Appl Microbiol Biotechnol (2006) 71: 473–479
DOI 10.1007/s00253-005-0166-8

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Isolation and characterization of a carotenoid oxygenase gene from *Chlorella zofingiensis* (Chlorophyta)

Received: 22 July 2005 / Revised: 30 August 2005 / Accepted: 31 August 2005 / Published online: 8 October 2005

Abstract The green alga *Chlorella zofingiensis* produces large amounts of the valuable ketocarotenoid astaxanthin under dark, heterotrophic growth conditions, making it potentially employable for commercial production of astaxanthin as feed additives, colorants, and health products. Here, we report the identification and characterization of a β-carotene oxygenase (CRTO) gene that is directly involved in the biosynthesis of ketocarotenoids in *C. zofingiensis*. The open reading frame of the *crtO* gene, which is interrupted by three introns of 243, 318, and 351 bp, respectively, encodes a polypeptide of 312 amino acid residues. Only one *crtO* gene was detected in the genome of *C. zofingiensis*. Furthermore, the expression of the *crtO* gene was transiently up-regulated upon glucose treatment. Functional complementation in *Escherichia coli* showed that the coding protein of the *crtO* gene not only exhibits normal CRTO activity by converting β-carotene to canthaxanthin via echinenone, but also displays a high enzymatic activity of converting zeaxanthin to astaxanthin via adonixanthin. Based on the bifunctional CRTO, a predicted pathway for astaxanthin biosynthesis in *C. zofingiensis* is described, and the CRTO is termed as carotenoid 4,4′-β-ionone ring oxygenase.

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Introduction

Carotenoids are important natural pigments that may be produced by many microorganisms and plants (Johnson and Schroeder 1995; Lee and Schmidt-Dannert 2002). The ketocarotenoid astaxanthin has recently attracted much attention due to its strong antioxidant properties that give it diverse biological capabilities such as protection against UV-light photooxidation, inflammation, cancer, and age-related diseases (Lorenz and Cysewski 2000; Guerin et al. 2003). Astaxanthin is commonly found in marine animals, e.g., crustaceans, shellfish, and salmonoids (Lorenz and Cysewski 2000). These animals do not possess the ability to synthesize the carotenoid pigment de novo. Instead, they acquire the pigment from their diet. Astaxathin has been exploited as a feed supplement for fish and shellfish.

The biosynthesis of astaxanthin is limited to some microorganisms, e.g., the yeast *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*), the freshwater alga *Haemoatococcus pluvialis*, and the marine bacteria *Agrobacterium aurantiacum* (Johnson and An 1991; Lorenz and Cysewski 2000). The final steps in astaxanthin formation are ketolation and hydroxylation of β-carotene. In all studied organisms except *X. dendrorhous*, the two modification reactions of β-carotene are independent. Two different pathways of astaxanthin biosynthesis were proposed: one starting from the oxidation of β-carotene producing intermediates of echinenone, canthaxanthin, and adonirubin, the other from the hydroxylation of β-carotene with β-cryptoxanthin, zeaxanthin, and adonixanthin as intermediates (Fig. 1) (Sandmann 1994; Cunningham and Gantt 1998; Margalith 1999). The first pathway has been demonstrated by functional analysis of the CrtW/BKT type diketolases from the marine bacteria *A. aurantiacum* and green alga *H. pluvialis*, in that the ketolases only accept β-carotene as substrate (Lotan and Hirschberg 1995; Lu et al. 1995; Misawa et al. 1995; Breitenbach et al. 1996; Linden 1999). The second pathway remains to be elucidated.

Although *H. pluvialis* is a potential commercial source of natural astaxanthin, the slow growth rate and relatively low growth temperature of this alga greatly hinder its application

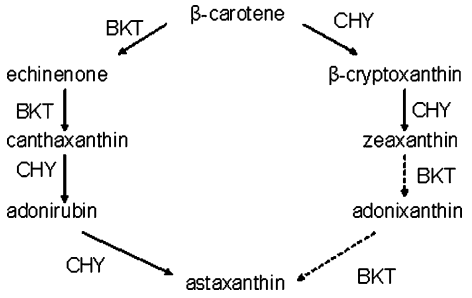

Fig. 1 Proposed pathway of astaxanthin biosynthesis in algae. *Solid arrows* represent documented reactions and *dash arrows* indicate speculated reactions in the biosynthesis pathway

on a large scale (Margalith 1999; Guerin et al. 2003). The chlorophycean microalga *Chlorella zofingiensis* was reported to accumulate high amounts of astaxanthin (up to 6.8 mg g^{-1}) and to reach very high values of biomass (up to 7 g dry weight l^{-1}) when grown photoautotrophically in batch culture (Orosa et al. 2001; Del-Campo et al. 2004). Moreover, this alga showed excellent growth (maximum specific growth rate 0.031 h^{-1}) and high yield of astaxanthin (10.3 mg l^{-1}) on glucose-supplemented medium in batch culture in the dark (Ip and Chen 2005), suggesting that the alga might be potentially employed for commercial production of astaxanthin on a large scale. However, *C. zofingiensis* contains much less astaxanthin than *H. pluvialis* (up to 40 mg astaxanthin g^{-1} dry weight) within the cell (Boussiba 2000). Information of astaxanthin biosynthesis on *C. zofingiensis* is limited. Compared with *H. pluvialis*, *C. zofingiensis* contains a relatively high amount of canthaxanthin (about 30% of total ketocarotenoids), indicating that canthaxanthin might be the end product of the oxygenation of β-carotene. Thus, in *C. zofingiensis*, astaxanthin might be synthesized via a different pathway from that in *H. pluvialis*: the oxygenation of zeaxanthin rather than the hydroxylation of canthaxanthin. This hypothesis has so far remained elusive. Based on the prediction, *C. zofingiensis* may consist of either two oxygenases (one for specifically converting β-carotene to canthaxanthin and the other for zeaxanthin to astaxanthin) or only one oxygenase with bifunctional activities of converting β-carotene to canthaxanthin as well as zeaxanthin to astaxanthin. The identification of the carotenoid oxygenase gene from *C. zofingiensis* is the key to revealing the astaxanthin biosynthesis in the alga.

The aim of this study is to isolate the carotenoid oxygenase gene from the important astaxanthin-producing green alga *C. zofingiensis*, characterize its function, and reveal its expression under conditions for ketocarotenoid accumulation. The putative pathway of astaxanthin biosynthesis in this alga is discussed.

Materials and methods

Strains and culture conditions

The green microalga *C. zofingiensis* (ATCC30412) was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The alga was maintained and cultured in a basal medium consisting (per liter) of 2 g yeast extract, 0.4 g L-asparagine, 1.2 g CH_3COONa, 0.2 g $MgC_{l2}{\cdot}6H_2O$, 0.02 g $CaCl_2{\cdot}2H_2O$, and 0.01 g $FeSO_4{\cdot}7H_2O$ (pH 6.8) (Kobayashi et al. 1993). *C. zofingiensis* was subcultured in the basal medium at 25°C under conditions of continuous illumination (30 μmol m^{-2} s^{-1}) for 5 days. To induce astaxanthin biosynthesis, glucose was added to the above culture at a final concentration of 20 g l^{-1}. Samples (15 ml) were collected at intervals (6, 12, 24, 36, and 48 h) and harvested by centrifugation.

Escherichia coli JM109 was used as a host for functional characterization of the *bkt* gene product from *C. zofingiensis*.

Genomic DNA and RNA isolation

DNA and RNA techniques were followed according to the standard methods described in Sambrook et al. (1989). DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Stewart and Via 1993). RNA was isolated from aliquots of about 10^8 cells harvested at different stages of astaxanthin accumulation using the TRI reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The concentration of total DNA and RNA was determined spectrophotometrically at 260 nm.

Cloning of *bkt* (*crtO*) cDNA and its correspondent gene

Degenerate primers were designed for the amplification of a partial *bkt* cDNA from *C. zofingiensis*. The primers were derived from the conserved nucleotide and amino acid sequences reported for the *bkt* genes from *H. pluvialis*, *A. aurantiacum*, and *Alcaligenes* PC-1 (Misawa et al. 1995; Kajiwara et al. 1995; Lotan and Hirschberg 1995). Primers dbktf1 (forward, 5′-GTTYYTNTAYACNGGNYTNTTYAT) and dbktr1 (reverse, 5′-CARRTANGTNCCRAARTARAA) were derived from motifs FLYTGLFI and FYFGTYLP. Primers dbktf2 (forward, 5′-CACIACICAYGAYGCIATG CAYGG) and dbktr2 (reverse, 5′-CCIYKRTGRAARTCI GGRTC) were derived from TTHDAMHG and DPD FHKG. One step reverse transcriptase–polymerase chain reaction (RT-PCR) with primers dbktf1 and dbktr1 was performed to amplify a portion of a *crtO* cDNA. Total RNA (100 ng) was from cells induced with 2% glucose for 24 h. RT-PCR conditions were 45°C for 15 min for cDNA synthesis, followed by PCR amplification (35 cycles of 94°C for 20 s, 42°C for 20 s, 72°C for 40 s). A nested PCR (with primers dbktf2 and dbktr2) was performed using the same PCR cycles. The nested PCR product was cloned into pTZ57R/T vector (Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. Positive clones were selected and sequenced in both directions using an automated ABI3100 sequencer. The sequence of putative insert was used for designing specific primers for 5′ and 3′ rapid amplification of cDNA ends (RACE) (5′-CGAC GGTTCCTGATGGCAATAGT and 5′-CAGCATGTCA

TAGTCAAACCAGGC for 5′ RACE; 5′-GCCTGGTTT GACTATGACATGCT and 5′-GAAGCACTGGGAGCAT CACAACT for 3′ RACE). RACE was performed using a 5′/3′ RACE kit according to the manufacturer's instructions (Roche, Mannheim, Germany). The RACE products were gel purified and sequenced. One pair of specific primers (forward, 5′-TTCCTGGGCCGTGCTGTATT; reverse, 5′-AACCTTGATTAAGTTACATGCT) was designed from the sequences of the 5′ and 3′ RACE fragments to amplify a full-length *bkt* cDNA and its correspondent gene.

Southern blot

Eight milligrams of genomic DNA was digested with *Kpn*I, *Bam*HI + *Eco*RI, and *Pst*I + *Xba*I, five restriction enzymes without recognition sites in the probed regions of the ketolase genes. The digested DNA was separated with an 0.8% agarose gel, transferred to a positively charged nylon membrane (Roche), and hybridized with digoxigenin (DIG)-labeled DNA probes in the presence of 50% (v/v) formamide at 47°C for 16 h. DNA probe was prepared by amplifying a 1,090-bp fragment of *bkt* gene with a pair of specific primers (forward, 5′-ATGGCTGCTGGCAAAT CA and reverse, 5′-GCCGCCAGAAAGACGCATA) and a plasmid containing the *bkt* gene template (30 cycles of 94°C for 20 s, 60°C for 20 s, 72°C for 1 min). Probe labeling and hybridization were carried out according to the instructions in the DIG Nonradioative Nucleic Acid Labeling and Detection System (Roche). After hybridization, the membrane was washed twice with 0.1× standard saline citrate (SSC) containing 0.1% sodium dodecyl sulfate (SDS) at 68°C for 15 min.

Functional analysis of *bkt* cDNA

The open reading frame of *bkt* was PCR amplified and cloned into the vector pBluescript II KS (Stratagene, USA) as an in-frame fusion to the *lacZ* gene resulting in plasmid pCzcrtO. *E. coli* strain JM109 was used as a host for complementation experiments by cotransformation of pCzcrtO with plasmids (either pACCAR16ΔcrtX or pACCAR25Δ crtX) that harbor the carotenoid biosynthesis genes for producing β-carotene or zeaxanthin, respectively (Misawa et al. 1995).

Pigment analysis

Carotenoids were extracted and analyzed according to McCarthy et al. (2004). *E. coli* cells were collected by centrifugation and freeze-dried. Extraction was carried out with a mixture of dichloromethane and methanol (25:75, v/v) until the cell debris was almost colorless. The combined extracts were evaporated to dryness and separated on a 5-μm ODS2 4.6×250-mm analytical column (Waters Spherisorb) with a Waters high-performance liquid chromatography (HPLC) system. Individual carotenoids were identified by absorption spectra and their typical retention times compared to standard samples of pure carotenoids.

RT-PCR assay

Total RNA (1 μg) extracted from samples treated with glucose for 6 to 48 h was reverse transcribed to cDNA with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) primed with oligo(dT) according to the manufacturer's instructions. PCR amplification was done using *bkt* forward 5′-ATGGCTGCTGG CAAATCA and reverse 5′-GCCGCCAGAAAGACGCA TA primers. *C. zofingiensis* actin primers (forward, 5′-TGCCGAGCGTGAAATTGTGAGG; reverse, 5′-CGTGA ATGCCAGCAGCCTCCA) were used to monitor equality of templates and loading. Amplification of the cDNA was done by conventional PCR [94°C for 2 min followed by 24 cycles (for *bkt* gene) or 22 cycles (for *actin* gene) of 94°C for 15 s, 59°C for 15 s, 72°C for 30 s]. PCR products were separated on 1.5% agarose gels and stained with ethidium bromide for photography (Biorad, USA).

Results

Cloning and characterization of the carotenoid oxygenase gene *crtO* from *C. zofingiensis*

Two pairs of degenerate primers were designed from the conserved motifs presenting among all known diketolases. RT-PCR with dbktf1 and dbktr1 (see "Materials and methods") could not generate clonable fragments (data not shown). However, a nested PCR with primers dbktf2 and dbktr2 generated a predicted 180-bp fragment (Fig. 2, lane 1). BLAST analysis showed that the sequence of the fragment was derived from a putative ketolase. With the sequence information, specific primers were designed for 5′ and 3′ RACE of the related gene. 5′ RACE generated a 600-bp fragment, and 3′ RACE produced a 1,400-bp fragment (Fig. 2). They were revealed by sequence as the 5′ and 3′ regions of a ketolase gene. RT-PCR with a pair of primers annealing to the most ends of the 5′ and 3′ RACE fragments generated a 2-kb fragment, confirming the

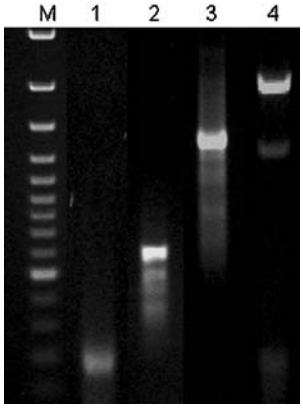

Fig. 2 PCR amplification of *C. zofingiensis crtO* gene. *Lane 1*, nested PCR product with primers dbktf2 + dbktr2; *lane 2*, product of 5′ RACE; *lane 3*, product of 3′ RACE; *lane 4*, RT-PCR product of full-length *crtO* cDNA; *M*, 100-bp DNA ladder plus (Fermentas)

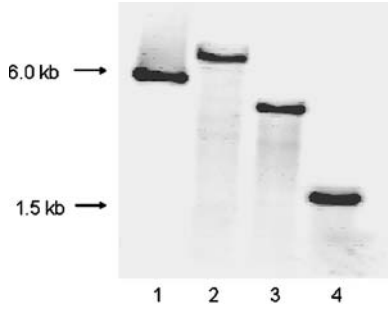

Fig. 3 Southern analysis of genomic DNA from *C. zofingiensis*. Genomic DNA (8 μg) was digested with *Kpn*I (*lane 2*), *Bam*HI + *Eco*RI (*lane 3*), or *Pst*I + *Xba*I (*lane 4*), respectively, electrophoretically separated on a 0.8% agarose gel, blotted onto a nylon membrane, and hybridized with a 928-bp *crtO* gene fragment amplified by PCR. Plasmids (400 pg) containing the *crtO* gene were cut by *Sac*I and used as a positive control (*lane 1*)

presence of the cDNA (Fig. 2, lane 4). The RT-PCR fragment was identified as a full-length *crtO*/*bkt* cDNA (GenBank accession no. AY772713). The open reading frame of this cDNA encodes a protein of 312 amino acids that shares 53% identical sequence with the *H. pluvialis* BKT (Kajiwara et al. 1995). The GC content of the *crtO* coding region is 51.1%, which is lower than that of the *bkt* from *H. pluvialis* (57.1%).

To characterize the correspondent gene of the *crtO* cDNA, genomic PCR was performed. A 3-kb fragment was generated and sequenced. Analysis of the obtained nucleotide sequence revealed that the product was the correspondent gene of the *crtO* cDNA (GenBank accession no. AY772714). The particular *crtO* gene consists of four exons and three introns of 243, 318, and 351 bp. Intron/exon splice sites of the *crtO* gene are strongly conserved. All introns start with GT and end with CAG. The first and last base of the exons is a G, which is strongly conserved as in the case of higher plants (Brown 1989). The sequence TGTAA, which was considered as a potential polyadenylation signal of green algae is located 13 bp upstream of the polyadenylation site. Similar to *Chlamydomonas* genes, the *crtO* gene is also characterized by a long 3′ noncoding region of about 1 kb in length.

To detect the number of *crtO* gene in the genome of *C. zofingiensis*, genomic DNA was digested with different restriction enzymes and subjected to Southern blot analysis. Using a 1-kb fragment of *crtO* as a probe, the homologous fragments showed strong hybridization signals (Fig. 3). Each of the three separate digests showed only one band, suggesting the presence of only one *crtO* gene in the genome of *C. zofingiensis*.

Functional analysis of the *crtO* cDNA in *E. coli*

To find out the enzymatic activity of the ketolase encoded by the *crtO* gene, the open reading frame of the gene was PCR amplified and inserted into the vector pBluescript II KS+ as an in-frame fusion to the *lacZ* gene, and the resulting plasmid (pCzcrtO) was cotransformed with pACCAR16ΔcrtX or pACCAR25ΔcrtX, respectively, into *E. coli* JM109. *E. coli* JM109 harboring plasmid pACCAR16ΔcrtX or pACCAR25ΔcrtX displays a yellow phenotype due to the accumulation of β-carotene or zeaxanthin. Transformants carrying pCzcrtO with pACCAR16ΔcrtX or pACCAR25ΔcrtX both display an orange phenotype, indicating the formation of new pigments. HPLC analysis of pigments from transformants is shown in Fig. 4. Compared with pigments extracted from *E. coli* harboring only the pACCAR16ΔcrtX that accumulates β-carotene (Fig. 4b), the *E. coli* containing pCzcrtO and pACCAR

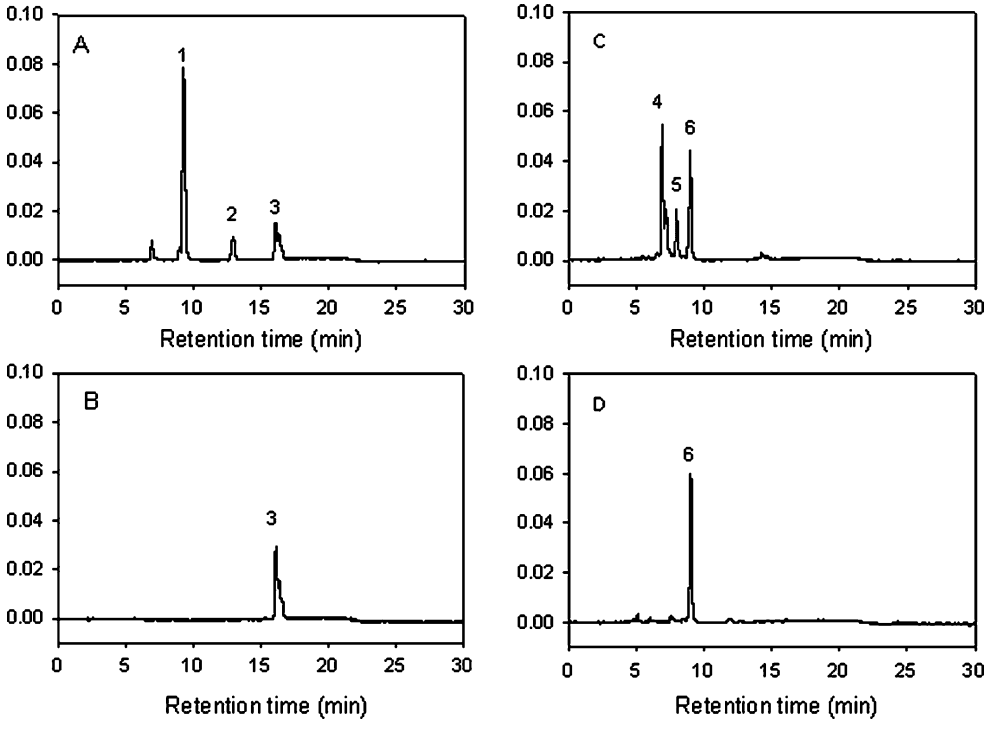

Fig. 4 HPLC chromatogram of carotenoid pigments extracted from *E. coli* cells carrying plasmid pACCAR16ΔcrtX and pCzcrtO (**a**), pACCAR16ΔcrtX (**b**), pACCAR25ΔcrtX and pCzcrtO (**c**), or pACCAR25ΔcrtX (**d**). Peaks were monitored at 480 nm and were identified as follows: *1* canthaxanthin, *2* echinenone, *3* β-carotene, *4* astaxanthin, *5* adonixanthin, *6* zeaxanthin

16ΔcrtX accumulated canthaxanthin (peak 1) and echinenone (peak 2) in addition to β-carotene (peak 3) (Fig. 4a). These results indicate that the *crtO* cDNA encodes a protein with enzymatic activity of converting β-carotene to echinenone and canthanxanthin. Thus, in this aspect, the ketolase from *C. zofingiensis* possesses the same enzymatic function as that from *H. pluvialis* and *A. aurantiacum*. Interestingly, besides zeaxanthin (Fig. 4c,d, peak 6), substantial amounts of astaxanthin (Fig. 4, peak 4) and adonixanthin (peak 5) were identified in the pigments from *E. coli* transformants carrying the pCzcrtO and pACCA R25ΔcrtX. These results demonstrate that the enzyme encoded by the *crtO* gene from *C. zofingiensis* is able to convert zeaxanthin to astaxanthin via adonixanthin. Coupled with Southern blot analysis, it is clear that *C. zofingiensis* only contains one carotenoid ketolase with bifunctional activities of converting β-carotene to canthaxanthin as well as zeaxanthin to astaxanthin.

Expression of the *Chlorella crtO* gene during the ketocarotenogenesis

C. zofingiensis was found to accumulate ketocarotenoids rapidly in response to high concentrations of glucose with or without light (Ip et al. 2004; Ip and Chen 2005). It is interesting to find out if glucose induces the expression of the *crtO* gene. Photoautotrophically grown cells at the exponential growth phase were treated with 2% glucose for 6 to 48 h. RT-PCR analysis showed that none-induced green cells had only small amounts of *crtO* mRNA, and the level of *crtO* mRNA remained stable in 48 h (Fig. 5, lanes 1 and 7), while the steady-state mRNA level was found increased upon the addition of 2% glucose, reaching its maximum at 24 h (Fig. 5, lanes 2, 3, and 4). The steady-state mRNA level began decreasing after 24 h upon onset of glucose (Fig. 5, lanes 5 and 6).

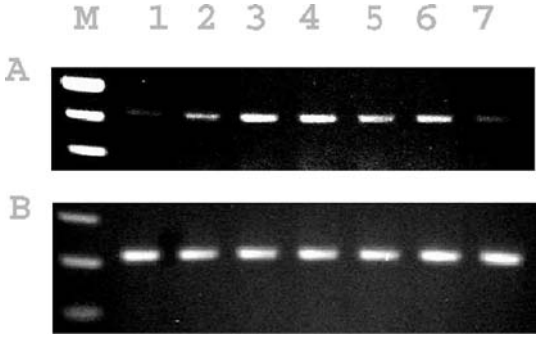

Fig. 5 Analysis of the differential expression of *crtO* gene in *C. zofingiensis* cells using RT-PCR. RT-PCR was performed using RNAs from cells grown in a medium without glucose for 6 h (*lane 1*), 48 h (*lane 7*), and with 2% glucose for 6 h (*lane 2*), 12 h (*lane 3*), 24 h (*lane 4*), 36 h (*lane 5*), and 48 h (*lane 6*); *M*, 100-bp DNA ladder plus (Fermentas). The 423-bp *crtO*-specific PCR product was separated on a 1.2% agarose gel (**A**), along with internal controls (actin) amplification (**B**)

Discussion

The carotenoid ketolases are enzymes that participate in the secondary carotenoid biosynthetic pathway to astaxanthin. These enzymes play an essential role in stress-dependent initiation of astaxanthin biosynthesis. Three types of carotenoid ketolases have been found from different organisms (Cunningham and Gantt 1998). The *bkt* one from *H. pluvialis* and *crtW* from *A. aurantiacum* and *Alcaligenes* PC-1 share highly homologous amino acid sequences (Lotan and Hirschberg 1995; Kajiwara et al. 1995; Misawa et al. 1995). This type of ketolase acts symmetrically—introducing two keto groups on both of the two β-ionone rings of β-carotene to generate canthaxanthin.

Interestingly, besides possessing such normal enzymatic activity as converting β-carotene to canthaxanthin, the *C. zofingiensis* ketolase exhibits a second function that can interact with the hydroxylase enzyme coded by the bacterial *crtZ* effectively, resulting in substantial amounts of adonixanthin and astaxanthin (Fig. 4c). In contrast, the ketolases from *H. pluvialis* and *A. aurantiacum* are very poor in the conversion of zeaxanthin to astaxanthin (Lotan and Hirschberg 1995; Breitenbach et al. 1996; Fraser et al. 1998). Recently, a ketolase from the cyanobacterium *Nostoc punctiforme* was detected also with an efficient conversion of zeaxanthin to astaxanthin (Steiger and Sandmann 2004). The ketolase described here is the first one that has been established with high efficient conversion of zeaxanthin to astaxanthin from eukaryote alga. Based on its function, the *C. zofingiensis* ketolase is termed carotenoid 4,4′-β-ionone ring oxygenase. The formation of adonixanthin in both transforming *E. coli* (Fig. 4c) and induced *C. zofingiensis* (data not shown) suggests that in *C. zofingiensis* astaxanthin is synthesized via the oxygenation of zeaxanthin (Fig. 1). Our preliminary study revealed that *C. zofingiensis* accumulated zeaxanthin when the cells were treated with glucose and diphenylalamine (DPA, an inhibitor specific to ketolase enzyme), indicating that the conversion of zeaxanthin to astaxanthin was blocked due to the inhibition of the ketolase by DPA (data not shown). Because substantial canthaxanthin (about 30% of total ketocarotenoids) is accumulated in *C. zofingiensis*, this pigment may represent the end product of oxygenated β-carotene (Rise et al. 1994). Thus, the carotenoid hydroxylase in this alga may not accept canthaxanthin as a substrate. The carotenoid hydroxylases from *Erwinia uredovora* (Breitenbach et al. 1996), *A. aurantiacum* (Missawa et al. 1995), and *H. pluvialis* (Linden 1999) are able to convert canthaxanthin into astaxanthin, while the hydroxylases from cyanobacteria are not able to accept echinenone or canthaxanthin as substrates (Albrecht et al. 2001). Thus, the enzymatic activities of carotenoid ketolase and hydroxylase are species specific and may decide the pathway of astaxanthin biosynthesis. Furthermore, their cooperation is crucial for the synthesis of substantial amounts of astaxanthin (Fraser et al. 1997; Steiger and Sandmann 2004). We predict that the astaxanthin pathway in *C. zofingiensis* occurs through the initial formation of zeaxanthin via adonixanthin, which is then converted to astaxanthin by *crtO* enzyme. Due to the bifunctional *crtO* enzyme, sub-

stantial canthaxanthin is also produced, which will accumulate because of the ineffective conversion of canthaxanthin to astaxanthin by the hydroxylase. However, the yet to be isolated hydroxylase gene from *C. zofingiensis* prevents the confirmation of this conversion.

Chlorella species can use glucose as carbon source (Tanner 1969; Shi et al. 1999). In a previous study, we found that glucose (0.5 to 4%) not only stimulated the growth of *C. zofingiensis* cells, but also enhanced the accumulation of ketocarotenoids in the alga cells (Ip et al. 2004; Ip and Chen 2005). In contrast, *H. pluvialis* usually accumulates secondary carotenoids after the cessation of cell growth (Margalith 1999; Boussiba 2000). Glucose triggers the transcription of mRNAs coding for such proteins as membrane transporters and glycolytic enzymes (Wolf et al. 1991; Stadler et al. 1995). Interestingly, we found that glucose coincidentally induced the expression of *crtO* gene in *C. zofingiensis* (Fig. 5). How glucose triggers the expression of the *crtO* gene and its correlation to astaxanthin accumulation are currently undertaken at our laboratory.

C. zofingiensis is an attractive candidate for mass production of high-value ketocarotenoids. It also represents a unique model system for studying the biosynthesis and regulation of ketocarotenoid. The cloning of the *crtO* gene from this alga and the illustration of its specific function provide valuable insight into the biosynthesis of astaxanthin in green microalgae. The *crtO* gene described here is suitable for the production of astaxanthin in such hosts that contain substantial amounts of zeaxanthin, e.g., in green algae or plants.

Acknowledgements We are grateful to Dr. K.W. Fan for technical assistance. This work was partially supported by a grant from the Research Grants Council of Hong Kong and the Outstanding Young Researcher Award of the University of Hong Kong, the Frontier Research Grant of the SCSIO, and the Hundred-Talents scheme of Chinese Academy of Science.

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