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Control of light-dependent keto carotenoid biosynthesis in *Nostoc* 7120 by the transcription factor NtcA

DOI 10.1515/znc-2016-0117
Received June 2, 2016; revised July 18, 2016; accepted July 19, 2016

Abstract: In *Nostoc* PCC 7120, two different ketolases, CrtW and CrtO are involved in the formation of keto carotenoids from β-carotene. In contrast to other cyanobacteria, CrtW catalyzes the formation of monoketo echinenone whereas CrtO is the only enzyme for the synthesis of diketo canthaxanthin. This is the major photo protective carotenoid in this cyanobacterium. Under high-light conditions, basic canthaxanthin formation was transcriptionally up-regulated. Upon transfer to high light, the transcript levels of all investigated carotenogenic genes including those coding for phytoene synthase, phytoene desaturase and both ketolases were increased. These transcription changes proceeded via binding of the transcription factor NtcA to the promoter regions of the carotenogenic genes. The binding was absolutely dependent on the presence of reductants and oxo-glutarate. Light-stimulated transcript formation was inhibited by DCMU. Therefore, photosynthetic electron transport is proposed as the sensor for high-light and a changing redox state as a signal for NtcA binding.

Keywords: β-carotene ketolases; canthaxanthin; high-light treatment; NtcA binding; transcriptional up-regulation.

Dedication: This work is dedicated to the memory of Professor Peter Böger. His determined studies on the action of bleaching herbicides aroused the interest of the first author and triggered his future work on carotenoid biosynthesis.

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1 Introduction

In cyanobacteria, carotenoids exclusively function as lipophilic antioxidative and UV- and light-protective pigments. Due to their polyene structure and appropriate substituents, they are able to quench triplet-state chlorophyll and singlet oxygen [1]. End products of cyanobacterial carotenoid pathways are mainly zeaxanthin (β,β-carotene-3,3′-diol) or canthaxanthin (β,β-carotene-4,4′-dione) [2, 3]. Both oxygenated β-carotene derivatives are highly photo protective which has been shown in different genetic engineering approaches. In *Synechococcus* with a higher zeaxanthin pool than the wild type, UV-induced damage of photosynthesis was prevented [4]. When the pathway was modified towards the synthesis of canthaxanthin at the expense of β-carotene, photosynthesis in this transformant was protected from UV-B and light inhibition as well as chlorophyll photo oxidation indicating the superior protection potential of canthaxanthin compared to zeaxanthin [5]. Apart from carotenoids acting as antioxidants in lipophilic environments, the mono keto β-carotene derivative echinenone (β,β-carotene-4-one) is the photo active component in a soluble carotenoid protein which dissipates excess energy from the light-harvesting phycobilisomes [6].

Depending on the type of strain, high-light conditions can stimulate the synthesis of zeaxanthin, canthaxanthin or myxoxanthophyll, thereby meeting the demand of these carotenoids for photo protection. Photo protection is an invasive process which may lead to lower carotenoid pools under high light through photo oxidation which up-regulated biosynthesis cannot fully compensate [7]. Increase of carotenoid biosynthesis under high-light conditions is caused by an up-regulation of the promotor of *crtB* in different zeaxanthin accumulating cyanobacteria [8, 9]. This gene encodes phytoene synthase, the gateway enzyme of the specific carotenoid pathway. A similar transcriptional regulation of this gene was also found in *Nostoc* PCC 73102 in which canthaxanthin is the photo protective carotenoid [10]. The ketolation reactions leading to diketo canthaxanthin occur in two steps with

monoketo echinenone as an intermediate accumulating in substantial amounts [11]. Most keto carotenoid-synthesizing cyanobacteria utilize two different ketolases. One is encoded by the bacterial *CrtW* gene [12], the other one by *crtO* which is found only in cyanobacteria and which in *Synechocystis* encodes a mono ketolase specific for the synthesis of echinenone [13]. In *Nostoc* PCC 7120 [14] and also in *Nostoc* PCC 73102 [10] both types of ketolase genes are present simultaneously. Under high light, the *crtO* gene is transcriptionally down-regulated and a *crtW* gene up-regulated resulting in take-over of canthaxanthin biosynthesis by the CrtW ketolase.

Regulation of gene transcription involves specific proteins with DNA binding domains which bind to the promoter region. One of these transcription factors in cyanobacteria is NtcA that is involved in several cellular processes including regulation of nitrogen metabolism [15] and in differentiation, including heterocyst formation [16]. Target promoters for NtcA possess a specific sequence for recognition and binding. As potential NtcA binding sites are recognizable in the promoter regions of *crtO*, *crtW* and *crtPB*, in *Nostoc* PCC 7120 (Cyanobase database at http://www.kazusa.or.jp/cyanobase/), we focused our investigation of light-regulated carotenoid synthesis on the transcriptional control of these carotenogenic genes by NtcA. As a result, we could demonstrate the involvement of NtcA as a regulator for increased carotenoid synthesis and for enhanced metabolization of β-carotene to canthaxanthin.

2 Experimental

2.1 Strains and cultivation

Nostoc PCC 7120 from the Pasteur Culture Collection, re-assigned as such by [17] and formerly designated *Anabaena* PCC7120, was grown on BG11 medium containing 18 μM sodium nitrate as nitrogen source [18]. To cultures of the deletion mutants of *Nostoc* PCC 7120 ΔcrtO (with inactivated *crtO* gene) and ΔcrtW (with inactivated *crtW* gene) [14], 30 μg/mL neomycin sulfate was added. Growth was at 28 °C under continuous white fluorescent light at an intensity of 40 μmol/m²·s (normal light condition) and constant gassing with 1.5% (v/v) CO_2 in air. High-light treatment was performed by transfer of cultures grown under normal light conditions to a light intensity of 400 μmol/m²·s from a halogen lamp. After harvesting the cells by centrifugation, they were used for carotenoid analysis and isolation of DNA and RNA. For inhibition of photosynthetic electron transport, 3-(3, 4-dichlorophenyl)-1,1-dimethyl urea (DCMU) was applied at a concentration of 1 μM before starting high-light treatment.

Escherichia coli DH5α was used for plasmid amplification and strain M15/pREP4 (Qiagen, Hilden, Germany) for heterologous expression of NtcA. Growth medium and conditions were as described [19].

2.2 Carotenoid analysis

Carotenoids from *Nostoc* PCC 7120 were extracted from freeze-dried cells. They were heated in methanol containing 6% KOH (w/v) at 60 °C for 20 min and the extract partitioned into 50% ether in petrol, bp 35–80 °C (v/v) [7]. 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-lm 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 and by comparison of their spectra. Authentic carotenoid standards for HPLC were generated by combinatorial biosynthesis in *E. coli* [20].

2.3 Genomic DNA isolation and plasmid construction

For the heterologous expression of the NtcA protein, the plasmid pQE30ntcA was constructed by PRC amplification of the open reading frame from plasmid pCSAM70 [21] with primers NtcAforSac and NtcArevSal (Table 1A). The resulting DNA fragment was cloned into the *Sac*I/*Sal*I sites of vector pQE30 (Qiagen, Hilden, Germany).

For the amplification of the promoter fragments of the different genes, genomic DNA was isolated from *Nostoc* 7120 after lysozyme treatment by the CTAB method followed by a phenol-chloroform purification step [19]. With this DNA, the promoter region fragments PRntcA, PRcrtO, PRcrtW1 and PRcrtW2 were amplified by PCR with the primers listed in Table 1B.

2.4 Transcript determination

Total RNA from *Nostoc* PCC 7120 was isolated with phenol and guanidine thiocyanate as described [22]. Residual

Table 1: Oligonucleotides for PCR amplification of NtcA (A), promoter fragments for gel retardation assay (B) and real time PCR (C).

(A)	
NtcAforSac	5′-GAGCTCATGATCGTGACACAAGATAAGG-3′
NtcArevSal	5′-GTCGACCTCCTGCTATTCTTCTTTAAGTG-3′
(B)	
GRA1-CrtWfor	5′-GCCAAAAAGTCAGTAAAAAC-3′
GRA1-CrtWrev	5′-TTTTCTGAATGCAGAGATGATG-3′
GRA2-CrtWfor	5′-TCTGAAGGGTGAGGGAAATC-3′
GRA2-CrtWrev	5′-TTGGCTAATTGTGTTCCATC-3′
GRA-CrtOfor	5′-ATGTCCAGACTGATATTT-3′
GRA-CrtOrev	5′-TTTAGTTACTAAGCCTTGA-3′
GRA-CrtPBfor	5′-TCTGAAGGGTGAGGGAAATC-3′
GRA-CrtPBrev	5′-TTGGCTAATTGTGTTCCATC-3′
(C)	
RT-CrtWfor	5′-GTCTTATTGGCGATGGAC-3′
RT-CrtWrev	5′-TTGTGGTAGCCGAAGTG-3′
RT-CrtOfor	5′-CGAGCGATTTCTGCACAAG-3′
RT-CrtOrev	5′-TGCAGCTACAACAGAGGAG-3′
RT-CrtPBfor	5′-TACGAAACCGGGTTACACG-3′
RT-CrtPBrev	5′-GCTCCCAGGTCAACATATC-3′
RT-NtcAfor	5′-TACGAGGCAGGAGAAGAG-3′
RT-NtcArev	5′-GAATCCGCGAAGACAGAC-3′

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, Sydney, Australia) with the Sensi-Mix SYBR No-ROX Kit (Bioline, Luckenwalde, Germany). The reference was a 277 bp fragment from the *rnpB* gene (RNAse subunit P) [23] which is constitutively expressed independent of illumination [24]. The oligonucleotides used in qRT-PCR are listed in Table 1C.

2.5 Isolation of the NtcA protein

Escherichia coli M15/pRep4/pQE30ntcA was harvested after growth on a shaker at 22 °C in SB medium [20] containing 3% ethanol to an OD_{600} of 0.6 before IPTG was added (0.1 mM) followed by a 3 h incubation. The harvested cells were re-suspended in 200 mM potassium phosphate buffer pH and 7.8 broken twice in a French Pressure Cell at 95 MPa. After centrifugation (10 min at 16,000 *g*), the supernatant was treated with Sigma (Taufkirchen, Germany) His-select Cobalt Affinity Gel equilibrated in 50 mM phosphate buffer containing 0.3 mM NaCl and 10 mM imidazole. After incubation with the protein extract, the gel was collected by centrifugation and washed with the same buffer. Finally, NtcA was eluted with the same buffer containing 250 mM imidazole and concentrated by precipitation with 6% TCA.

2.6 Gel retardation assay

DNA fragments of the promoter regions were amplified by PCR. About 100–200 ng DNA was incubated with NtcA (0.3 µM or as indicated in Section 3 – Results) for 15 min in 10 mM Tris-HCl buffer pH 7.5 containing 40 mM KCl, 0.1 mM $MnCl_2$, 0.5 mg/mL BSA, 1 mM DDT and 10% glycerol. 2-Oxoglutarate (0.6 mM), $MgCl_2$ and ATP were added as indicated in Results. The DNAs and DNA-NtcA complexes were separated by native SDS-polyacrylamide gel electrophoresis on a 7% gel. Their bands were stained in a 0.5 mg/mL ethidium bromide solution.

3 Results

3.1 Carotenoids under high light

In *Nostoc* PCC 7120 wild type under low light conditions, β-carotene is the major carotenoid. The second highest concentration was found for monoketo echinenone followed by diketo canthaxanthin (Figure 1). Upon transfer to high light, the amount of canthaxanthin was five-fold increased already after 6 h, whereas the concentrations of β-carotene and echinenone, both precursor and intermediate in the pathway to canthaxanthin, decreased concurrently. After 24 h, most of the higher canthaxanthin level was retained. In the low-light control, the concentration changes for all three carotenoids over 24 h were negligible.

The *Nostoc* PCC 7120 mutant with the inactivated *crtW* gene showed the same tendency of higher canthaxanthin synthesis under high light but lower levels were reached (Figure 1). Instead, the echinenone concentration was higher than in the wild type and a similar light-dependent decrease was observed. In the *crtO* deletion mutant, canthaxanthin synthesis was completely blocked and formation of echinenone, the only keto carotenoid in this strain, was only 20% of echinenone synthesis in the wild type. However, in contrast to wild type, a doubling of the echinenone content occurred under high light.

3.2 Transcript changes upon high-light treatment

Changes of transcript levels after transfer to high light, relative to the levels in low light, were determined for the ketolases CrtW and CrtO and for the phytoene synthase CrtB (Figure 2). In wild type, a strong increase of the transcripts of all carotenogenic genes was observed,

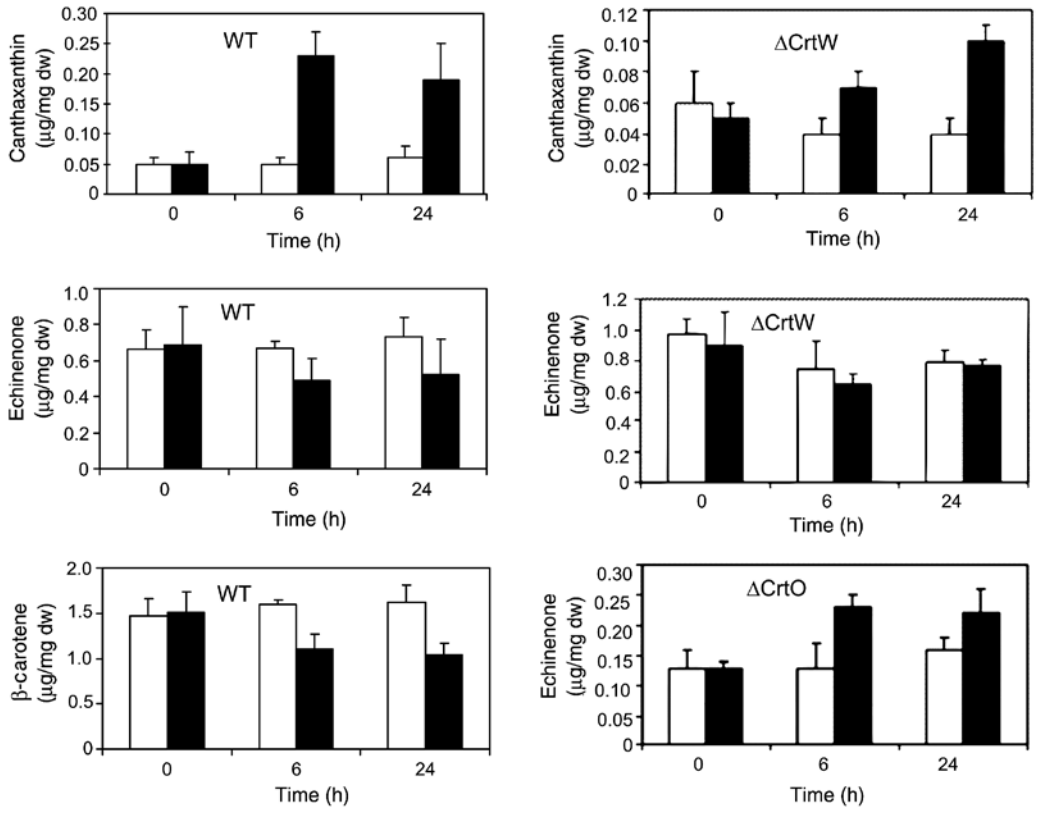

Figure 1: Change of carotenoid concentrations in *Nostoc* 7120 wild type (WT) and deletion mutant ΔcrtW and ΔcrtO after transfer from low light (open bars) to high light (filled in bars).

in contrast to the *ntcA* transcript which did not change significantly (data not shown). For *crtW*, transcript level was seven-fold higher after 6 h treatment and 15 fold higher after 24 h. The increase of the *crtO* transcript was slower but reached a similar relative increase after 24 h. The *crtB* gene is light-regulated in other cyanobacteria [9, 10]. Therefore, we also looked for transcription changes of this gene. Its transcript exhibited transient kinetics with a maximum of an almost five-fold higher amount followed by a slight decrease from 6 to 24 h.

In the mutant with inactivated *crtO*, the transcript of *crtW* was increased similar to the wild type, but this relative increase was only threefold after 24 h of high-light treatment (Figure 2). In the mutant with inactivated *crtW*, the *crtO* transcript was increased as observed for the wild type. However, this increase of 30-fold higher than the initial value was twice as high as in the wild type. Application of the inhibitor of photosynthetic electron transport, DCMU, prevented a relative increase of the *crtO* transcript in *Nostoc* PCC 7120 upon transfer to high light (Figure 2). In contrast to *crtO*, the transcripts of *crtW* and *crtP* decreased strongly after DCMU addition regardless of light treatment (data not shown).

3.3 Prediction of NtcA binding sites in carotenogenic genes

As NtcA is a universal transcription factor with known binding sites, we searched for NtcA binding sites in the promoter regions of *crtW*, *crtO* and the operon *crtPB* (Figure 3). Analysis of the *crtW* gene from *Nostoc* PCC 7120 (alr3189) revealed a typical –10 consensus region of a σ^{70} promoter at position –12 from the translation start codon (Figure 3, top). Instead of a missing –35 element, a potential NtcA binding region at position –58 which is 46 nucleotides upstream from the –10 region, was identified. This NtcA binding site consists of the consensus motif $GTAN_7TAC$ [25]. Further upstream at position –320 another potential NtcA binding site $GTAN_8AAC$ was found at position –320.

The promoter of *crtO* (all3744) contains a –10 σ^{70} region at position –28 (Figure 3, middle). Again, the –35 region is replaced by a putative NtcA binding motif $GTAN_8TAC$, 14 nucleotides further upstream. The *crtP* (alr1832) and the *crtB* (alr1833) genes are transcribed in all analyzed cyanobacteria as an operon with a common promoter region [8, 26]. In this region, a –10 element is found at position –47 (Figure 3, bottom). At position –263, a NtcA box with a slightly modified

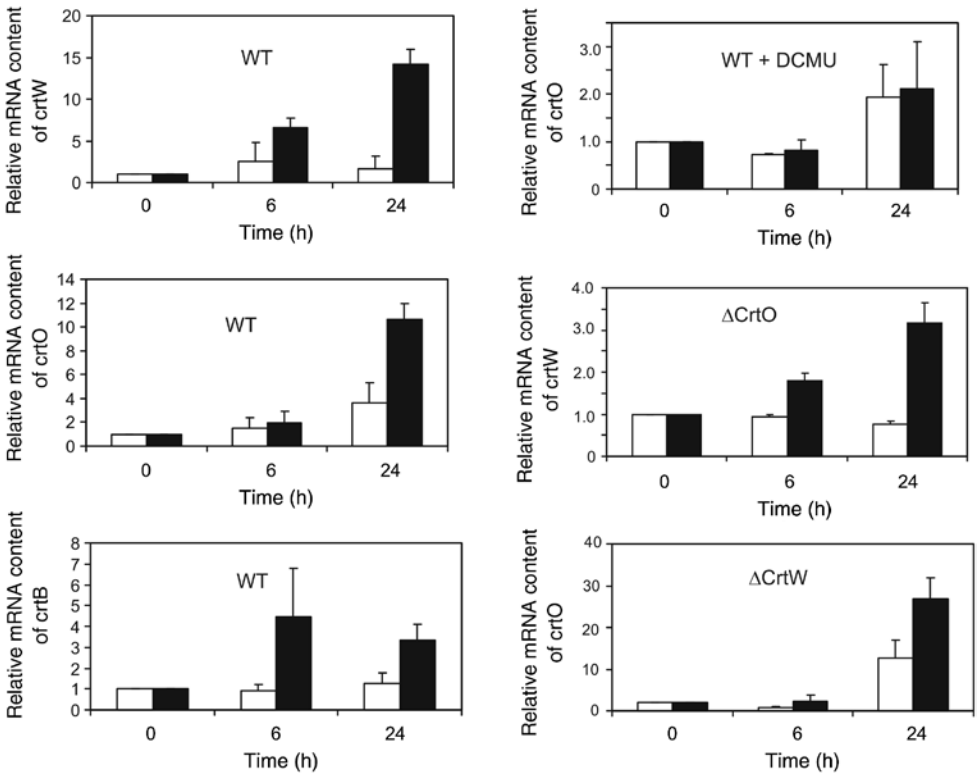

Figure 2: Transcript ratios of the genes of phytoene synthase *crtB* and the ketolases *crtO* and *crtW* in *Nostoc* 7120 grown in high light (open bars) versus low light (filled in bars). Transcript of *crtO* was also determined from a culture treated with the inhibitor of photosynthetic electron transport DCMU.

consensus motif of $GTGN_8GAC$ could be predicted. This is 110 nucleotides downstream of the –10 promoter element.

3.4 Analysis of NtcA binding

Binding activity of purified NtcA to DNA was tested with a fragment of the glutamine synthetase gene *pglnA* from *Nostoc* 7120 which covers the NtcA binding site [27]. Gel retardation assays were carried out with different NtcA concentrations (Figure 4). A doubling of the NtcA concentration resulted in a doubling of the DNA–Ntca complex. A 296 bp DNA fragment from vector pMON38201 without the NtcA binding motif was used as negative DNA control. In this case, no DNA protein complex was observed.

Two individual *crtW* promoter regions with sizes of 144 bp for PRcrtW1 and 154 bp for PRcrtW1 (as specified in Figure 3) were used in this assay (Figure 4). As indicated by the intensity of the DNA-NtcA bands on top of both gels, binding was observed starting at 0.01 μM NtcA and increased continuously when higher concentrations were applied. With 0.3 μM, almost 100% binding was reached for PRcrtW2. For PRcrtW1 binding at this concentration was 73%. The 144 bp DNA fragment PRcrtO of *crtO* (as specified in Figure 3) covering the predicted NtcA binding site also exhibited an NtcA complexed band in the gel retardation assay. However, this binding was less intense reaching only 42% with 0.3 μM NtcA (Figure 4). The promoter region upstream of *crtP* is responsible not only for the transcription of this gene but also for *crtB* which is part of the *crtPB* operon. The 137 bp DNA fragment from the promoter region PRcrtPB (as specified in Figure 3) formed a complex with NtcA binding 86% of the DNA at 0.3 μM (Figure 4).

In the gel retardation assay, we further analyzed the conditions for NtcA binding to the promoter regions of the carotenogenic genes (Table 2). In addition, the binding strength of the DNA–NtcA complex is positively affected by oxo-glutarate and negatively affected by Mg ions. For NtcA binding to all carotenogenic gene promoters, reduced conditions were essential.

4 Discussion

As in other *Nostoc* strains [11], echinenone is the major keto carotenoid in *Nostoc* PCC 7120 accompanied by

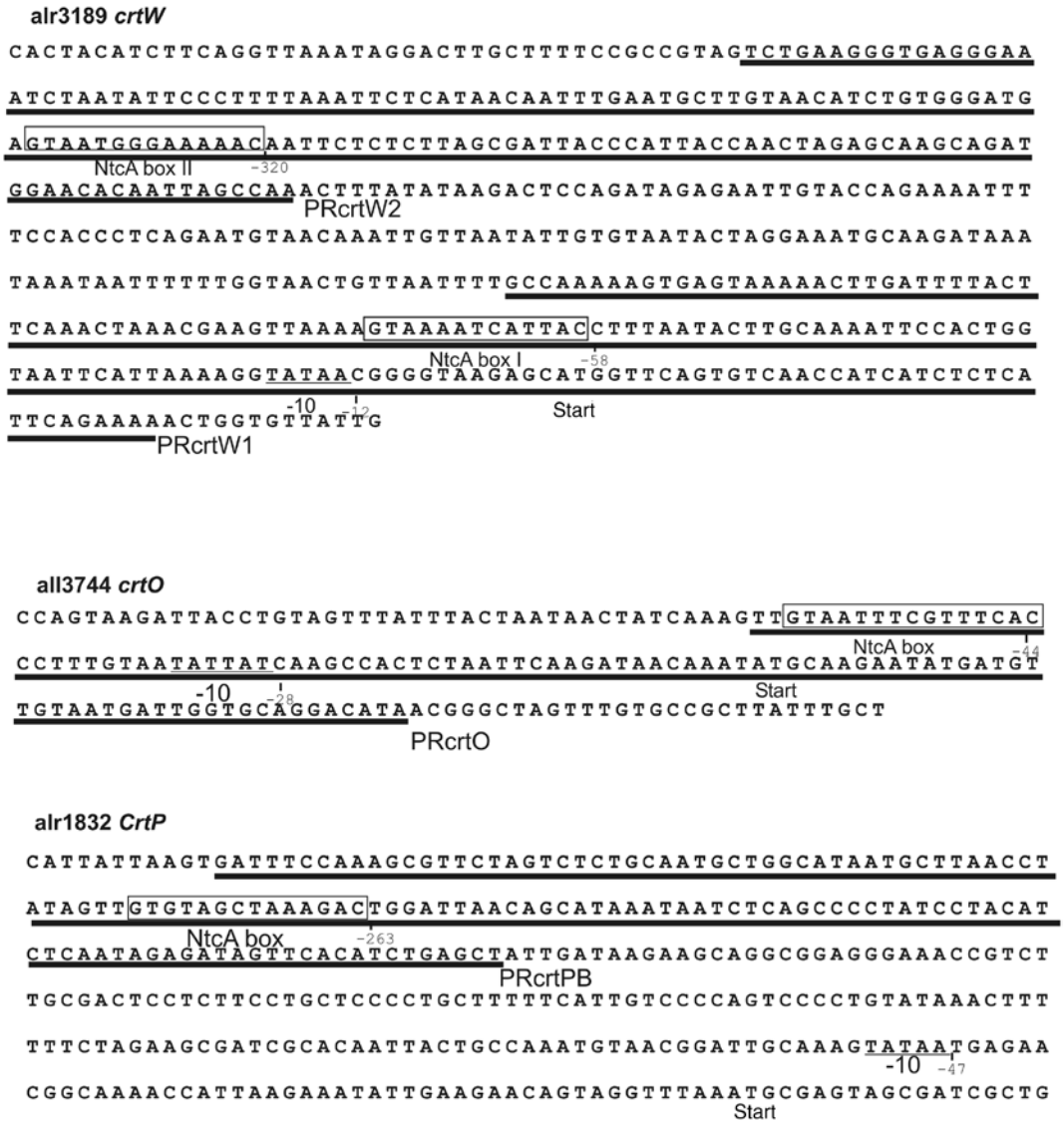

Figure 3: Promoter regions of the *crtW*, *crtO* genes and the *crtPB* operon of *Nostoc* 7120. Predicted NtcA binding regions are boxed, the –10 promoter elements are marked and the DNA fragments used in the gel retardation assay are underlined.

diketo canthaxanthin (Figure 1). Their relative abundance is shifted towards the synthesis of canthaxanthin with increasing light intensity at the expense of the non-ketolated precursor β-carotene as already reported for *Nostoc* PCC 73102 [10]. The photo protective activity of canthaxanthin is superior to that of echinenone [5, 28]. The enhanced canthaxanthin synthesis strengthens the ability to survive high-light conditions. In addition, the transcriptional up-regulation of phytoene synthase and desaturase (Figure 2) supplies precursor carotenoids for the synthesis of canthaxanthin and compensates loss of carotenoids by photo oxidation [10]. Both phytoene synthase [4] and phytoene desaturase [26] are limiting enzymes in carotenoid biosynthesis of cyanobacteria. There they are jointly expressed through a common promoter finally resulting in higher carotenoid formation.

Two different ketolases are involved in the formation of keto carotenoids in cyanobacteria. Generally, CrtW catalyzes the double ketolation of β-carotene to canthaxanthin [29]. In contrast, CrtO acts as a mono ketolase synthesizing echinenone with only one keto group in *Synechocystis* [13]. However, CrtO of *Nostoc* PCC 73102 is a diketolase involved in the synthesis of canthaxanthin [10]. The carotenoid profiles of the *crtO* deletion mutant of *Nostoc* 7120 reveal a loss of canthaxanthin synthesis (Figure 1). This finding demonstrates that only CrtO can catalyze canthaxanthin formation in *Nostoc* PCC 7120. These functional diversities demonstrate the high species-dependent variability

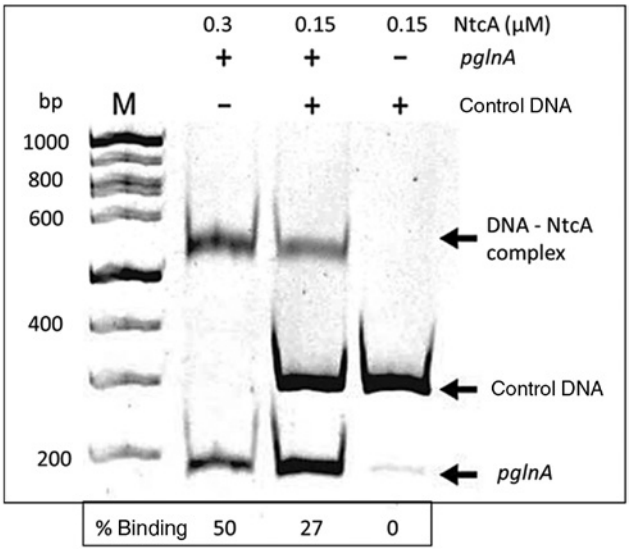

Figure 4: Gel retardation assay to assess the NtcA binding activity with *pglnA* DNA as positive control (167 bp fragment including the NtcA binding site) and a 296 bp fragment from vector pMON38201 as negative DNA control. The percentage of retarded DNA is indicated at the bottom.

Table 2: Binding of NtcA at promoters of the carotenogenic genes *crtPB*, *crtO* and *crtW*.

Additions (mM)	Binding (%)			
	PRcrtW1	PRcrtW2	PRcrtO	PRcrtPB
Oxoglu				
0	51	48	29	36
0.2	68	79	52	71
0.4	85	97	46	87
0.6	85	94	57	89
$MgCl_2$				
0	100	89	68	31
1	89	90	25	56
2	19	54	11	33
5	7	17	4	0
ATP				
0	24	28	19	29
0.1	22	30	15	38
0.2	20	23	20	32
0.5	21	24	14	34
DDT				
0	0	0	0	0

The DNA regions are indicated in Figure 3. Concentrations of 2-oxoglutarate (Oxoglu) and DDT were 0.6 mM and 1 mM, respectively, unless stated otherwise.

of both ketolases to accept echinenone as a substrate and convert it to canthaxanthin. In contrast, CrtW possesses a low affinity for echinenone and directs precursors only in support of β-carotene utilization half way to canthacanthin (Figure 6). In a previous publication [14], it has been shown that CrtW can ketolate myxoxanthophyll which, due to a single β-ionone ring in its molecule, can only be converted to a mono keto product. Under our growth condition of *Nostoc* PCC 7120, the myxoxanthophyll concentration was below detection.

Up-regulation of canthaxanthin biosynthesis from β-carotene via echinenone (Figure 6) is transcriptionally regulated in *Nostoc* PCC 7120 (Figure 3). For both ketolase genes, *crtW* and *crtO*, higher transcript levels were found under high light. This is in contrast to *Nostoc* PCC 73102 in which only one of the two *crtW* genes present therein exhibited increased transcription, whereas the *crtO* transcript is unchanged providing the basic catalytic activity regardless of light conditions [10]. As *crtP* is transcribed together with the phytoene synthase gene *crtB* from the same promoter, higher transcript levels of *crtB* indicate the simultaneous up-regulation of phytoene desaturase.

The promoters of all the analyzed carotenogenic genes can be assigned as type 2 σ^{70} promoters [30]. They possess only the –10 hexamer, and the –35 region is replaced by a binding site for a transcriptional activator protein. The most universal transcription factor in cyanobacteria is NtcA. Upon the screening of the promoter

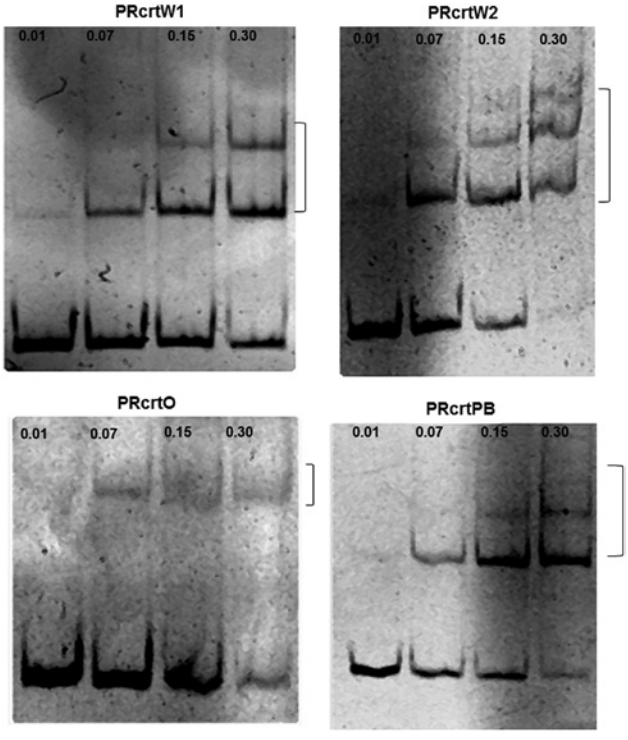

Figure 5: Gel retardation assays with DNA fragment of the promoter region of carotenogenic genes of *Nostoc* 7120 and NtcA. NtcA concentrations (µM) are indicated on top. The region of the DNA-NtcA complexes is marked as the right side of each gel.

region of the ketolase genes and the *crtPB* operon, NtcA binding site were predicted (Figure 4). Only the one for *crtO* was strictly canonical. Both binding motifs downstream of *crtW* and the one of *crtPB* were modified either by a spacing region one nucleotide shorter or by the TAC region modified to AAC or GAC. These modifications were previously found for other *Nostoc* PCC 7120 genes [25]. In our case, using the promoter region DNA of *crtW*, *crtO* and *crtPB*, we could demonstrate that these varied NtcA motifs are genuine biding sites as demonstrated in the gel shift assays (Figure 5). This indicates that NtcA is a positive regulator for all the investigated carotenognic genes of *Nostoc* 7120, enhancing their basal transcription activity in a coordinated way. Binding of oxo-glutarate to NtcA is essential for the establishment of the right protein conformation for DNA binding [31]. As the NtcA transcript did not increase under high light (data not shown), it is unlikely that the mode of NtcA activation is concentration-driven.

DCMU, the inhibitor of photosynthetic electron transport, also inhibits the increase of the transcripts of the carotenogenic genes under high-light whereas reduced conditions are favorable for NtcA-binding to their promoters. This is an indication that high-light sensing proceeds via photosynthesis rather than being a direct effect of photooxidation or triggering by reactive oxygen species. It has been shown that changes in the cellular redox state of *Nostoc* PCC 7120 affect binding of NtcA to DNA which depends on free thiol residues of the NtcA protein [32]. Therefore, we propose for the light-dependent transcriptional activation of the respective carotenogenic genes a sensing of high light through the photosynthetic electron transport system (Figure 6). This may lead to a change in the redox state with a reduced plastoquinone pool or accumulation of reductants e.g. by the ferredoxin-thioredoxin reaction which both could be the signal for NtcA binding. A similar activation mechanism involving the photosynthetic electron transport was suggested for several other light-regulated and NtcA-activated genes in *Synechocystis* [33].

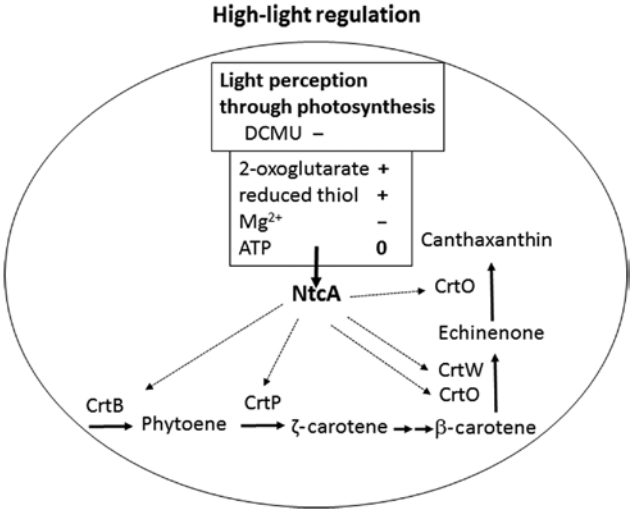

Figure 6: Light perception, activation of NtcA binding and involvement in up-regulation of carotenogenic genes under high-light conditions in *Nostoc* 7120. Boxes on top of NtcA indicate activation factors for NtcA binding, dashed arrows NtcA-dependent transcriptional up-regulation of *crt* genes.

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