Biosynthesis of ketocarotenoids in transgenic cyanobacteria expressing the algal gene for β-C-4-oxygenase, *crtO*

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Abstract The ketocarotenoid astaxanthin is produced by a number of marine bacteria and microalgae. It is synthesized from β-carotene by the addition of two keto groups to carbons C4 and C4′ and two hydroxyl groups to C3 and C3′. The gene, *crtO*, encoding β-C-4-oxygenase which converts β-carotene to canthaxanthin was cloned from the green alga *Haematococcus pluvialis*. We transferred *crtO* to the cyanobacterium *Synechococcus* PCC7942, which contains a β-carotene hydroxylase gene and normally accumulates β-carotene and zeaxanthin. The genetically engineered cyanobacterium produced astaxanthin as well as other ketocarotenoids. The results confirm that *crtO* can function in cyanobacteria in conjunction with the intrinsic carotenoid enzymes to produce astaxanthin. Specifically, this finding indicates that β-carotene hydroxylase, which normally converts β-carotene to zeaxanthin, can also function in the biosynthesis of astaxanthin. These results provide the first evidence of genetic manipulation of a plant-type carotenoid biosynthesis pathway toward the production of novel carotenoids.

Key words: Carotenoid; Astaxanthin; Gene transfer; *Synechococcus*; *Haematococcus pluvialis*

1. Introduction

Carotenoids constitute a major class of natural pigments. Over 600 of them have been identified in plants, animals, bacteria and fungi [1,2]. In photosynthetic cells carotenoids serve as accessory pigments in light harvesting and in protecting against photo-oxidative damage [3,4]. In higher plants they provide distinctive pigmentation of fruits and flowers and in certain animals they contribute to the colors of the skin or feathers.

The ketocarotenoid astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is produced by a number of microorganisms including the green alga *Haematococcus pluvialis* [5,6] and the heterobasidiomycetous yeast *Phaffia rhodozyma* [7]. Astaxanthin is responsible for imparting the pinkish color to the flesh of many marine animals such as salmonids and crustaceans, and to the feathers of some birds, i.e. flamingoes [8]. Animals cannot synthesize astaxanthin de novo but must acquire it from their diets. Astaxanthin has been shown to be an extremely efficient antioxidant that provides protection against oxygen free radicals [9,10], acts as an anti-cancer agent [11] and stimulates the immune system [12].

Early steps in the biosynthesis of carotenoids include the head-to-head condensation of two molecules of geranylgeranyl diphosphate to produce phytoene. Phytoene then undergoes a series of sequential dehydrogenation reactions to give phytofluene, ζ-carotene (or its 'unsymmetrical' isomer 7,8,12,13-tetrahydrolycopene), neurosporene and finally lycopene. At each stage a new double bond is introduced and the conjugated polyene chain is extended by two double bonds. Once a carotenoid has reached the lycopene level of desaturation cyclization can take place at each end of the linear molecule resulting in the formation of a six-carbon β, ε, or γ-ring [13]. The species-specific introduction of oxygen functionalities into acyclic and cyclic carotenes gives rise to the large structural diversity observed in the xanthophylls.

It has been postulated that in the green alga *H. pluvialis* astaxanthin is synthesized from β-carotene through echinenone, canthaxanthin and adonirubin [14,15]. We have recently cloned from *H. pluvialis* a cDNA, *crtO*, which encodes the enzyme β-C-4-carotene oxygenase [16]. By functionally expressing this gene in *Escherichia coli* which produced β-carotene it was established that this enzyme catalyzes the formation of canthaxanthin from β-carotene via echinenone as an intermediate step [16]. Cloning a gene with a similar function was reported by Kajiwara et al. [17].

Expressing *crtO* in β-carotene producing *E. coli* cells which contained in addition the gene *crtZ*, encoding β-carotene hydroxylase from *Erwinia herbicola*, resulted in the synthesis of a range of ketocarotenoids, including (3*S*,3′*S*) astaxanthin (unpublished data). A similar result was reported with the *crtZ* gene of *Erwinia uredovora* [17]. However, it has not been determined in what order these enzymes act in the pathway, i.e. whether the keto groups are introduced first into β-carotene at C4 and C4′ by the *crtO* gene product, or the hydroxyl groups are introduced first at C3 and C3′ by *CRTZ*.

In this report we describe the use of gene transfer technology to alter the carotenoid biosynthesis pathway in cyanobacteria (blue-green algae) towards the production of astaxanthin and related ketocarotenoids. The capacity of *crtO* expression in the cyanobacterium *Synechococcus* PCC7942 to produce astaxanthin and the various ketocarotenoid intermediates that are found indicates that β-carotene hydroxylase, which normally converts β-carotene to zeaxanthin, can also function in astaxanthin synthesis.

2. Materials and methods

2.1. Organisms and growth conditions

The unicellular cyanobacterium *Synechococcus* sp. PCC7942 (for-

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Abbreviations: Ap, ampicillin resistance gene; Cm, chloramphenicol resistance gene; *crtO*, β-C-4-oxygenase gene; *crtZ*, β-carotene hydroxylase gene; ORI, origin of DNA replication; *psbA*, the *psbA-I* gene of *Synechococcus* PCC7942; Tc, tetracycline resistance gene

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merly *Anacystis nidulans*) was grown on BG11 medium [18] at 35°C under a light intensity of 50 μM/m²/s.

2.2. Plasmids

The full length cDNA of *crtO* from *H. pluvialis* [16] (accession number X86782), was inserted into the *PstI* site in the β-lactamase gene of the plasmid pAN35T (Fig. 1A) which contains the *psbA-I* gene of *Synechococcus* sp. PCC7942 [19]. The nucleotide sequence of *crtO* is significantly diverged from the prokaryotic gene *crtW* [20] and the *bkt* cDNA [17]. The recombinant plasmid in which *crtO* was inserted in the same orientation as the β-lactamase gene was designated pANKETO-A (Fig. 1B), and the plasmid in which *crtO* was inserted in the reverse orientation was designated pANKETO-B (Fig. 1C). Transcription of the cDNA of *crtO* in transgenic cyanobacterial cells transformed with pANKETO-A is driven by the promoter of the β-lactamase gene. However, in the pANKETO-B transformed strain the *crtO* is not transcribed and thus it serves as a control for expression of *crtO* in vivo. No prokaryotic-specific ribosome binding site was inserted upstream to the presumed initiation codon. These plasmids were used in the transformation of the *Synechococcus* PCC7942 cells.

2.3. Transformation of cyanobacteria

Synechococcus sp. PCC7942 cells were transformed by a modification of the procedure of Van den Hondel et al. [21]. Cells of *Synechococcus* sp. PCC7942 were harvested at the mid-log stage of growth in suspension culture by centrifugation at 13 000×*g* for 10 min at room temperature. The cells were then suspended in 1/20 volume of fresh BG-11. Aliquots of this suspension (1 ml) were mixed with 0.3 μg of plasmid DNA in a 10 ml test tube. The transformation mixture was incubated for 24 h at 35°C in the light, with constant aeration. The contents of each tube were then plated on solid BG-11 medium containing 7.5 μg/ml chloramphenicol. Colonies of chloramphenicol-resistant cells appeared after incubation for 7 days.

2.4. DNA blot hybridization analysis

Cyanobacterial DNA was purified as described by Williams and Szalay [22]. The DNA was digested with restriction endonucleases and fractionated by electrophoresis in a 0.9% agarose gel. DNA blotting and hybridization were carried out according to the protocol described by Sambrook et al. [23]. The coding sequence of *crtO* or *psbA-I* were labeled in vitro with ^{32}P and served as molecular probes.

2.5. Pigment analysis

Aliquots of *Synechococcus* sp. PCC7942 cells were harvested by centrifugation at 13 000×*g* for 10 min and washed once in water. After removing the water cells were resuspended in 100 μl of acetone and incubated at 25°C for 15 min in the dark. The samples were centrifuged again at 13 000×*g* for 10 min and the acetone supernatant containing the pigments was placed in a clean tube. More than 99% of the carotenoids were extracted by this procedure as determined by re-extraction after breaking and grinding the samples. The pigment extract was blown to dryness under a stream of N_2 and stored at −20°C until required for analysis.

Chlorophyll concentration was determined using a Shimadzu UV-160A spectrophotometer and calculated as $OD_{665}/74.5$ = [mg/ml]. Total carotenoid concentration was determined as described in [14].

Normal phase HPLC of the pigment extracts was carried out using a Spherisorb ODS-2 column (silica 5 μm 4.6 mm×250 mm) (Phenomenex). The HPLC procedure followed the protocol described by Schüep and Schierle [24] with minor modifications. Prior to analysis the column was washed with 1 g H_3PO_4 in 100 ml of methanol for 60 min at 1.0 ml min^{-1} and then equilibrated with the eluting solvent for 90 min at a rate of 1.5 ml min^{-1}. The mobile phase was pumped by triphasic Merck-Hitachi L-6200A high pressure pumps at a flow rate of 1.5 ml min^{-1}. The mobile phase consisted of an isocratic solvent system which comprised hexane/dichloromethane/isopropyl alcohol/triethylamine (88.5:10:1.5:0.1, v/v). Peaks were detected in the range of 200–600 nm using a Waters 996 photodiode-array detector. All

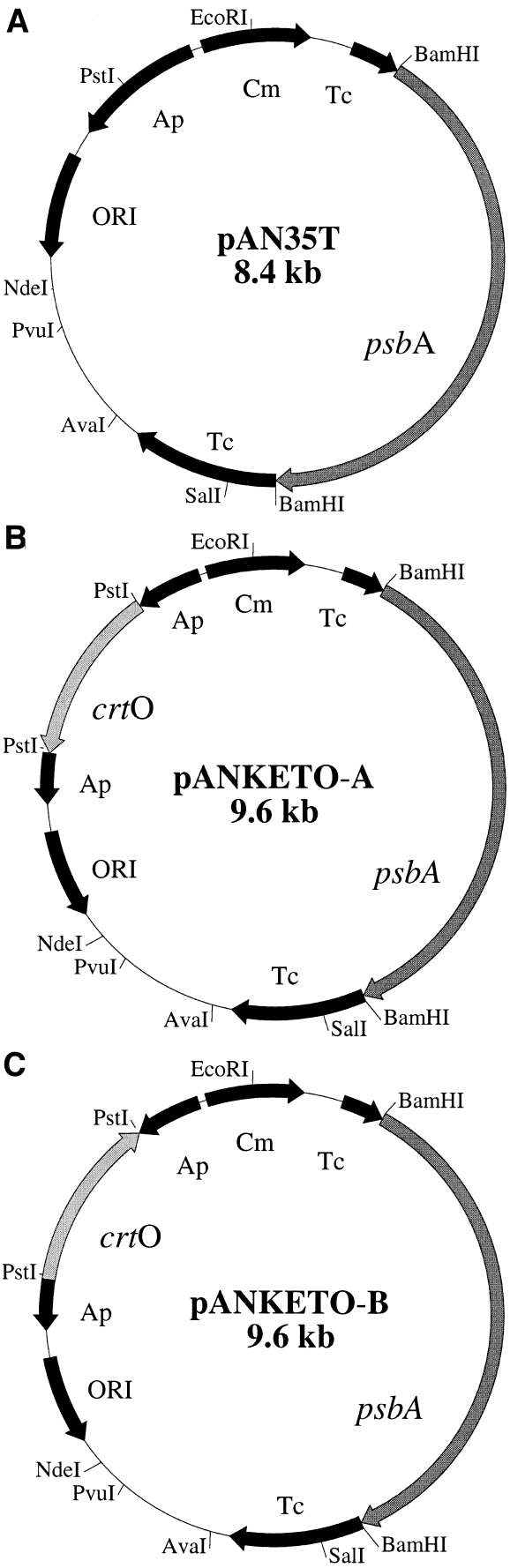

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Fig. 1. Map of plasmids pAN35T (A); pANKETO-A in which *crtO* was inserted in the β-lactamase gene of pAN35T (B); and pANKETO-B where *crtO* is inserted in the same site but in the opposite orientation (C).

spectra were recorded in the eluting HPLC solvent as was the spectral fine structure. Wavelengths given in parentheses denote shoulders. The degree of spectral fine structure is expressed as the ratio of the peak heights of %III/II where the zero value is taken as the minimum between the two absorption peaks, the peak height of the longest wavelength absorption wavelength is designated III, and that of the middle absorption wavelength as II [14]. In the case of conjugated ketocarotenoids, such as astaxanthin, which exhibits a single absorption peak [25] with no fine structure, the %III/II value is zero.

Thin-layer chromatography (TLC) was carried out using silica gel 60 F_{254} plates (Merck), using ethyl acetate/hexane (40:60, v/v) as the eluent.

Individual pigments were identified by their typical retention times, absorption spectra and R_f values as compared to standard samples of chemically pure β-carotene, zeaxanthin, echinenone, canthaxanthin, adonirubin and astaxanthin (the latter four were kindly provided by Dr. Andrew Young from Liverpool John Moores University). Other carotenoids are tentatively identified according to their chromatographic and spectral characteristics compared with published data [14].

The following carotenoids were isolated from the strains of *Synechococcus* PCC7942: β-carotene, R_f 0.92, R_t 1.7 inseparable from authentic (β-carotene), λ_{max} nm: (428), 455, 480, %III/II = 22. Echinenone, R_f 0.90, R_t 2.0 inseparable from authentic (echinenone), λ_{max} nm: 462, %III/II = 0. Adonirubin, R_f 0.82, R_t 2.3 inseparable from authentic (adonirubin), λ_{max} nm: 475, %III/II = 0. Canthaxanthin, R_f 0.87, R_t 2.4 inseparable from authentic (canthaxanthin), λ_{max} nm: 472, %III/II = 0. 3′-Hydroxyechinenone, R_f 0.80, R_t 3.0, λ_{max} nm: 463, %III/II = 0. β-Cryptoxanthin, R_f 0.83, R_t 3.5, λ_{max} nm: (428), 453, 479, %III/II = 27. Astaxanthin, R_f 0.79, R_t 4.7 inseparable from authentic (astaxanthin), λ_{max} nm: 479, %III/II = 0. Adonixanthin, R_f 0.72. R_t 6.4, λ_{max} nm: 465, %III/II = 0. Zeaxanthin, R_f 0.65, R_t 11.0 inseparable from authentic (zeaxanthin), λ_{max} nm: (428), 455, 484, %III/II = 45.

3. Results

3.1. Transformation of cyanobacteria with crtO

DNA of the plasmids pANKETO-A and pANKETO-B was transfected to cells of the cyanobacterium *Synechococcus* sp. PCC7942 and chloramphenicol-resistant transformants were isolated. Such colonies could have originated by the insertion of the whole plasmids into the cyanobacterial chromosome following a single recombination event between homologous DNA sequences as illustrated in Fig. 2.

To confirm this possibility, a DNA blot analysis of the transformed strains was carried out. A 1.2 kb *PstI-PstI* DNA fragment, which included the entire *crtO* sequence, was used as a molecular probe in the Southern blot analysis of genomic DNA extracted from transformed and wild-type strains of *Synechococcus* PCC7942. As can be seen in Fig. 3, the pANKETO-A transformed strain contained a single *crtO* sequence that was inserted into the cyanobacterial chromosome at the site of the gene *psbA-I*, as indicated by the hybridization patterns with the DNA of plasmid pAN35T which contained the *psbA-I* gene. Similar results were observed with pANKETO-B transformed strain (data not shown).

3.2. Carotenoid content in transformed cyanobacteria

Cyanobacterial strains which were transformed with either pANKETO-A or pANKETO-B were grown in liquid BG-11 medium containing chloramphenicol until mid-logarithmic phase and their pigments were examined. Cells which were transformed with pANKETO-A contained 0.58% carotenoid, whilst those transformed with pANKETO-B contained 0.47% carotenoid on a dry weight basis. Table 1 and Fig. 4A demonstrate that *Synechococcus* cells which were transformed with pANKETO-B showed the same composition of carotenoids as the wild-type strain where β-carotene accounted for nearly 50% of the carotenoid content, zeaxanthin for 45% and the rest was β-cryptoxanthin. No ketocarotenoids were detected in these cells. In contrast, cells of the pANKETO-A transformed strain accumulated various ketocarotenoids which constituted 15% of the total carotenoid content in the cells (Table 1 and Fig. 4B). The predominant ketocarotenoid encountered was echinenone, with adonixanthin and astaxanthin also present at appreciable levels in the cells. Other ketocarotenoids detected were canthaxanthin, 3′-hydroxyechinenone

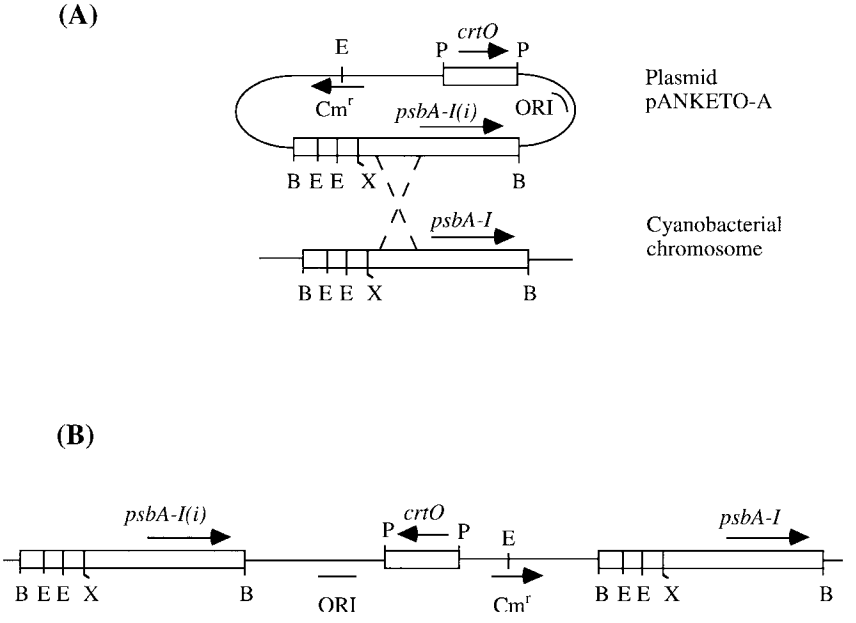

Fig. 2. Scheme of possible recombination event between homologous DNA sequences in plasmid pANKETO-A and in the cyanobacterial chromosome in the *psbA-I* region (A). A single crossing-over event leads to the insertion of the whole plasmid into the cyanobacterial chromosome and creates a duplication of the *psbA-I* sequence (B). Arrows indicate directions of the coding sequences. B, *Bam*HI; E, *Eco*RI; P, *Pst*I; X, *Xho*I.

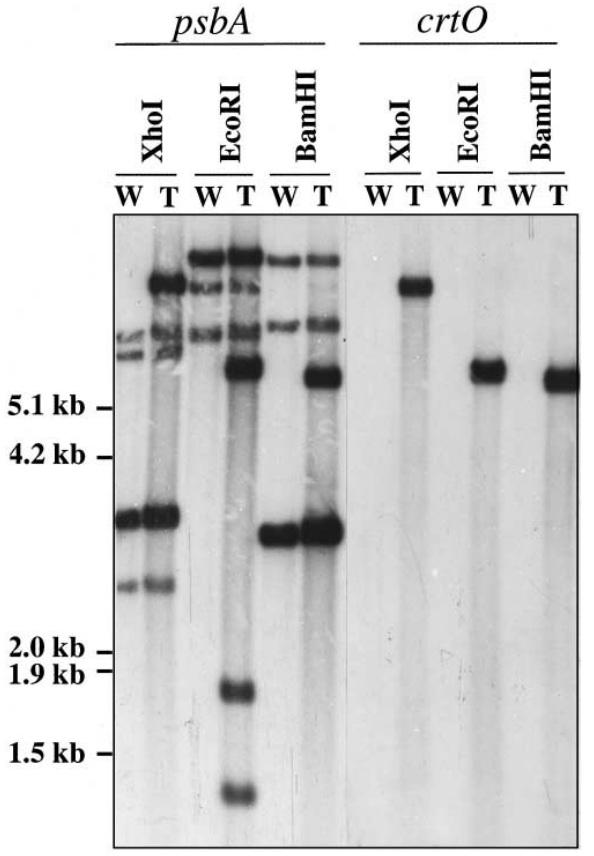

Fig. 3. DNA blot analysis of cyanobacterial DNA. DNA of wild type (W) and chloramphenicol-resistant strain that contained pANKETO-A (T) was digested with *Xho*I, *Eco*RI and *Bam*HI and separated on agarose gel electrophoresis. DNA blot hybridization was carried out with either a *psbA-I* DNA probe or *crtO* DNA probe, as indicated.

and adonirubin all of which were present at relatively low levels. Identification of carotenoids by HPLC was based on typical retention times and absorption spectra (not shown) as compared with chemically pure standards (Fig. 4C).

4. Discussion

The results of the DNA blot hybridization (Fig. 3) clearly demonstrate that a single *crtO* gene was inserted into the genomic DNA of *Synechococcus* PCC7942 in each strain of the strains- pANKETO-A and PANKETO-B. Since the altered carotenoid composition was found in pANKETO-A (Table 1), which expresses *crtO*, but neither in pANKETO-B, which expresses the anti-sense of *crtO* nor in the wild-type strain, it is concluded that the phenotype of ketocarotenoid biosynthesis in pANKETO-A is dictated by the *crtO* gene product.

Expression of *crtO* in the cyanobacterial cells resulted in the production of a range of ketocarotenoids not normally synthesized in *Synechococcus* PCC7942, including the economically important carotenoid astaxanthin. The ketocarotenoids which were detected in the cells are all biosynthetically related and represent possible intermediates in the astaxanthin biosynthesis pathway (Fig. 5).

The exact pathway of astaxanthin synthesis in the cyanobacterial cells cannot be determined from the results acquired in the present investigation. From the experiments carried out in *E. coli* we know that the *crtO* gene product, β-C-4-oxygenase (*CRTO*), fully converts β-carotene to canthaxanthin by oxidations at C4 and C4′ [16]. In cyanobacteria β-carotene is normally converted to zeaxanthin by β-carotene hydroxylase which adds hydroxyl groups at C3 and C3′. These two enzymes are active in the transgenic cyanobacteria, as evident by the presence of detectable amounts of both zeaxanthin and canthaxanthin. The existence in the transformed cyanobacteria of adonixanthin, adonirubin and astaxanthin clearly indicates that at least one of the enzymes, and possibly both, can utilize a carotenoid substrate which already possesses an oxygen-containing group. Only the biochemical analyses of the enzymes will allow the route of astaxanthin biosynthesis in these cells to be determined.

The marine bacterium *Agrobacterium aurantiacum*, which synthesize astaxanthin, contains a gene, *crtZ*, for β-carotene hydroxylase which is highly conserved with the *crtZ* from *Erwinia* species where it catalyzes the formation of zeaxanthin [26]. In addition, it contains a gene, *crtW*, that encodes an enzyme with β-C-4-oxygenase activity [20]. It has been suggested that in *A. aurantiacum* each of the enzymes can utilize a carotenoid with an oxygenated β-ring. The gene for β-carotene hydroxylase from cyanobacteria has not been cloned yet but recently the plant cDNA for this enzyme was isolated [27]. It is likely that, similar to other carotenoid biosynthesis enzymes [28], the cyanobacterial β-carotene hydroxylase will be highly conserved with the plant homolog. Therefore, it is likely that transgenic plants that express *crtO* will be capable of producing astaxanthin.

The overall level of ketocarotenoids detected in the transformed cells did not exceed 15% of the total carotenoid composition. However, they did constitute 30% of the xanthophylls. This may have been caused by several reasons. Most of the β-carotene in cyanobacteria is located in the photosynthetic reaction centers [28]. This steady-state level is essential for autotrophic growth. Indeed, the level of β-carotene does not change in the pANKETO-A transformed strain. The exact function of zeaxanthin in cyanobacteria has not been determined. However, it is probable that this xanthophyll performs an indispensable function. Therefore, severe depletion of zeaxanthin and β-carotene in favor of ketocarotenoids is not expected in autotrophic growth conditions as it will be detrimental to the cells. In addition, there is a limit to the

Table 1
Carotenoid content of *Synechocccus* PCC7942 transformed strains

Carotenoid	Carotenoid composition of transformed *Synechococcus* (% of total carotenoids)	
	pANKETO-A	pANKETO-B
β-Carotene	49.6 ±0.56	50.3 ±1.61
Echinenone	6.8 ±0.10	–
Adonirubin	0.7 ±0.04	–
Canthaxanthin	1.1 ±0.03	–
3′-Hydroxyechinenone	0.5 ±0.02	–
β-Cryptoxanthin	1.9 ±0.16	4.4 ±0.35
Astaxanthin	2.7 ±0.12	–
Adonixanthin	3.0 ±0.05	–
Zeaxanthin	33.7 ±0.39	45.3 ±1.68
Total chlorophyll (mg l^{-1} culture)	19.6 ±0.19	19.5 ±0.18
Total carotenoid (mg l^{-1} culture)	5.3 ±0.11	4.7 ±0.02
Carotenoids as % dry weight	0.58 ± 0.04	0.47 ± 0.01

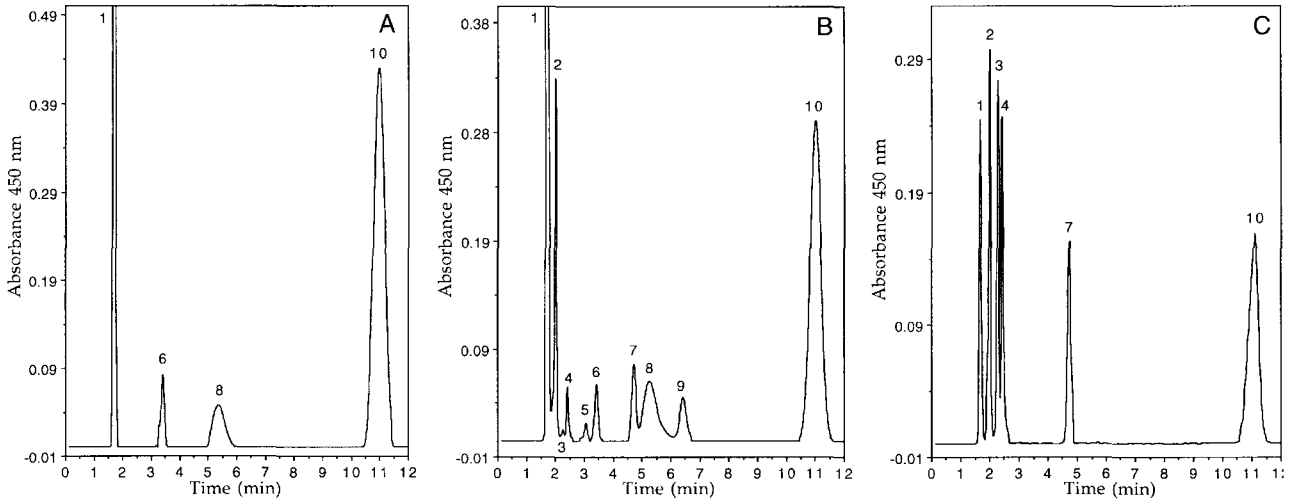

Fig. 4. HPLC chromatogram of pigments extracted from *Synechococcus* cells transformed with pANKETO-B (A) or pANKETO-A (B), and of chemically pure standards (C). Peaks were monitored at 450 nm and are identified as follows: 1, β-carotene; 2, echinenone; 3, adonirubin; 4, canthaxanthin; 5, 3′-hydroxyechinenone; 6, β-cryptoxanthin; 7, astaxanthin; 8, chlorophyll *a*; 9, adonixanthin; 10, zeaxanthin.

concentration of carotenoids that can accumulate in the cyanobacterial cells. In the transformed cells there was an increase of approx. 15% in the total carotenoid content which is due to the production of ketocarotenoids whilst β-carotene and zeaxanthin remain at their normal intracellular concentration. The location of the ketocarotenoids in the cyanobacteria was not investigated nor their chirality determined, but they appeared in a free form and not esterified as in *H. pluvialis*. In the alga the ketocarotenoids accumulate in lipid globules in the cell cytoplasm [29,30]. In the absence of such structures in cyanobacteria it could be that the ketocarotenoids synthesized in the cells are not stable and are subject to a relatively high rate of degradation in relation to other carotenoids normally found in the cells.

The results of the present investigation demonstrate that the β-C-4-oxygenase from an algal source can function in a heterologous system, cyanobacteria in this case, without the requirement of an additional specific factor. It utilizes the natural substrates and physiological environment (membrane) provided by the cyanobacterial cells, and is able to function

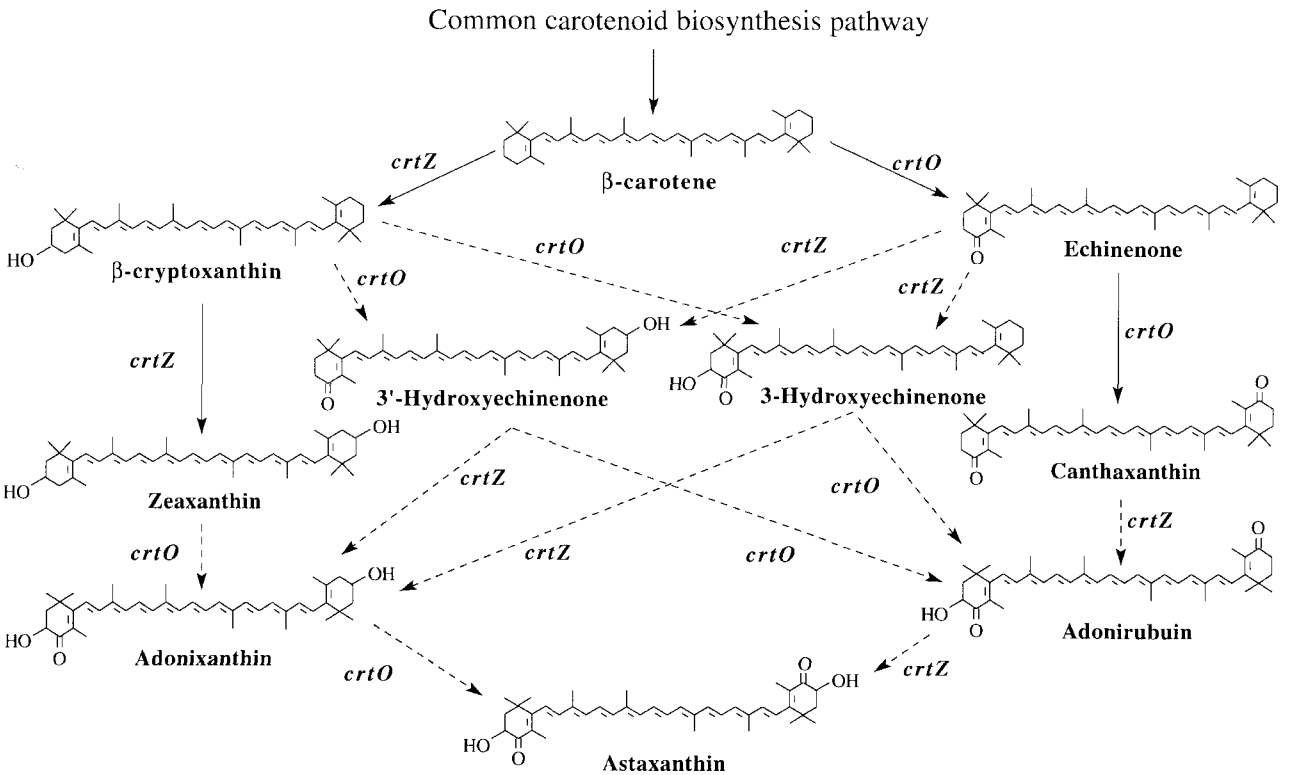

Fig. 5. Possible pathways for astaxanthin biosynthesis in *Synechococcus* cells transformed with pANKETO-A. Solid arrows represent documented reactions and dotted arrows indicate speculated reactions in the biosynthesis pathway.

in conjunction with the cyanobacterial β-carotene hydroxylase to form a number of ketocarotenoids. The implications of this finding are that the introduction of *crtO* into eukaryotic cells, i.e. higher plants, which already possess β-C-3-hydroxylase should result in the formation of a similar set of ketocarotenoids including the economically valuable carotenoid astaxanthin. This finding can now be used to develop transgenic plants where the expression of *crtO* will result in the production of astaxanthin, particularly where the carotenoid substrate, i.e. β-carotene and zeaxanthin are already present at high concentrations in the plant tissues. This process will be facilitated where efficient transformation systems and cloning vectors already exist. These novel carotenoids will impart a pinkish/red color to the tissues, thereby improving the nutritional and economic value of the product.

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