and as inactive enzyme; however, when the transformants were cultured at 30°C, a significant portion of the recombinant proteins were insoluble and accumulated at levels of 5–10% of the total cytosolic proteins as active forms.

The optimal induction time of the recombinant CrtW, CrtZ, CrtY, CrtI, CrtB, and CrtE proteins was achieved at 6, 3, 6, 3, 3, and 3 h after induction, respectively. The expressed recombinant CrtW, CrtZ, CrtY, CrtI, CrtB, and CrtE proteins were purified by affinity chromatography as described in the Materials and methods section and the molecular weights of the purified protein were determined as 27, 18, 42, 55, 33, and 30 kDa in a single band, respectively. These Crt proteins tagged with His residues were confirmed by Western blot analysis (Fig. 3). The concentration of Crt proteins purified after affinity chromatography was typically about 0.5 mg/ml.

We have also provided the molecular characteristics and expression of our newly identified astaxanthin biosynthesis genes. We determined the functions of the astaxanthin biosynthesis enzymes through chromatographic and spectroscopic analysis of pigments produced from the transformed *E. coli* with individual genes. To test the biological activity of the purified carotenoid biosynthesis enzymes, enzymatic assays were performed on the purified proteins. As summarized in Table 2, individual gene products of the carotenoid biosynthesis genes purified from the *E. coli* transformants displayed catalytic activity. High-level expressions of individual genes from the *P. haeundaensis* carotenoid biosynthesis genes were achieved by introducing each gene into the *E. coli* cells. Each gene product of the carotenoid biosynthesis genes was purified to homogeneity, and the purified protein displayed enzymatic activity. These data will provide a wider base of knowledge on the primary structure of the astaxanthin biosynthesis gene cluster at the molecular level and biotechnological applications of carotenoids. The observations and genetic manipulations of the astaxanthin biosynthesis enzymes from *P. haeundaensis* make this species a very useful model in which to study the mechanism of astaxanthin biosynthesis. In addition, the results of this study can be used to enhance the production of astaxanthin through the manipulation of carotenoid biosynthesis genes in *P. haeundaensis*, an important application since astaxanthin is a pigment of high economic value.

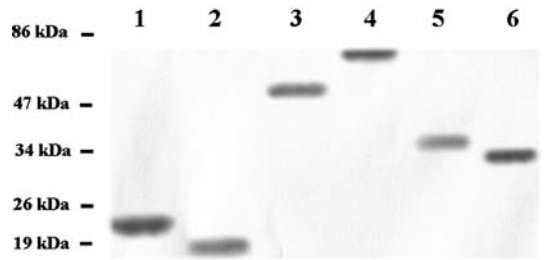

Fig. 3 Western blot analysis of the purified carotenoid biosynthesis proteins. Lane 1, CrtW; lane 2, CrtZ; lane 3, CrtY; lane 4, CrtI; lane 5, CrtB; lane 6, CrtE

Table 2 Enzymatic activities of the carotenoid biosynthesis enzymes CrtE, CrtB, CrtI, CrtY, CrtZ, and CrtW

Carotenoid biosynthesis enzymes	Substrate	Enzyme activity (pmol/h/mg protein)
CrtE	FPP	15
CrtB	GGPP	210
CrtI	Phytoene	11
CrtY	Lycopene	21
CrtZ	β-carotene	13
CrtW	Zeaxanthin	30

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