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ORIGINAL ARTICLE

Multiple ketolases involved in light regulation of canthaxanthin biosynthesis in *Nostoc punctiforme* PCC 73102

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Abstract In the genome of *Nostoc punctiforme* PCC 73102, three functional β-carotene ketolase genes exist, one of the *crtO* and two of the *crtW* type. They were all expressed and their corresponding enzymes were functional inserting 4-keto groups into β-carotene as shown by functional pathway complementation in *Escherichia coli*. They all synthesized canthaxanthin but with different efficiencies. Canthaxanthin is the photoprotective carotenoid of *N. punctiforme* PCC 73102. Under high-light stress, its synthesis was enhanced. This was caused by up-regulation of the transcripts of two genes in combination. The first *crtB*-encoding phytoene synthase is the gate way enzyme of carotenogenesis resulting in an increased inflow into the pathway. The second was the ketolase gene *crtW148* which in high light takes over β-carotene conversion into canthaxanthin from the other ketolases. The other ketolases were down-regulated under high-light conditions. CrtW148 was also exclusively responsible for the last step in 4-keto-myxoxanthophyll synthesis.

Keywords Canthaxanthin synthesis · β-Carotene ketolases · Carotenoid composition · Cyanobacteria · High-light stress · Transcriptional regulation

Abbreviations
HL High light
LL Low light

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Introduction

Carotenoids are essential components of the photosynthetic apparatus. They prevent the generation of singlet oxygen by quenching and dissipating the energy as heat (Frank and Cogdell 1996). Furthermore, they protect photo-system II from photoinhibition (Niyogi 1999). Thus, carotenoids become vital as membrane-integrated antioxidants. Most cyanobacteria synthesize either ketolated or hydroxylated β-carotene derivatives (Goodwin 1980; Takaichi and Mochimaru 2007). They are not only integral components of photosynthetic pigment protein complexes but also act as protecting antioxidants for the photosynthetic apparatus. For unicellular *Synechococcus* PCC 7942, the photoprotective property of zeaxanthin is well established (Götz et al. 1999). Formation of zeaxanthin is light-dependent (Masamoto and Furukawa 1997). The up-regulation of entire carotenoid biosynthesis as a response to light stress has been investigated in detail showing that the genes of the initial reactions of the carotenogenic pathway are under transcriptional control (Schäfer et al. 2006). Under strong light conditions, the activity of the promoter of the phytoene desaturase/synthase operon was increased.

In *Nostoc*-related filamentous cyanobacteria, the ketocarotenoids, echinenone and canthaxanthin are dominating (Arima et al. 2012), functionally replacing zeaxanthin as photoprotective carotenoid as shown in a cyanobacterial model system (Albrecht et al. 2001). Ketolation of β-carotene to monoketo echinenone and diketo canthaxanthin can be catalyzed by two different ketolases: the CrtW type (Misawa et al. 1995) which is present in several bacteria and the CrtO type of cyanobacteria first identified in *Synechocystis* PCC 6803 (Fernandez-Gonzalez et al. 1997). Both show no significant homology to each other.

The genomes of several *Nostoc*-related species have been sequenced. By gene comparison, the presence of both ketolase gene types was confirmed (Takaichi et al. 2005). First evidence that they contribute differently to keto carotenoid biosynthesis has been obtained with deletion mutants of *Nostoc* (*Anabaena*) PCC 7120 due to their distinct substrate preference (Mochimaru et al. 2005). As a special feature, *Nostoc* PCC 73102 carries two copies of the *crtW* ketolase gene (Steiger and Sandmann 2004) in addition to one copy of *crtO*. Heterologous complementation analysis showed that all three genes are active. However, their contribution to the formation of echinenone and canthaxanthin in cells of *Nostoc* PCC 73102 remains open. Light stress and photoprotection in cyanobacteria are destructive for the carotenoids. Therefore, an enhanced supply of these pigments under high light conditions is important (Steiger et al. 1999). Light-dependent up-regulation on demand may be a general feature in cyanobacteria. In ketocarotenoid forming cyanobacteria, the two types of ketolases may play a different role in light response.

In the present study, we investigated the carotenoid composition and their formation under high light conditions, the contribution of the ketolase genes, *crtO* and two copies of *crtW* to ketocarotenoid synthesis and their light-dependent regulation.

Materials and methods

Growth of organisms

Nostoc punctiforme PCC 73102 from the Pasteur Culture Collection, Paris (=ATCC 29133) was grown in BG11 medium (Rippka et al. 1979) at 28 °C gassed with 1.5 % CO_2-enriched air. Standard light conditions were 50 photons cm^{-2} s^{-1} and high light had an intensity of 300 photons cm^{-2} s^{-1}. In the high-/low-light experiments, a 5-day-old culture was diluted about 1:2 to a chlorophyll concentration of 3 µg/ml and divided. Then, $t = 0$ samples were taken from individual cultures before they were all exposed to the light intensities mentioned above. To parallel cultures, the phytoene desaturase inhibitor difunon (Sandmann and Böger 1989) was added in a concentration of 100 µM directly before transfer to light.

Gene complementation experiments were carried out in *Escherichia coli* DH5α grown for 48 h in LB medium at 28 °C with added antibiotics ampicillin (100 µg/ml), kanamycin (34 µg/ml) and chloramphenicol (34 µg/ml) according to their plasmids. They were co-transformed with plasmids generating carotenoid backgrounds to be used as substrates for the products of the ketolase gene introduced in a second compatible plasmid.

Plasmids

Plasmids used were pACCAR16ΔcrtX for a β-carotene background, pACCAR16ΔcrtX for a zeaxanthin background (Misawa et al. 1995) and pRARE from Novagen (Darmstadt, Germany) encoding six rare t-RNAs for *E. coli*. The whole tRNA cassette was PCR amplified from the latter plasmid subcloned into the T-overhang vector pMON38201 (Borovkov and Rivkin 1997), cut out with *Sac*I/*Sal*I and ligated into the *Sac*I/*Sal*I sites of plasmid pBBR1MCS2 (Kovach et al. 1995), resulting in plasmid pBBR1MCS2Rosetta. Plasmids containing the *crtW* gene from *Nostoc* PCC 73102, pPQE30-CrtW148 with gene *crtW148* (JGI accession number Npun_4798) and pPQE30-CrtW38 with gene *crtW38* (Npun_5919) were described before (Steiger and Sandmann 2004). Plasmid pPQE30-CrtO expressing the *crtO* gene (Npun_3745) was constructed by PCR amplification of the entire reading frame (forward primer GGTACCATGCAAGAGTATGATGTT GTG, reverse primer CTGCAGCTAATTAATTCCAAA GACTGACTC) and cloning into T-overhang vector pMON38201. The gene was cut out with the restriction enzymes *Pst*I and *Acc*65I and cloned into the *Pst*I and *Acc*65I site of plasmid pPQE30 (Stickforth and Sandmann 2011).

Carotenoid analysis

Carotenoids from *Nostoc* and *E. coli* were extracted from freeze-dried cells. They were heated in methanol at 65 °C for 20 min and the extract partitioned into 50 % ether in petrol. After addition of water for complete phase separation, the upper phase was collected and the solvent evaporated. Carotenoids were separated and quantified by HPLC on a Nucleosil C18, 3-µm column with a mobile phase of acetonitrile/2-propanol/methanol (85:5:10, by vol.). Spectra were recorded on-line with a Kontron DAD 440 diode array detector (Kontron Instruments, Neufahrn, Germany). Peaks were identified by co-chromatography with reference compounds together with their spectra. Authentic carotenoid standards for HPLC were generated by combinatorial biosynthesis in *E. coli* (Sandmann 2002).

Transcript determination

RNA isolation from *Nostoc* PCC 73102 low- and high-light cultures was carried out according to Singh et al. (2010) by suspending the cells in Trizol solution, breaking by freezing, thawing and vortexing with glassbeads, chloroform treatment and precipitation with 2-propanol. RNA integrity was proofed by formaldehyde-agarose-gel electrophoresis. Residual DNA was removed by treatment with RNAse-free DNAse. Complementary cDNAs were synthesized using RevertAid Transcriptase (Fermentas, St. Leon-Roth,

Germany). qRT-PCR was carried out in the Rotor Gene PCR Cycler (Corbett Life Science) with the Sensi-Mix SYBR No-ROX Kit (Bioline, Luckenwalde, Germany) for the phytoene synthase gene *crtB*, the ketolase genes *crtW38*, *crtW148*, *crtO* and the constitutively expressed *trpA* gene for tryptophan synthase, α-subunit as reference (Soule et al. 2009). The PCR cycles were 1 cycle of denaturation at 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 15 s, annealing at 48 °C for 15 s, and extension at 72 °C for 15 s and a final melt curve with range from 60 to 95 °C. Comparative quantification (McCurdy et al. 2008) was used to compare the relative expression versus the reference after calculating the $2^{-\Delta\Delta C_t}$ value according to Livak and Schmittgen (2001).

Reproducibility

The HPLC diagrams represent typical results which have been obtained several times. Carotenoid values resulted from 5 to 8 determinations and are given as mean values ± standard deviations. Transcript ratios are from three independent cultures and are given as mean values ± standard deviations.

Results

The carotenoid composition of *Nostoc* PCC 73102 was quantified from cultures grown either at a light intensity of 50 photons cm^{-2} s^{-1} (LL) or 300 photons cm^{-2} s^{-1} (HL) (Table 1a). In LL, β-carotene is the dominating carotenoid. The keto carotenoid of highest concentration is the monoketo echinenone which is almost fivefold increased over the diketo canthaxanthin. Other carotenoids in low concentrations are myxoxanthophyll and 4-keto-myxoxanthophyll. After dilution of the culture 1:2 and growth under the same LL condition, most of the carotenoids accumulated at higher concentrations except for myxoxanthophyll. This increase in total carotenoids in the LL experiments can be explained for this culture by less shading which means higher light exposition per cell due to a lower cell density. Under HL, all carotenoids are increased compared to LL except for β-carotene. The sum of all carotenoids is only slightly lower in HL than in LL. Looking at the percentage of canthaxanthin, it started with 6.5 % of total carotenoids (calculated from Table 1a) and reached 18.3 % after 24-h HL treatment compared to 6.8 % after 24-h LL. From these vales, a threefold increase of canthaxanthin synthesis can be roughly estimated when disregarding differences in oxidation susceptibilities of individual carotenoids. 4-Ketomyxoxanthophyll shows a similar higher percentage in HL compared to LL.

In untreated *Nostoc* PCC 73102, phytoene is completely converted into subsequent carotenoids and therefore not detectable (Table 1b). By blocking the conversion of phytoene with the phytoene desaturase inhibitor difunon, phytoene accumulation after transfer either to LL or HL indicates the activity of phytoene synthesis which determines the degree of carotenogenesis. One feature for identification of phytoene during HPLC is its typical spectrum with shoulders and maxima at 278, 285 and 296 nm is shown in Fig. 1a. Over 6 h and 25 h, accumulation of phytoene was measured. At the end of this period, the almost constant rates of phytoene synthesis were one to sevenfold higher in HL than in LL.

The ketolase genes *crtW38* and *crtW148* from *Nostoc* PCC 73102 are highly homologous with a similarity of 73 %. Both *crtW* genes are unrelated to *crtO* with similarities of 11 % or less. These genes were cloned, inserted into an expression vector and co-transformed into *E. coli* with a second plasmid generating either a β-carotene or a zeaxanthin background. HPLC separations of extracts from these transformants are shown in Fig. 2. CrtW148 completely converted β-carotene into one product (peak 1, Fig. 2a) which co-chromatographs with canthaxanthin and shows the typical symmetrical bell-shaped spectrum with a maximum at 475 nm (Fig. 1b). In the complementation experiment of CrtW38, less than half of the two β-carotene isomers, all-*trans* (peaks 3, spectrum in Fig. 1d) and a *cis* isomer (peak 3′) were utilized to canthaxanthin (peak 1). In addition, echinenone (peak 2 all-*trans*, peak 2′ a *cis* isomer), the monoketo product of β-carotene, was formed as an intermediate. It exhibits an asymmetrical bell-shaped spectrum with a maximum at 465 nm (Fig. 1c).

Upon transformation of a β-carotene producing *E. coli* with pPQE30-CrtO, no ketolase activity was observed. Analysis of the CrtO sequence revealed the presence of rare codons for *E. coli* especially for arginine. Therefore, a third plasmid, pBBR1MCS2Rosetta expressing several tRNAs for arginine, was co-expressed for the β-carotene conversion experiment and also later when zeaxanthin conversion was investigated. The complementation experiment with CrtO (Npun_3745) showed a carotenoid pattern (Fig. 2c) similar to CrtW38 except for a less efficient conversion of echinenone to canthaxanthin. We also assayed the product of gene Npun_0229 from *Nostoc* PCC 73102, which exhibited a lower degree of homology to the first functionally characterized *crtO* gene from *Synechocystis* PCC 6803 compared to Npun_3745, in the same way for ketolase activity. Although it exhibits a similarity of 73 % to the functional *crtO* gene, it was non-functional as a ketolase.

Formation of 4-keto myxoxanthophyll from myxoxanthophyll (Takaichi et al. 2005) involves a C-4 ketolation at

Table 1 Carotenoid composition (μg/g dry weight) in *Nostoc punctiforme* PCC 73102 grown at low light (LL, 50 photons cm^{-2} s^{-1}) or high light (HL, 300 photons cm^{-2} s^{-1}) (a), and in the presence of difunon (b)

Five to eight determinations, means ± standard deviation

	t_0	6-h LL	6-h HL	24-h LL	24-h HL
a					
KetoMyx	4.0 ± 0.8	5.7 ± 0.8	6.1 ± 1.4	4.2 ± 0.8	19.9 ± 2.8
Myx	26.8 ± 3.0	21.1 ± 6.5	33.7 ± 9.7	25.9 ± 8.8	94.8 ± 20.4
Canth	44.2 ± 3.1	64.3 ± 6.6	105.5 ± 7.7	63.8 ± 11.2	168.8 + 17.3
Ech	202.3 ± 36.5	271.5 ± 85.9	176.8 ± 35.8	308.0 + 38.4	214.6 ± 10.6
β-Car	405.4 ± 75.8	487.1 ± 107.1	426.5 ± 67.1	531.0 ± 73.4	422.1 ± 19.3
Sum of car	682.7	849.7	748.5	933.00	920.1
b					
Phyt	0	38.00 ± 7.9	50.0 ± 9.2	120.1 ± 12.5	205.8 ± 44.8

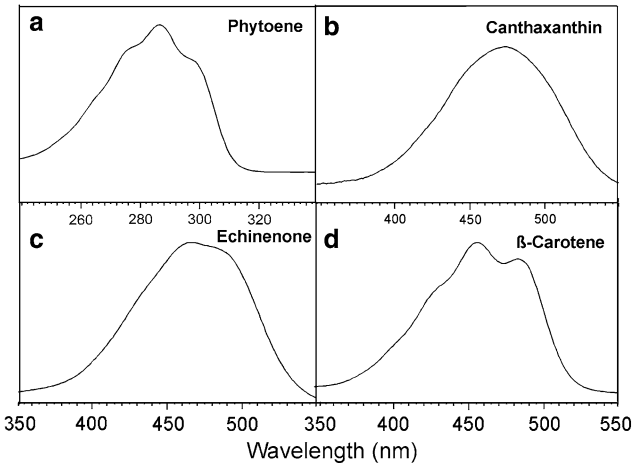

Fig. 1 a–d Spectra of carotenoids synthesized during functional complementation of ketolase genes in *Escherichia coli* and of accumulating phytoene after treatment of *Nostoc punctiforme* PCC 73102 with the phytoene desaturase inhibitor difunon in the HPLC mobile phase acetonitrile/2-propanol/methanol (85:5:10)

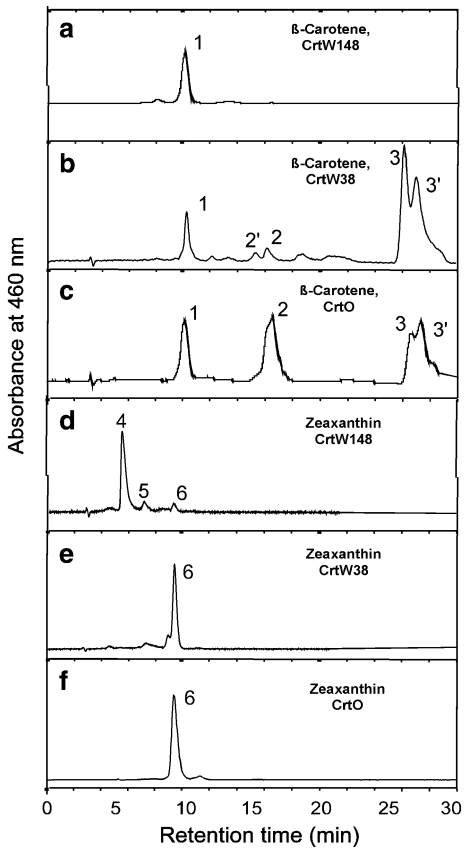

Fig. 2 HPLC separation and identification of products resulting from functional complementation of ketolase genes from *Nostoc punctiforme* PCC 73102 in *Escherichia coli*. **a** Co-transformation of pPQE30crtW148 into a β-carotene background. **b** Co-transformation of pPQE30crtW38 into a β-carotene background. **c** Co-transformation of pPQE30crtO into a β-carotene background. **d** Co-transformation of pPQE30crtW148 into a zeaxanthin background. **e** Co-transformation of pPQE30crtW38 into a zeaxanthin background. **f** Co-transformation of pPQE30crtO into a zeaxanthin background. Peak 1 canthaxanthin peak 2 echinenone (2′ *cis* isomer), peak 3 β-carotene with *cis* isomer 3′, peak 4 astaxanthin, peak 5 adonixanthin and peak 6 zeaxanthin

a 3-hydroxylated β-ionone ring. Therefore, complementation was carried out also in *E. coli* with a background of zeaxanthin [=3,3′-$(HO)_2$-β-carotene]. When zeaxanthin (peak 6) was generated in *E. coli* as a substrate for the ketolases, CrtW38 and CrtO were unable to ketolate this dihydroxy carotenoid (Fig. 2e, f). However, CrtW148 efficiently converted zeaxanthin to astaxanthin, the 3,3′-$(HO)_2$-4,4′-diketo β-carotene derivative (peak 4, same chromophore and same spectrum as canthaxanthin, Fig. 1b). As an intermediate, adonixanthin (=4-keto zeaxanthin) (peak 5, same chromophore and same spectrum as echinenone, Fig. 1c) was detectable in small amounts. This result indicated that CrtW148 is the only ketolase in *Nostoc* PCC 73102 which can insert a keto group at position 4 of a 3-hydroxy-β-ionone ring.

The differential transcriptional regulation of the ketolase genes under HL was analyzed together with the gene *crtB* for phytoene synthase, the enzyme which produced more phytoene under HL (Table 1b). In Fig. 3, transcript ratios HL versus LL for these four genes related to the *trpA* transcript as internal standard are shown. In case of *crtB*, a transient increase was observed with a 1.7-fold higher relative amount 6 h after start of the HL treatment. After 24 h, the transcript ratios leveled down near to one

indicating then an equal expression of *crtB* in LL and HL. Another gene with a higher transcript amount in HL relative to LL of almost fourfold is *crtW148*. Again, the time course is transient with a maximum at 6 h. In contrast to *crtW148*, the other two ketolase genes *crtW*38 and *crt*O exhibit lower transcript amounts in HL than in LL.

Discussion

Nostoc PCC 73102 synthesizes the keto carotenoids, 4-keto-myxoxanthophyll, echinenone and canthaxanthin (Table 1b) as outlined in Fig. 4. These are the typical keto carotenoids found in *Nostoc* species (Takaichi et al. 2005). Canthaxanthin is a superior antioxidant protecting against photooxidation by energy dissipation (Albrecht et al. 2001). Under high light, this process is highly invasive leading to destruction of protecting carotenoids (Steiger et al. 1999). For zeaxanthin-containing cyanobacteria, compensation of the oxidized carotenoids has been demonstrated by a light-dependent transcriptional up-regulation not only of the entire pathway but also of the conversion of the pool of non-protective β-carotene to zeaxanthin (Schäfer et al. 2006). It could be shown for *Nostoc* PCC 73102 that also this canthaxanthin-forming species shows a similar feature, the up-regulation of phytoene synthesis (Table 1b) and a preferential formation of the end-product canthaxanthin. Although carotenogenesis is up-regulated in HL versus LL, total carotenoids are even slightly lower. The concentrations in Table 1a resemble the carotenoid pool sizes. They are determined not only by biosynthesis which is increased in HL but also by photo-degradation which is enhanced. Obviously, biosynthesis cannot fully compensate the photooxidative loss of carotenoids.

In contrast to other cyanobacteria, three distinct ketolases are present in *Nostoc* PCC 73102, two of the *crtW* type and one of the *crtO* type. They all catalyze the formation of canthaxanthin via echinenone from β-carotene (Fig. 2a–c). Nevertheless, they differ in their catalytic properties and their role in high-light regulation. CrtW148 is highly efficient for conversion of β-carotene via echinenone to canthaxanthin (Fig. 4). In contrast, CrtW38 exhibits a much lower affinity for β-carotene utilization. We have shown that CrtO is not a monoketolase as in *Synechocystis* PCC 6803 (Fernandez-Gonzalez et al. 1997) but works as a canthaxanthin-forming diketolase in *Nostoc* PCC 73102 (Fig. 2c). Its lack of specificity for 3-HO-β-ionone end groups resembles CrtO from *Rhodococcus erythropolis* (Choi et al. 2007). This catalytic property can be found for a few CrtW ketolases including CrtW148 (but not for CrtW38) from *Nostoc* PCC 73102 (Fig. 2d) or for CrtW from *Brevundimonas* sp. (Choi et al. 2007). Therefore, CrtW148 is the exclusive ketolase for the formation of 4-keto-myxoxanthophyll either in LL or HL. This is also the case for CrtW from *Nostoc* PCC 7120 (Mochimaru et al. 2005). In addition, formation of canthaxanthin in this species is mainly catalyzed by CrtW rather than by CrtO.

All the investigated carotenogenic genes are transcriptionally regulated (Fig. 3). Up-regulation of the phytoene

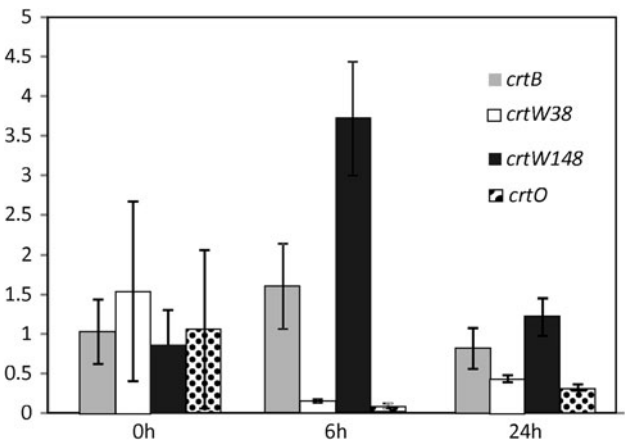

Fig. 3 Transcript ratios of the phytoene synthase gene *crtB* and the ketolase genes *crtO*, *crtW48* and *crtW148* in *Nostoc punctiforme* PCC 73102 grown in high light versus low light

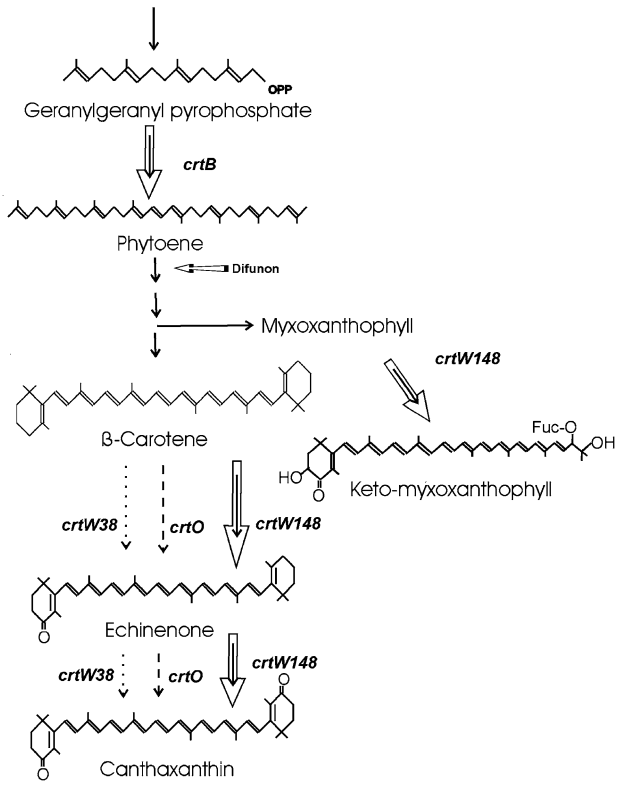

Fig. 4 Overall picture of the carotenoid biosynthesis pathway in *Nostoc punctiforme* PCC 73102 and its photoregulation. Investigated genes are indicated: *wide arrows* stand for up-regulation in high light, *dashed arrow* for down-regulation, *dotted arrow* for low activity and down-regulation. The inhibition site of difunon is pointed out

synthase gene *crtB* coincides with increased phytoene synthesis in the HL culture (Table 1b). In a similar way, the transcript of *crtW148* is also higher in HL than in LL leading to increased canthaxanthin formation (Table 1a). Therefore, it is valid to assume that higher transcript levels reflect higher amounts and activities of the corresponding enzymes. In contrast to CrtW148, the other ketolases, CrtW38 and CrtO, are down-regulated under the same HL conditions. All results obtained lead to the conclusion that under LL CrtO is mainly responsible for keto carotenoid formation with a minor contribution by CrtW38 due to its low activity and CrtW148 due to a relative low expression level. Since in HL CrtO and CrtW38 are down-regulated and CrtW148 up-regulated, the latter takes over the ketolase function from the other enzymes. Under high-light stress, up-regulation of CrtB provides an increased inflow into the carotenogenic pathway. In combination with the up-regulated CrtW148, high amounts of photoprotective canthaxanthin are synthesized (Table 1a). These properties of the biosynthesis pathway are outlined in Fig. 4.

It seems that provision of protective carotenoids on demand during photooxidation under high-light stress is a common feature in cyanobacteria regardless whether canthaxanthin or zeaxanthin is formed. The synthesis of the latter was inherited to photosynthetic prokaryotes including some regulatory properties. For pepper leaves, it has been shown that zeaxanthin synthesis is light–dark regulated (Simkin et al. 2003). The development of oxygenic photosynthesis and the oxygen atmosphere built up by ancient cyanobacteria made it necessary to adapt the carotenogenic pathway towards synthesis of protective carotenoids. In the course of evolution, formation of canthaxanthin which is found in a very few other prokaryotes became wide-spread in many groups of cyanobacteria (Takaichi and Mochimaru 2007) and became light-regulated. The others species which rely on zeaxanthin as photoprotective carotenoid use a CrtR-type β-carotene hydroxylase exclusively found in cyanobacteria (Masamoto et al. 1998) which may have evolved from CrtW due to their close sequence similarity. This may suggest a common origin of up-regulation of canthaxanthin and zeaxanthin under light stress among cyanobacteria.

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