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Carotenoid biosynthesis in *Calothrix* sp. 336/3

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Abbreviations: dHex, deoxyhexose; GGPP, geranylgeranyl pyrophosphate; OCP, orange carotenoid protein; ROS, reactive oxygen species.

Abstract (max 250 words)

The carotenoid composition of the filamentous heterocystous N_2-fixing cyanobacterium *Calothrix* sp. 336/3 was investigated under three conditions: in full medium (non-diazotrophic growth); in the absence of combined nitrogen (diazotrophic growth); and after long-term H_2 photoproduction (diazotrophic medium and absence of nitrogen in atmosphere). *Anabaena* sp. PCC 7120 and its Δ*hupL* mutant with disrupted uptake hydrogenase were used as reference strains. Analysis of identified carotenoids and enzymes involved in carotenogenesis showed the presence of three distinct biosynthetic pathways in *Calothrix* sp. 336/3. The first one is directed towards biosynthesis of myxoxanthophylls, such as myxol 2'-methylpentoside and 2-hydroxymyxol 2'-methylpentoside. The second pathway results in production of hydroxylated carotenoids, such as zeaxanthin, caloxanthin and nostoxanthin, and the last pathway is responsible for biosynthesis of echinenone and hydroxylated forms of ketocarotenoids, such as 3'-hydroxyechinenone and adonixanthin. We found that carotenogenesis in filamentous heterocystous cyanobacteria varies depending on the nitrogen status of the cultures, with significant accumulation of echinenone during diazotrophic growth at the expense of *β*-carotene. Under the severe N-deficiency and high CO_2 supply, which leads to efficient H_2 photoproduction, cyanobacteria degrade echinenone and *β*-carotene and accumulate glycosylated and hydroxylated carotenoids, such as myxol (or ketomyxol) 2'- methylpentosides, 3'-hydroxyechinenone and zeaxanthin. We suggest that the stability of the photosynthetic apparatus in *Calothrix* sp. 336/3 cells under N-deficiency and high carbon conditions, which is also appeared as the partial recovery of the pigment composition by the end of the long-term (~1 month) H_2 photoproduction process, might be mediated by a high content of hydroxycarotenoids.

Keywords: Carotenoid biosynthesis, carotenogenic genes, dizaotrophic growth, hydrogen photoproduction, photoinhibition, oxidative stress

Introduction

Carotenoids in photosynthetic organisms play three major roles. First, they primarily serve as the accessory pigments in the light-harvesting complexes of photosynthetic apparatus (reviewed in Frank and Cogdell 1996). By absorbing light in the range complementary to other pigments, carotenoids efficiently transfer the energy to chlorophyll (Chl) via the singlet-singlet energy transfer mechanism (Naqvi 1980). Second, carotenoids as a major part of the complex antioxidant system play an essential role in the protection of photosynthetic cells against photoinhibition and oxidative damage. The photoprotective role of carotenoids is linked to quenching the excited triplet-state Chl that prevents formation of toxic singlet oxygen and to their ability of scavenging singlet oxygen directly (Cogdell et al. 2000). They are so important that an inability to form carotenoids is lethal in cyanobacteria under photoautotrophic conditions (Sozer et al. 2010). Third, carotenoids play a role in the structural stabilization of membranes, transmembrane proteins and protein complexes. The role of *β*-carotene in stabilization of photosystem II (PSII) has been known for a long time (Trebst 2003, Bautista et al. 2005). The glycosylated carotenoids (myxoxanthophylls) of cyanobacteria together with cholesterol and other membrane-spanning lipids may contribute to stabilization of membranes and affect their fluidity and polarity (Mohamed et al. 2005).

The protective functions of carotenoids could become especially important in N_2-fixing cyanobacteria that experience a permanent conflict of their highly oxygen sensitive nitrogenase system with O_2-evolving photosynthesis. While the water-splitting and O_2-generating photosynthetic apparatus provides the cells with energy and reducing equivalents that are needed for cell metabolism and also N_2-fixation (Böhme 1998, Bothe et al. 2010), the repair of photosynthetic apparatus and other protein complexes could not be sustained without a constant supply of fixed nitrogen needed for protein biosynthesis. It is not surprising, therefore, that this delicate balance is very sensitive to any changes in the environmental conditions. Nevertheless, the data on the role of carotenoids in diazotrophic cyanobacteria and, in particular, in diazotrophic, heterocyst-forming species is limited (Kelman et al. 2009, Onishi et al. 2015, López-Igual et al. 2016).

In heterocystous strains, the conflict between N_2-fixation and oxygenic photosynthesis is partly hindered by a localization of O_2-sensitive nitrogenase system in specialized cells, heterocysts, which provide microoxic environment (reviewed in Böhme 1998, Bothe et al. 2010, Allahverdiyeva et al. 2014). It has been suggested that an elevated rate of respiration together with a thick cell wall and a

lack of active PSII complexes ensure low levels of oxygen inside heterocysts. In the light, elimination of O_2 is also driven by the heterocyst-specific flavodiiron protein Flv3B, which is crucial for protection of nitrogenase under the diazotrophic growth condition (Ermakova et al. 2013, Ermakova et al. 2014). Nevertheless, permeation of O_2 to heterocysts via narrow terminal pores that connect heterocysts with vegetative cells (Walsby 2007) and concomitant production of ROS cannot be avoided. Therefore, it is not unexpected that heterocysts possess ROS scavenging mechanisms similar to vegetative cells (Banerjee et al. 2013). The contribution of carotenoids to the protection of nitrogenase and photosynthetic complexes in heterocysts has not been studied well. Their participation in the light harvesting for N_2-fixation is still not resolved (Staal et al. 2003). However, there is comprehensive information on the role of carotenoids in the protection of photosynthetic apparatus in general (Britton 1995, Trebst 2003, Kusama et al. 2015) and on carotenogenesis in cyanobacteria (Takaichi and Mochimaru 2007, Takaichi 2011).

Efficient H_2 photoproduction in filamentous heterocystous cyanobacteria is driven by the nitrogenase enzyme and occurs under the condition of N-limitation and high CO_2 supply (Leino et al. 2012) that results in an imbalanced C/N ratio in cyanobacteria and affects the cell fitness (Kosourov et al. 2014). In the latter work we found that the long-term H_2 photoproduction condition led to the decreased PSII yield (YII), degradation of Chl *a* and a noticeable visual change in the pigment composition of cyanobacteria embedded in Ca^{2+}-alginate films. The Chl *a* degradation and visual color changes were pronounced in the films with entrapped cells of the Δ*hupL* mutant of *Anabaena* sp. PCC 7120 that showed the highest H_2 photoproduction yield due to inactivation of the uptake hydrogenase enzyme located in heterocysts. Another good H_2-producing strain, *Calothrix* sp. 336/3, which was isolated from a local lake in Finland (Sihvonen et al. 2007; Allahverdiyeva et al. 2010), showed more stable photosynthetic activity under the same condition: Y(II) was higher than in *Anabaena* wild-type and Δ*hupL* strains, and the Chl *a* content even increased by the end of ~600 h experiment (Kosourov et al. 2014). These observations required further detailed study. Although carotenogenesis in *Anabaena* sp. PCC 7120 was very well characterized (Takaichi et al. 2005, Graham and Bryant 2009), information about carotenoids in *Calothrix* was limited to only one report in *Calothrix parietina* (Stransky and Hager, 1970).

In the present work, we investigate the composition of carotenoids in heterocystous filamentous cyanobacterium *Calothrix* sp. 336/3 (*i)* grown on the full medium (non- diazotrophic

growth), (*ii*) grown in the absence of combined nitrogen (diazotrophic growth) and (*iii*) after long-term H_2 photoproduction (absence of nitrogen in the medium and atmosphere). This study reports the presence of three distinct carotenoid biosynthesis pathways in the *Calothrix* sp. 336/3. The first pathway leads to production of myxoxanthophylls, the second to accumulation of hydroxycarotenoids and the third pathway to production of ketocarotenoids and their hydroxylated derivatives. Under the conditions favorable to efficient H_2 photoproduction, the carotenoid compositions of *Calothrix* sp. 336/3 and the Δ*hupL* mutant of *Anabaena* sp. PCC 7120, demonstrate a shift towards accumulation of glycosylated and hydroxylated carotenoids.

Results

Identification of carotenoid pigments in Calothrix sp. *336/3*

The N_2-fixing filamentous heterocystous model cyanobacterium, *Anabaena* sp. PCC 7120, with a well characterized carotenoid profile (Takaichi et al. 2005), was selected as a reference strain in this study. For long-term H_2 photoproduction experiments, it was replaced with the Δ*hupL* mutant, which shows high H_2 photoproduction yields due to disruption of large subunit of [Ni-Fe] uptake hydrogenase in heterocysts (Masukawa et al. 2002). The carotenoid compositions of these two strains were almost identical (Table 1 and 2). As shown in Fig. 1A, the HPLC elution profile for the methanol extracted pigments from *Anabaena* sp. PCC 7120 that was obtained with the solvent system 1 (see Material and Methods) demonstrates the presence of 6 major carotenoids (peaks 1a – 6a): ketomyxol 2'-fucoside, myxol 2'-fucoside, 3'-hydroxyechinenone, canthaxanthin, echinenone and all-trans-*β*-carotene. The cells also contained *cis* forms of ketomyxol 2'-fucoside and myxol 2'-fucoside (peaks 1a' and 2a'). Differently from the data reported by Takaichi et al. (2005), the HPLC elution profile also showed the presence of 9-*cis*-*β*-carotene (peak 6a') and a trace of 13-*cis*-*β*-carotene that appeared as a shoulder (6a") on the 9-*cis*-*β*-carotene peak. The *cis* forms of *β*-carotene had the absorption maxima slightly shifted in the blue region (Fig. S1 in the supplemental material) and demonstrated an increased intensity (%D_B/D_{II}) of the *cis*-peak absorbance at ~340 nm (Liaaen-Jensen 1995), which was more pronounced in 13-*cis*-*β*-carotene. In addition, *Anabaena* sp. PCC 7120 samples contained a small amount of zeaxanthin, which was negligible in diazotrophically-grown cells.

The benthic cyanobacterium, *Calothrix* sp. 336/3, demonstrated a much wider range of carotenoids compared to *Anabaena* sp. PCC 7120 (Fig. 1B). The HPLC elution profile obtained with

the solvent system 1 showed the presence of 9 major (peaks 1b - 9b) and 5 minor (peaks 2b', 3b', 6b', 9b' and 9b'' shoulder) carotenoid products. Similar to *Anabaena* sp. PCC 7120, *Calothrix* sp. 336/3 synthesized all-*trans*-*β*-carotene (peak 9b; λ_{max}: 450, 476 nm), 9-*cis*-*β*-carotene (peak 9b'; λ_{max}: 445, 471 nm) and 13-*cis*-*β*-carotene (9b'' shoulder; λ_{max}: 443, 467 nm). Their absorption spectra are presented in Fig. S1. As expected, the molecular mass of the *β*-carotene protonated molecule $[M+H]^+$ was *m/z* 537. The all-*trans*-*β*-carotene peak also has the same retention time as the *β*-carotene standard from DHI Lab Products.

Another major carotenoid extracted from *Calothrix* sp. 336/3, which has the same retention time as the standard, was echinenone (peak 8b). This carotenoid has one keto group at the C-4 position and shows a broad absorption at around 460 nm (Fig. S2). The following introduction of another keto group in the second ionone ring of echinenone should produce canthaxanthin. This carotenoid was present in *Anabaena* sp. PCC 7120 (Fig. 1A, peak 4a), but only traces were found in some samples of *Calothrix* sp. 336/3. Canthaxanthin in the samples was positively identified by the retention time, its absorption spectrum with λ_{max} at 473 nm (Fig. S2) and molecular mass of protonated molecule $[M+H]^+$ at *m/z* 565. Instead of canthaxanthin, *Calothrix* sp. 336/3 accumulated 3'-hydroxyechinenone, a hydroxylated ketocarotenoid at the C-3' position (Fig. 1B, peak 7b). In the solvent system 1 for HPLC separation, it was co-eluted partly with a minor amount of *cis*-caloxanthin (peak 6b'), but the solvent system 2 gave a better resolution in that case. In both systems, the retention times and spectra (λ_{max}: 460 nm) were identical to 3'-hydroxyechinenone extracted from *Anabaena* sp. PCC 7120. Under the LC-MS condition, the mass spectrum of this carotenoid peak showed the presence of a protonated molecule at *m/z* 567 $[M+H]^+$ and intense fragment ion at *m/z* 549 $[M+H-18]^+$. Another ketocarotenoid product was found in the peak 4b. It had almost the same broad absorption with the maximum at 460 nm as echinenone (Fig. S3), but slightly shifted to the red region. The mass spectrum of this carotenoid peak showed the presence of protonated molecule at *m/z* 583 $[M+H]^+$, and fragment ions 565 $[M+H-18]^+$ and 547 $[M+H-36]^+$. The only known carotenoid with molecular mass of 582 Da that has two hydroxyl groups (as determined by the mass spectrum) and at least one keto group (as determined by the reduced spectral fine structure) is adonixanthin (4-ketozeaxanthin). Adonixanthin could be a product of either 3'-hydroxyechinenone hydroxylation at the C-3 position or C-4 ketolation of zeaxanthin that are both present in the *Calothrix* sp. 336/3.

In contrast to *Anabaena* sp. PCC 7120, *Calothrix* sp. 336/3 synthesized three major pigments of the hydroxycarotenoid group: zeaxanthin, caloxanthin and nostoxanthin (Fig. 1B, peaks 6b, 5b and 3b, respectively). They had *β*-carotene-like spectra (Fig. S2, S3) with three major absorption maxima at 428 (shoulder), 451, 478 nm in the solvent system 1 (427 (shoulder), 450, 475 nm in the solvent system 2). Despite similar absorption maxima, these carotenoids showed slightly different spectral fine structures (%III/II) and most importantly they differed by the size and the number of major fragments in the mass spectra, as expected (Bharti et al. 2014). The zeaxanthin peak showed a protonated molecule at *m/z* 569 $[M+H]^+$ and fragment ions 551 $[M+H-18]^+$ and 533 $[M+H-36]^+$, caloxanthin similarly had *m/z* 585 $[M+H]^+$, 567 $[M+H-18]^+$ and 549 $[M+H-36]^+$, and the nostoxanthin peak showed a protonated molecule at *m/z* 601 $[M+H]^+$ and three ions from losses of water: 583 $[M+H-18]^+$, 565 $[M+H-36]^+$ and 547 $[M+H-54]^+$. The peak 6b (Fig. 1B) also had the same retention time and spectral properties as the zeaxanthin standard from DHI Lab Products. In addition, the *cis* forms of nostoxanthin (peak 3b') and caloxanthin (peak 6b') were found in *Calothrix* sp. 336/3 samples. These two carotenoids showed a noticeable increase of the *cis*-peak absorbance at ~340 nm and shifted maxima of three major absorption peaks to the blue region, but the mass spectra were the same as that obtained for the corresponding *trans* forms. Despite the fact that *β*-cryptoxanthin is considered as an intermediate product of *β*-carotene hydroxylation to zeaxanthin (Takaichi and Mochimaru 2007), this carotenoid was not detected in *Calothrix* sp. 336/3 samples in the current setup.

The polar carotenoids in *Calothrix* sp. 336/3 were presented by 2-hydroxymyxol 2'-methylpentoside, myxol 2'-methylpentoside and *cis* form of myxol 2'-methylpentoside (Fig. 1B, peak 1b, 2b and 2b‘, respectively). The myxol 2'-methylpentoside peak and the peak of its *cis* form had exactly the same retention times and the spectral properties as the myxol 2'-fucoside and its cis form extracted from *Anabaena* sp. PCC 7120. The absorption maxima of peaks 2a and 2b (Fig. 1) in the solvent system 1 were typical for myxoxanthophylls: 296, 367, 452, 475 and 506 nm (Fig. S2), and the spectral fine structure of %III/II was around 54-55. The *cis* form (Fig. 1B, peak 2b‘) had the absorption maxima at 301, 365, 448 (shoulder), 472, 502 nm (%III/II=30) with significant gain of the absorbance at ~365 nm. The mass spectrum of myxol 2'-methylpentoside showed the presence of a protonated molecule at *m/z* 731 $[M+H]^+$ and the loss of water fragment ions at 713 $[M+H-18]^+$ and 695 $[M+H-36]^+$ and three major fragment ions at *m/z* 567 $[M+H-dHex]^+$, 549 $[M+H-dHex-18]^+$ and 531 $[M+H-dHex-36]^+$. Thus, the neutral loss of 164 Da (dHex) indicates a deoxyhexose, presumably fucose, rhamnose

or chinovose that are typical conjugates for cyanobacterial myxoxanthophylls (Takaichi and Mochimaru 2007). In contrast to *Anabaena* sp. PCC 7120, *Calothrix* sp. 336/3 did not produce a 4-ketomyxol analogue and accumulated 2-hydroxymyxol 2'-methylpentoside. This myxoxanthophyll had almost the same absorption maxima as myxol 2'-methylpentoside, but a slightly reduced spectral fine structure: 297, 368, 450, 475, 506 nm (%III/II=51). It also showed the presence of a protonated molecule and five fragment ions in the mass spectrum, but of different sizes: *m/z* 747 $[M+H]^+$, 729 $[M+H-18]^+$, 711$[M+H-36]^+$, 583 $[M+H-dHex]^+$, 565 $[M+H-dHex-18]^+$ and 547 $[M+H-dHex-36]^+$.

Identification of putative genes encoding enzymes of the carotenoid biosynthesis pathways

The availability of the genome sequence of *Calothrix* sp. 336/3 (Isojärvi et al. 2015), offers an opportunity for further identification of genes and enzymes involved in biosynthesis of carotenoids in this cyanobacteria. A homology search through the *Calothrix* sp. 336/3 genome sequence database (NCBI reference sequence: NZ_CP011382.1) for the genes encoding enzymes with confirmed functions revealed several candidates (Table 3). These candidates include the genes of early steps of carotenoid biosynthesis, such as geranylgeranyl pyrophosphate synthase (*crtE*), phytoene synthase (*crtB*), 15-*cis*-phytoene desaturase (*crtP*) and ζ-carotene desaturase (*crtQ*). The products of *crtB*, *crtP* and *crtQ* demonstrate a high sequence identity (65 – 75%) to the products of the similar genes from *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942. Additionally, the *cis*-carotene isomerase (CrtH) homolog showing 74% identity to the CrtH from *Synechocystis* sp. PCC 6803 was also found in *Calothrix* sp. 336/3.

The *Calothrix* sp. 336/3 contains two CruA-type lycopene cyclases, the CruA and the parologous CruP, that are 64 and 55% identical to the CruA and CruP from *Synechococcus* sp. PCC 7002, respectively (Table 3). Interestingly, *Calothrix* sp. 336/3 also possesses *cruF* and *cruG* most probably encoding 1',2'-hydratase and 2'-O-glycosyltransferase, respectively. Similar to *Anabaena* sp. PCC 7120 that produces myxol 2'-fucoside (Fig. 1A, peak 2a), *Calothrix* sp. 336/3 contains the homologous *wcaG* gene, the product of which showed 86% identity (Table 3) to the All4826 from the *Anabaena* sp. PCC 7120 with the confirmed GDP-fucose synthase activity in this strain (Mochimaru et al. 2008).

The production of hydroxylated carotenoids, in *Calothrix* sp. 336/3 apparently depends on two available hydroxylases: *β*-carotene hydroxylase (CrtR) and 2,2´-*β*-hydroxylase (CrtG). The CrtR

hydroxylase from *Calothrix* sp. 336/3 is 78% identical to CrtR from *Anabaena* sp. PCC 7120 and 71% identical to the CrtR from *Synechocystis* sp. PCC 6803 (Table 3). The *crtG* is not available in the *Anabaena* sp. PCC 7120, but common for the strains producing caloxanthin and nostoxanthin. Besides *Calothrix* sp. 336/3, orthologs of this enzyme exist in several cyanobacteria including *Chlorogloeopsis fritschii* sp. PCC 6912 (73% identity to CrtG from *Calothrix* sp. 336/3), *Thermosynechococcus elongatus* BP-1 (36% identity), *Synechococcus* sp. PCC 7942 (52% identity), *Synechococcus* sp. PCC 7335 (44% identity).

The *Calothrix* sp. 336/3 has one CrtO-type *β*-carotene ketolase (WP_035158015.1, Table 3), which is 64% identical to Slr0088 from *Synechocystis* sp. PCC 6803, 83% identical to Gll0394 from *Gloeobacter violaceus* PCC 7421 and 84% identical to All3744 from *Anabaena* sp. PCC 7120. The *Calothrix* sp. 336/3 does not contain any CrtW-type *β*-carotene ketolases.

The carotenoid composition of cyanobacteria grown on full medium and under N_2-fixing conditions

In accordance with previous data reported for model *Anabaena* and *Nostoc* species (Takaichi et al. 2005), during active growth *Anabaena* sp. PCC 7120 and its uptake hydrogenase Δ*hupL* mutant accumulate primarily four carotenoids: *β*-carotene, echinenone, myxol 2'-fucoside and 4-ketomyxol 2'-fucoside (Table 1, Z8 and Z8x samples; Table 2, +N_2 sample). When grown on the full medium, the shares of these carotenoids in the wild-type strain were about 36, 16, 19 and 12%, respectively. Under N_2-fixing conditions (diazotrophic growth), the share of *β*-carotene declined to 20% and the share of echinenone rose to 28% (Table 1). In the Δ*hupL* strain grown under N_2-fixing conditions, there was a noticeable increase of myxol 2'-fucoside at the expense of *β*-carotene (Table 2; 32 and 13%, respectively). Independently of the growth condition, *Anabaena* sp. PCC 7120 and Δ*hupL* cells also contained marked amounts of canthaxanthin (3 - 5%), *cis* forms of *β*-carotene (0.6 - 6%) and some other carotenoids. However, their contents, except zeaxanthin, did not vary significantly in samples.

Similar to *Anabaena* sp. PCC 7120, *Calothrix* sp. 336/3 accumulates *β*-carotene, echinenone and myxol 2'-methylpentoside (either fucoside, rhamnoside or chinovoside) as the major carotenoids, but does not synthesize 4-ketomyxol analogues (Table 1 and 2). Instead of the keto form, the cells accumulate hydroxylated myxoxanthophyll, 2-hydroxymyxol 2'-methylpentoside. The shares of *β*-carotene, echinenone and myxol 2'-methylpentoside in cells pre-grown for two weeks in full medium

were 36, 15 and 11%, respectively. The share of 2-hydroxymyxol 2'-methylpentoside did not exceed 6% and did not vary significantly with the growth condition (Table 1 and 2). *Calothrix* sp. 336/3 grown on the full medium also contained ~28% hydroxylated carotenoids, which include 10% zeaxanthin, 12% caloxanthin and 6% nostoxanthin. Under N_2-fixing conditions, the share of hydroxylated carotenoids slightly increased, mostly due to accumulation of 14% zeaxanthin and 8% nostoxanthin. Similarly to *Anabaena* sp. PCC 7120, the share of echinenone in diazotrophically grown *Calothrix* sp. 336/3 cells was significantly higher than in cells grown on the full medium (Table 1, 15 and 20%, respectively). At the same time, the share of *β*-carotene in diazotrophic cells declined to 20 - 27% (Table 1 and 2). Independently of the growth condition, *Calothrix* sp. 336/3 had 2 - 3% 3'-hydroxyechinenone, 1 - 3% *cis* forms of *β*-carotene and some other minor carotenoids.

The difference in carotenoid composition in cultures grown on full medium and under the diazotrophic condition led to differences in the *in situ* absorption spectra (Fig. 2A). As expected, both strains grown under N_2-fixing conditions had a higher absorbance in the 460 – 510 nm region, which corresponds to the carotenoid absorption band (Fig. 2B). Indeed, both diazotrophic cultures showed a higher carotenoid / Chl *a* ratio as compared to the cells grown on the full medium (Table 1). Interestingly, *Calothrix* sp. 336/3 did not demonstrate any significant changes in the phycocyanin (PC) peak at ~630 nm (Fig, 2A), but diazotrophically grown *Anabaena* sp. PCC 7120 showed a strong decline of absorbance in this region. In contrast to *Anabaena*, *Calothrix* sp. 336/3 also possessed a very prominent level of phycoerythrin (PE) with a peak absorbance at ~570 nm (Fig, 2A) that did not depend on the growth condition.

Changes in the pigment composition under long-term H_2 photoproduction

For efficient H_2 photoproduction, diazotrophically pre-grown cyanobacteria were entrapped in Ca^{2+}-alginate microfilms and placed in diazotrophic medium under an Ar atmosphere supplemented with 3 - 6 % CO_2. Thus, N was completely eliminated both from the medium and from the vial atmosphere, allowing efficient production of H_2 via the nitrogenase enzyme located in heterocysts (Leino et al. 2012). In the long-term experiments, however, N-depletion led to a gradual loss of photochemical activity in vegetative cells and, therefore, to a gradual decline of O_2 and H_2 photoproduction rates overtime (Kosourov et al. 2014).

The pigment composition of cyanobacteria entrapped in Ca^{2+}-alginate microfilms changed after their long-term incubation under an Ar atmosphere supplemented with 3 and 6 % CO_2 (Table 2). Under N-depletion, cyanobacteria demonstrated significant degradation of Chl *a*. Since Chl *a* declined more dramatically than carotenoids, the carotenoids to Chl *a* ratio increased in all samples. The most noticeable increase in the ratio was observed in *Calothrix* sp. 336/3 films after 340 h incubation under an Ar atmosphere supplemented with 6% CO_2 (Table 2, 340 h sample). Extending the incubation to 600 h, however, led to a recovery of both Chl *a* and the total carotenoid contents in *Calothrix* sp. 336/3 cells, whilst the carotenoids to Chl *a* ratio was still slightly higher than in the beginning of the experiment (Table 2, 600 h sample). A similar recovery was not observed in the Δ*hupL* mutant of *Anabaena* sp. PCC 7120 throughout the 780 h H_2 photoproduction period.

The most noticeable change in the carotenoid composition, which was observed both in the *Calothrix* sp. 336/3 and Δ*hupL* cells entrapped in microfilms, was a decline of echinenone (Table 2). The decline was more pronounced in cyanobacteria treated with 6% CO_2 and was noticeable even in the *Calothrix* sp. 336/3 films, where partial recovery of carotenoids occurred (Table 2, 600 h sample). On the contrary, the share of 3'-hydroxyechinenone rose slightly but significantly ($P < 0.05$) in all samples. Similarly, the overall content of myxoxanthophylls increased in cyanobacteria after long-term incubation without N and in the presence of CO_2. For example, the share of 4-ketomyxol 2'-fucoside in the Δ*hupL* cells increased up to 41%. In *Calothrix* sp. 336/3 cells that did not produce ketomyxol, the major change was detected for myxol 2'- methylpentoside, whose share rose up to 30%. In the Δ*hupL* mutant, there was a noticeable increase in the share of another hydroxylated carotenoid, zeaxanthin. The relative amounts of other carotenoids did not change in either strain.

Discussion

Three distinct carotenogenesis pathways in Calothrix sp. *336/3*

The carotenoid biosynthetic pathways in the *Calothrix* sp. strain 336/3 proposed in this study are very diverse (Fig. 3) but show some similarities to carotenogenesis in other cyanobacteria, especially for the most conserved early reactions of phytoene biosynthesis and its conversion to lycopene (Armstrong et al. 1990, Liang et al. 2006). The biosynthesis of phytoene in *Calothrix* sp. 336/3 is catalyzed by geranylgeranyl pyrophosphate (CrtE) and phytoene (CrtB) synthases. The following reaction, the four-step desaturation of phytoene to lycopene, is driven by two enzymes in most

cyanobacteria: 15-*cis*-phytoene desaturase (CrtP) and ζ-carotene desaturase (CrtQ). In the plant-type desaturation of phytoene to lycopene, besides CrtP and CrtQ desaturases, the third enzyme, *cis*-carotene isomerase (CrtH) is needed for isomerization of poly-*cis*-prolycopene to all-*trans*-lycopene (Takaichi and Mochimaru 2007). The genes encoding the above mentioned enzymes are available in *Calothrix* sp. 336/3 (Table 3).

Similar to other cyanobacteria, *Calothrix* sp. 336/3 does not accumulate lycopene and converts it to other carotenoids. Cyclization of lycopene to *β*-carotene in this strain seems to be driven by the CruA-type lycopene cyclases (CruA and CruP, Table 3). Among cyanobacteria, they were first identified and functionally confirmed in *Synechococcus* sp. PCC 7002 (Maresca et al. 2007). We determined that *Calothrix* sp. 336/3 contains two genes encoding proteins with accession numbers WP_035155193.1 and WP_035157658.1 showing high identity to CruA and CruP of *Synechococcus* sp. PCC 7002, respectively. Though, their lycopene cyclase function in *Calothrix* should be confirmed further. The role of *cruA* orthologues in *Anabaena* sp. PCC 7120 is also under question. While Alr3524 (CruA) did not show lycopene cyclase activity (Mochimaru et al. 2008), Alr0920 (CruP) has not been checked yet.

Similarly to *Anabaena* sp. PCC 7120, *Calothrix* sp. 336/3 possesses a biosynthetic pathway towards production of myxol and its derivatives (myxoxanthophylls) (Fig. 3). At the first step of myxoxanthophyll production, lycopene is converted to myxol, which requires formation of the *β*-ionone ring, desaturation at the C-3',4' position and introduction of hydroxyl groups at C-3, C-1' and C-2'. These reactions are proposed to be driven by the CruA-type lycopene cyclase, CrtR hydroxylase and CruF hydratase (Graham and Bryant 2009). In the next step, the hydroxyl group at the C-2' position is glycosylated to form myxoxanthophyll. In *Synechococcus* sp. PCC 7002, 2'-O-glycosyltransferase (CruG) is responsible for this reaction (Graham and Bryant 2009). Since *Calothrix* sp. 336/3 has *cruA*/*cruP*, *crtR, cruF* and *cruG* homologs (Table 3), as well as *wcaG* encoding GDP-fucose (methylpentose) synthase, the biosynthesis of myxoxanthophylls in this strain most likely occurs in a way similar to *Synechococcus* sp. PCC 7002. In contrast to *Anabaena* sp. PCC 7120, *Calothrix* sp. 336/3 does not produce a ketomyxol form (Fig. 1). Instead, *Calothrix* sp. 336/3 accumulates 2-hydroxymyxol 2'-methylpentoside, most probably due to the presence of CrtG (2,2´-*β*-hydroxylase) capable of introducing the hydroxyl group at the C-2 position. In line with that, the deletion mutant of *Thermosynechococcus elongatus* BP-1, which was the first cyanobacterial strain where the functions

of CrtG were characterized, did not produce 2-hydroxymyxol 2'-fucoside in contrast to the wild-type strain (Iwai et al. 2008). On the other hand, the mutant of *Synechococcus* sp. PCC 7002 with overexpressed CrtG homolog from *Synechococcus* sp. PCC 7942 started producing 2-hydroxymyxol 2'-fucoside (Graham 2008).

The production of hydroxycarotenoids such as caloxanthin, nostaxanthin and 3'-hydroxyechinenone in *Calothrix* sp. 336/3 is driven by two distinct hydroxylases, *β*-carotene hydroxylase (CrtR) and 2,2'-*β*-hydroxylase (CrtG), that were positively identified in the genome sequence database (Table 3). The CrtR enzyme is responsible for the accumulation of carotenoids hydroxylated at C-3 and C-3' positions. In *Calothrix* sp. 336/3, this enzyme has a high capacity for converting *β*-carotene to zeaxanthin, which under our experimental conditions, showed up to 20% of the total carotenoid share (Tables 1 and 2). In contrast, *Anabaena* sp. PCC 7120 produced much less zeaxanthin under all tested conditions. The latter assumes different substrate specificity of CrtRs in these two strains. Cyanobacteria with CrtR also produce *β*-cryptoxanthin from *β*-carotene, 3'-hydroxyechinenone from echinenone and myxol from plectaniaxanthin (Makino et al. 2008, Graham and Bryant 2009). The 2,2'-*β*-hydroxylase enzyme in *Calothrix* sp. 336/3 is likely responsible for accumulation of carotenoids hydroxylated at C-2 and C-2' positions, such as 2-hydroxymyxol 2'-methylpentoside (as described above), caloxanthin and nostaxanthin.

The presence of ketocarotenoids, such as echinenone and 3'-hydroxyechinenone in the HPLC elution profile (Fig. 1 B, peaks 7b and 8b) indicated the availability of ketolases in the strain. Indeed, we found that *Calothrix* sp. 336/3 has one CrtO-like *β*-carotene ketolase (Table 3) that is a typical enzyme for echinenone-accumulating strains, such as *Anabaena* sp. PCC 7120 (Fig. 1 A, peak 5a), *Synechocystis* sp. PCC 6803 (Fernández-González et al. 1997) and *Gloeobacter violaceus* PCC 7421 (Steiger et al. 2005). Similar to *Synechocystis* sp. PCC 6803 and in contrast to *Anabaena* sp. PCC 7120, *Calothrix* sp. 363/3 does not contain the CrtW-like *β*-carotene ketolase. Interestingly, this cyanobacterium did not produce ketomyxol and only traces of canthaxanthin were detected in some samples (identified by retention time, absorption spectrum and molecular weight). This strain also showed the presence of adonixanthin (Fig. 1 B, peak 4b), which might be a product of either zeaxanthin ketolation or hydroxylation of 3'-hydroxyechinenone.

In our experimental setup we could not identify synechoxanthin in the *Calothrix* sp. 363/3 and *Anabaena* sp. PCC 7120 samples. Although this aromatic xanthophyll has been detected in *Anabaena*

(Maresca et al. 2008), we assume that *Calothrix* sp. 363/3 does not produce it as the genes encoding CruE and CruH enzymes responsible for the synechoxanthin biosynthesis (Graham et al. 2008, Maresca et al. 2008) are not found in the genome of *Calothrix* sp. 363/3.

Thus, carotenogenesis in *Calothrix* sp. 363/3 shows three distinct biosynthetic pathways (Fig. 3). The first one is directed towards the biosynthesis of myxoxanthophylls, which leads to accumulation of myxol 2'-methylpentoside and 2-hydroxymyxol 2'-methylpentoside in cells. The second pathway results in production of hydroxylated carotenoids, such as zeaxanthin, caloxanthin and nostoxanthin. The last pathway begins from ketolation of *β*-carotene to echinenone with minor production of canthaxanthin as a result of a second ketolation and ends with the biosynthesis of hydroxylated forms of ketocarotenoids, such as 3'-hydroxyechinenone and adonixanthin.

The ketocarotenoid echinenone may play a role in N_2-fixation in heterocystous cyanobacteria

Under N_2-fixing conditions, all three strains studied here accumulated echinenone at the expense of *β*-carotene (Table 1 and 2). Accumulation of echinenone may reflect the possible contribution of this pigment to light harvesting for N_2-fixation and/or its involvement in photoprotection under this particular condition. In agreement with the first assumption, *in situ* absorption spectra of diazotrophically grown *Anabaena* sp. PCC 7120 and *Calothrix* sp. 336/3 cultures showed increased absorbance of the carotenoid band (Fig. 2, 460 – 510 nm). Since the share of *β*-carotene in all diazotrophic samples declined and the level of echinenone rose accordingly, the contribution of the latter into the overall carotenoid absorbance should increase. However, the effect was less pronounced in *Calothrix* cells, where the rise of hydroxylated carotenoids, such as zeaxanthin and nostoxanthin under N_2-fixing conditions was also prominent (Table 1).

In *Anabaena* sp. PCC 7120, the increase of the carotenoid band absorbance occurred simultaneously with degradation of phycobilisomes (PBSs) (Fig. 2, 590 – 650 nm) that confirms significant rearrangements within photosynthetic antenna complexes. Degradation of PBSs under the condition of N-deficiency is a well documented phenomenon, which reduces absorption of light energy, provides the cell with substrate for protein biosynthesis and allows them to overcome stressed situations (Baier et al. 2004). On the contrary, *Calothrix* sp. showed more stable photosynthetic antennae that may reflect a different acclimation strategy to N-deficiency (or imbalance in C:N ratio).

In agreement with the light harvesting assumption, Staal and co-authors (2003) showed, by analyzing the light action spectra of N_2-fixation in several heterocystous cyanobacteria, the contribution of echinenone and *β*-carotene to nitrogenase activity and concluded that these pigments are present in the heterocysts. At the same time, the involvement of other carotenoids in photoenergy transfer to nitrogenase was not as significant (Staal et al. 2003). The latter, though, may simply reflect the lower overall content of other carotenoids as compared to echinenone and *β*-carotene (Table 1). The changes in the carotenoid content on N-depletion were also confirmed spectrophotometrically for *Anabaena variabilis* ATCC 29413, in which a reduction in the relative amount of *β*-carotene was observed (Onishi et al. 2015).

In this context, we suggest that the enhanced level of echinenone in *Anabaena* sp. PCC 7120 / Δ*hupL* and in *Calothrix* sp. 336/3 may reflect the contribution of this pigment to the N_2-fixation process. Interestingly, the diazotrophically pre-grown Δ*hupL* strain, which is affected in H_2 recycling activity in the heterocyst, showed higher echinenone / *β*-carotene ratio as compared to the wild-type strain (Table 1 and 2, *Anabaena* sp. PCC 7120 vs. Δ*hupL*, + N_2 condition only). Though, the effect of this mutation on carotenoid composition is not direct. The H_2 uptake activity in *Anabaena* is linked to respiration, which contributes to O_2 removal in heterocysts (Tamagnini et al. 2007). Thus, the absence of uptake hydrogenase may affect the O_2-senstitive nitrogenase enzyme and intensify N-deficiency. This indirect effect of the H_2 uptake activity in heterocysts on photosynthetic apparatus in vegetative cells (Kosourov et al. 2014) may lead to the change of carotenoid composition both in vegetative cells and heterocysts. Further investigations are needed for understanding the possible role of echinenone and other carotenoids in N_2-fixing cyanobacteria.

Conditions favorable to H_2 photoproduction affect the carotenoid content in heterocystous cyanobacteria

Immobilization of the cells together with N-deficiency and high CO_2 supply restrict culture growth, prolong and enhance H_2 photoproduction yields in heterocystous cyanobacteria (Leino et al. 2012). However due to a strong N-depletion and reduced cell fitness, photochemical activity and H_2 photoproduction rates of the cells decreases overtime (Kosourov et al. 2014). The entrapment of cyanobacteria in thin films also ensures uniform distribution of light to the cells within the

immobilization matrix (Gosse et al. 2010, Tsygankov and Kosourov 2014) and allows a more precise evaluation of light effects on carotenoid biosynthesis.

As expected for the N-depleted condition, we observed essential degradation of Chl *a* in the Δ*hupL* mutant of *Anabaena* sp. PCC 7120 throughout the experiment (Table 2, *Anabaena* 780 h films) and in the *Calothrix* sp. 336/3 but only in the middle of the H_2 photoproduction phase (Table 2, 340 h samples). The loss of Chl *a* in the films with N-depleted cyanobacteria is not surprising since N-deficiency has been known for a long time to cause degradation of PSII reaction centers and re-arrangement of PBSs (Onishi et al. 2015). In alginate films, the effect might be enhanced by additional oxidative damage due to possible accumulation of O_2 inside the immobilization matrix, especially under high CO_2 levels. Since a part of *β*-carotene molecules is directly associated with PSII and PSI reaction centers (Jordan et al. 2001, Umena et al. 2011), degradation of these centers caused by the oxidative stress should result in the partial loss of *β*-carotene in cells. A significant decline of *β*-carotene ($P < 0.05$) was indeed observed in the Δ*hupL* mutant and in 340 h *Calothrix* sp. 336/3 films (Table 2). An increase of Chl *a* allomers in our samples relatively to Chl *a*, but not the Chl *a* epimer to Chl *a* (data not shown) could be another sign of oxidative stress.

After the initial degradation, the Chl *a* content recovered in *Calothrix* sp. 336/3 (Table 2, 600 h films) but not in the Δ*hupL* mutant of *Anabaena* sp. PCC 7120 where all pigments progressively declined (Table 2, 780 h samples). The films with entrapped *Calothrix* sp. 336/3 cells did not show any significant decrease in the total carotenoid level throughout the experiment. Moreover, the level even increased by the end of H_2 photoproduction period (Table 2, 600 h films). Despite the partial recovery of the pigment composition by 600 h, *Calothrix* sp. 336/3 films still showed, as compared to 0 h samples, a significantly higher level of myxol 2'-methylpentoside and declined level of echinenone. A similar pattern was observed in the Δ*hupL* strain, where cells accumulated 4-ketomyxol 2'-fucoside and decreased the share of echinenone (Table 2). In contrast to *Calothrix* sp., the increase in the level of 4-ketomyxol 2'-fucoside in the Δ*hupL* films occurred on the background of the overall pigment degradation. The significant increase in the share of myxoxanthophylls in both strains under H_2 photoproduction conditions is unclear at this point. They are essential for normal membrane organization and may contribute to the stabilization of thylakoid membrane under high light conditions, thus, participating in photoprotection (Muhamed et al. 2005, Takaichi 2011).

Interestingly, both strains contained less echinenone under long-term H_2 production conditions when compared to diazotrophic growth in the suspension culture, but showed an increased level of 3'-hydroxyechinenone (Table 2). The decreased level of echinenone is in agreement with possible involvement of this carotenoid in light harvesting during active growth, which is not needed to the same extent under the H_2 photoproduction condition. In the absence of active growth, the excess of light energy reaching photosynthetic reaction centers should be dissipated more efficiently. In cyanobacteria this is achieved by re-arrangement of PBSs (Onishi et al. 2015) and excitation energy quenching triggered by the orange carotenoid protein (OCP) (Wilson et al. 2008, Punginelli et al. 2009). In addition, OCPs have another photoprotective function serving as singlet oxygen quenchers (Sedoud et al. 2014). Since 3'-hydroxyechinenone is a major carotenoid of OCPs (Kerfeld et al. 2003), the increase of its level under the H_2 photoproduction condition at the expense of echinenone is not surprising.

The *Calothrix* sp. 336/3 possesses two genes encoding OCPs with accession numbers WP_035153924.1 (75% identity with All3149 of *Anabaena* sp. PCC 7120 and 78% identity with P83689.1 of *Arthrospira maxima*) and WP_035155465.1 (65% and 68% identity with All3149 and P83689.1, respectively). Interestingly, the gene encoding WP_035155465.1 is localized within the *hupL* element (Leino et al. 2014), which is excised during heterocyst maturation by XisC recombinase (Carrasco et al. 2005). The significance of this fact for acclimation of *Calothrix* sp. 336/3 to diazotrophic conditions remains unclear (Leino et al. 2014). In addition, *Calothrix* sp. 336/3 has two genes encoding NTD-OCP like proteins WP_035157925.1 and WP_035152692.1, and one gene encoding a homolog of the C-terminal domain of the OCP (WP_035157922.1). López-Igual and co-authors (2016) have recently demonstrated the contribution of NTD-OCP like proteins in photoprotection in *Anabaena* sp. PCC 7120. While All4941 can interact with the antenna and induce permanent thermal energy dissipation, All3221 and Alr4783 have been shown to be very good singlet oxygen quenchers. WP_035157925.1 and WP_035152692.1 of *Calothrix* sp. 336/3 are 76 and 54% identical to All4941 and Alr4783 of *Anabaena* sp. PCC 7120, respectively. Thus, the OCPs-mediated photoprotection mechanisms also seem to play an important role in *Calothrix* sp. 336/3.

After long-term H_2 photoproduction, the Δ*hupL* films showed an enhanced level of another hydroxylated carotenoid, zeaxanthin, which also increases from the trace level to above 1% (Table 2). The effect, however, was not pronounced in the *Calothrix* sp. 336/3 strain, where it might be masked

by the high share of this carotenoid in the cells. Zeaxanthin is involved in protection of the repair cycle of photodamaged PSII centers by depressing the levels of singlet oxygen (Kusama et al. 2015).

Oxygenated carotenoids (ketocarotenoids and hydroxycarotenoids) act as efficient antioxidants in the protection of membranes from photooxidation and radical-mediated lipid peroxidation (McNulty et al. 2007), and they do that to a greater degree than apolar carotenes (Graham 2008). The *Calothrix* sp. 336/3 strain, in general, has a much wider range of oxygenated carotenoids than *Anabaena* sp. PCC 7120 and shows more stable photosynthetic apparatus that is capable of partial recovery even under adverse environmental conditions, such as N-depletion, partial anaerobiosis and high CO_2 levels. We, therefore, suggest that accumulation of hydroxylated carotenoids in *Calothrix* sp. 336/3 may contribute to the stability of this strain to photoinhibition and oxidative stress. Though, more experimental evidence is needed to prove this hypothesis.

Material and Methods

Strains and growth conditions

The *Calothrix* sp. 336/3, *Anabaena* sp. PCC 7120 and the Δ*hupL* mutant of *Anabaena* sp. PCC 7120 were cultivated in 500-ml Erlenmeyer flasks containing 300 ml of either Z8 (full medium) or Z8x medium (without combined nitrogen sources) in the growth chamber at 22°C. The Erlenmeyer flasks were illuminated from two sides with 50 μmol photons m^{-2} s^{-1} cool-daylight fluorescence light (Lumilux T8 15W/865 lamps; Osram) and sparged continuously with sterile air. The *Calothrix* sp. 336/3 was obtained from the University of Helsinki Cyanobacteria Collection (Helsinki, Finland), the wild-type *Anabaena* sp. strain PCC 7120 was obtained from the Pasteur Culture Collection (Paris, France), and the Δ*hupL* mutant of *Anabaena* sp. PCC 7120 was kindly provided by Prof. H. Sakurai. The Δ*hupL* mutant is the strain with interrupted large subunit (accession number AAC79878.1) of [Ni-Fe] uptake hydrogenase (Masukawa et al. 2002) and, thus, affected in H_2 uptake activity in heterocysts that allows high H_2 photoproduction yields.

Cell immobilization and H_2 photoproduction experiments

The cultures for immobilization were washed once in Z8x medium and harvested by centrifugation. The cells were entrapped in thin Ca^{2+}-alginate films according to the protocol developed by Kosourov and Seibert (2009), which was later adapted for immobilization of cyanobacterial cells (Leino et al.

2012). Briefly, the cell pellets were re-suspended in small amount of MQ water and mixed thoroughly with sterile 4% alginate in water (Na-alginate from brown algae, 71238, Sigma-Aldrich, St. Louis, MO), using the formulation ratio: 1 g wet cell weight, 0.5 ml H_2O and 1 ml 4% alginate. The mix was smeared on the top of the template to form a microfilm, which was polymerized by applying 50 mM $CaCl_2$ solution. For precise comparison, the cells were entrapped in alginate microfilms even for the time zero point, which represents the cultures grown diazotrophically.

After immobilization, the 3-cm^2 Ca^{2+}-alginate strips were transferred into 23-ml vials containing 5 ml of Z8x medium. To initiate efficient H_2 photoproduction by the strips, the headspace of the vials was purged with Ar for 20 min, and then supplemented with 3 and 6% CO_2. The vials were placed in a growth chamber at 26°C under continuous overhead illumination with cool-white fluorescent lamps (150 μmol photons m^{-2} s^{-1}; Philips Master TL-D T8 15W/840). The gas phase in the headspace of the vials was re-charged every 6th day with a new portion of Ar supplemented with 3 and 6% CO_2.

Extraction, purification and identification of pigments

The Ca^{2+}-alginate matrices with entrapped cells of cyanobacteria were destroyed in 50 mM Na-EDTA solution (pH 7.0). The cells were washed once with Z8x medium by centrifugation. The photosynthetic pigments were extracted twice from the pelleted cells with 100% methanol in the dark cold room at 4°C. The third extraction confirmed the residual quantities of Chl *a* and major carotenoids in the samples. The extracts were combined or processed independently. The cell debris was removed by centrifugation. The supernatants were filtered through a 0.2-μm polytetrafluoroethylene syringe filters. They were not concentrated by drying and transferred immediately into dark amber vials with glass inserts (250 μl volume capacity) for HPLC analysis. In the case of suspension cultures, the same extraction procedure was applied.

Pigments were separated by the high-performance liquid chromatography (HPLC, Agilent 1100 Series, Agilent Technologies, Palo Alto, CA) equipped with a diode array detector and the reverse phase C18 column (4 × 125 mm, 5 μm, LiChroCART, Merck KGaA, Darmstadt, Germany). In the first approach (solvent system 1) followed after the method of Gilmore and Yamamoto (1991), two solvents A and B were applied consecutively at a constant flow rate of 0.5 ml min^{-1}. An isocratic run with solvent A consisted of acetonitrile/methanol/0.1 M Tris-HCl buffer adjusted to pH 8.0 (72:8:3, v/v) for 4 min was followed by a linear gradient of solvent B from 0 to 100% for 15 min. The solvent B

consisted of methanol/hexane (4:1, v/v). After the gradient, the isocratic run of solvent B was applied for another 26 min. The column was re-equilibrated between samples for a minimum of 10 min with solvent A. To compare our results with the described in Takaichi et al. (2005) we used the second separation system (solvent system 2), where the pigments were eluted with methanol/water (9 : 1, v/v) for 20 min and then with 100% methanol at a flow rate of 0.5 ml min^{-1}. In our case, the first separation system gave better peak resolution and, therefore, was used as the main system for pigment separation. The pigments were detected at 440 nm.

Carotenoids and chlorophyll pigments in samples were identified by the retention time and by the peculiarities of absorption and mass spectra in comparison to standard compounds and reported data. The absorption spectra were collected from HPLC runs of at least three independent samples and then normalized. For LC-MS (solvent system 3), the pigments were separated using a high-performance liquid chromatograph mass spectrometer (Agilent 1100 Series LC/MSD Trap XCT Plus, Agilent Technologies, Palo Alto, CA). Methanol extracts were injected into a Luna C8 (2) column (2 × 150 mm, 5 µm, Phenomenex) and eluted 0.15 ml min^{-1} with different gradients of isopropanol + 0.1% formic acid in water at 40°C. Mass spectra were acquired using electrospray ionization in the positive mode. The standards of myxoxanthophyll (ε_{478} 216 L g^{-1} cm^{-1}), zeaxanthin (ε_{450} 254 L g^{-1} cm^{-1}), canthaxanthin (ε_{476} 208 L g^{-1} cm^{-1}), Chl *a,* echinenone (ε_{460} 215 L g^{-1} cm^{-1}) and *β*-carotene (ε_{454} 250 L g^{-1} cm^{-1}) were purchased from DHI Lab Products (Hørsholm, Denmark) and used for quantification of identified carotenoids. For quantitative analysis, the molar extinction coefficients of each carotenoid group were assumed to be the same: 2-hydroxymyxol 2'-methylpentoside, myxol 2'-methylpentoside, 4-ketomyxol 2'-fucoside and myxol 2'-fucoside (group 1); nostoxanthin, caloxanthin and zeaxanthin (group 2); 3'-hydroxyechinenone and echinenone (group 3), canthaxanthin (group 4) and *β*-carotenes (group 5). The level of Chl *a* in 100% methanol extracts was confirmed spectrophotometrically at 665 nm (ε_{665} 12.9447 L mg^{-1} cm^{-1}, Ritchie 2006).

Absorbance measurements with cell suspensions

In situ suspension absorbance spectra (370–750 nm) were obtained in the 8 mL integrating cavity of the OLIS CLARiTY 17 UV/VIS/NIR spectrophotometer (On Line Instrument Systems, Inc., Bogart, GA, USA). Raw apparent absorbance data were converted to absorbance values using Fry's method (Fry et al. 1992).

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Disclosures

The authors have no conflicts of interest to declare.

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Table 1. Composition of carotenoids in the wild-type *Anabaena* sp. PCC 7120 and *Calothrix* sp. 336/3 strains placed under different growth conditions. The cultures were grown on the full Z8 medium or on the medium without combined nitrogen sources (Z8x). Carotenoids were extracted from the pellets harvested at the late log phase. Values represent an average of 3 – 5 independent experiments ± standard deviations. Asterisks indicate statistically significant differences between Z8 and Z8x samples for each strain. The group of other carotenoids includes *cis* forms of ketomyxol 2'-fucoside, myxol 2'-fucoside for *Anabaena* sp. PCC 7120, and *cis* forms of myxol 2'- methylpentoside, nostoxanthin and caloxanthin for *Calothrix* sp. 336/3. Blue and red backgrounds show decreased and increased carotenoid shares, respectively.

Carotenoids, %	*Anabaena* sp. PCC 7120		*Calothrix* sp. 336/3	
	Z8, Air	Z8x, Air	Z8, Air	Z8x, Air
2-hydroxymyxol 2'-methylpentoside[a]	-	-	5.6 ± 1.4	2.9 ± 2.4
4-ketomyxol 2'-fucoside	12.4 ± 1.3	14.1 ± 2.5	-	-
myxol 2'-fucoside	19.3 ± 1.0	22.7 ± 6.5	10.7 ± 1.1[b]	6.8 ± 1.2**[b]
nostoxanthin	-	-	5.5 ± 0.4	7.7 ± 2.7*
adonixanthin	-	-	0.7 ± 0.3	0.6 ± 0.1
caloxanthin	-	-	11.5 ± 1.0	13.2 ± 3.2
zeaxanthin	1.0 ± 0.0	*traces***	10.2 ± 0.4	14.1 ± 1.6***
3'-hydroxyechinenone	1.0 ± 0.1	0.6 ± 0.6	3.6 ± 0.3	2.1 ± 0.9**
canthaxanthin	5.1 ± 0.3	4.1 ± 1.1	-	-
echinenone	15.7 ± 1.9	27.7 ± 6.6*	14.7 ± 0.8	20.1 ±3.5**
β-carotene	36.4 ± 0.8	20.2 ± 10.1*	36.0 ± 2.2	27.0 ± 7.5**
9-*cis*-*β*-carotene	4.0 ± 0.4	5.5 ± 1.4	0	3.2 ± 0.3***
13-*cis*-*β*-carotene	2.1 ± 0.1	1.7 ± 0.4	1.6 ± 0.3	1.3 ± 0.4
Other carotenoids	~3	~4	0	~0.9
Carotenoids / Chl *a*	0.17	0.23	0.22	0.24

*, P < 0.05; **, P < 0.01; ***, P < 0.001

[a]fucoside, rhamnoside or chinovoside

[b]myxol 2'-methylpentoside (fucoside, rhamnoside or chinovoside)

Table 2. Pigments composition of the Δ*hupL* mutant of *Anabaena* sp. PCC 7120 and the *Calothrix* sp. 336/3 under diazotrophic growth condition (Z8x, + N_2) and after long-term H_2 photoproduction (Z8x, -N_2). The experiments were done with cyanobacteria entrapped in thin Ca^{2+}-alginate films. The pigments were extracted in the beginning, in the middle and in the end of H_2 photoproduction stage. Values represent an average of 3-6 independent experiments ± standard deviations. Asterisks indicate statistically significant differences between Z8x, + N_2 and Z8x, -N_2 samples. Blue and red backgrounds show decreased and increased carotenoid shares, respectively.

Carotenoids, %	Δ*hupL* (*Anabaena* sp. PCC 7120)			*Calothrix* sp. 336/3			
	Growth stage Z8x, +N_2	H_2 photoproduction Z8x, -N_2		Growth stage Z8x, +N_2	H_2 photoproduction Z8x, -N_2		
	Air	Ar + 3% CO_2 780 h	Ar + 6% CO_2 780 h	Air	Ar + 3% CO_2 340 h	Ar + 6% CO_2 340 h	Ar + 6% CO_2 600 h
2-hydroxymyxol 2'-methylpentoside[a]	-	-	-	2.8 ± 0.1	4.3 ± 0.4	4.6 ± 1.3	2.6 ± 0.5
4-ketomyxol 2'-fucoside	13.5 ± 3.6	39 ± 5.8**	41.0 ± 2.0***	-	-	-	-
myxol 2'-fucoside	32.0 ± 3.1	26.7 ± 4.7	29.7 ± 2.5	7.0 ± 0.3[b]	29.6 ± 1.7***[b]	24.7 ± 2.0**[b]	16.0 ± 4.7*[b]
nostoxanthin	-	-	-	9.3 ± 0.1	4.7 ± 0.4*	7.7 ± 1.0	9.6 ± 0.9
adonixanthin	-	-	-	0.6 ± 0.02	0.8 ± 0.1	1.1 ± 0.1	0.7 ± 0.1
caloxanthin	-	-	-	14.7 ± 0.7	8.8 ± 0.6***	12.7 ± 0.7**	14.0 ± 0.4
zeaxanthin	*traces*	0.7 ± 0.03***	0.7 ± 0.05**	14.6 ± 0.9	12.9 ± 1.5	20.7 ± 3.8	15.0 ± 0.8
3'-hydroxyechinenone	0.4 ± 0.2	1.3 ± 0.3*	1.1 ± 0.07**	1.4 ± 0.1	2.3 ± 0.2*	2.0 ± 0.1*	2.3 ± 0.4*
canthaxanthin	3.4 ± 0.5	4.3 ± 1.2	4.1 ± 0.3	*traces*	*traces*	*traces*	*traces*
echinenone	28.8 ± 3.9	14.3 ± 6.4*	12.1 ± 2.5***	19.7 ± 0.7	9.6 ± 0.3**	4.1 ± 0.4**	9.8 ± 1.7**
β-carotene	12.7 ± 3.2	7.1 ± 1.4*	3.2 ± 1.7*	24.2 ± 1.8	20.1 ± 1.1*	16.5 ± 0.5**	22.5 ± 5.0
9-*cis*-*β*-carotene	4.5 ± 0.4	1.9 ± 1.6	2.2 ± 0.5**	3.1 ± 0.4	3.2 ± 0.2	3.2 ± 0.6	3.7 ± 0.4
13-*cis*-*β*-carotene	0.6 ± 0.4	1.5 ± 0.2*	0.9 ± 0.3	1.1 ± 0.4	1.6 ± 0.3	0.7 ± 0.8	0.9 ± 0.2
Other carotenoids	3.6 ± 0.9	3.3 ± 1.2	5.0 ± 0.7	1.4 ± 0.4	2. 2 ± 0.1	2.1 ± 0.1	2.8 ± 1.9
Total carotenoids, mg m^{-2}	28.8 ± 13.0	16.7 ± 5.5	18.8 ± 1.7	58.1 ± 1.7	56.4 ± 15.8	42.5 ± 0.9	123.9 ± 18.6***
Chl *a*, mg m^{-2}	100.1 ± 20.6	33.8 ± 18.6**	35.3 ± 6.8**	234.4 ± 45.6	88.2 ± 39.7***	47.8 ± 8.2***	295.4 ± 49.2
Carotenoids / Chl *a*	0.29	0.50	0.53	0.25	0.64	0.89	0.42

*, P < 0.05; **, P < 0.01; ***, P < 0.001

[a]fucoside, rhamnoside or chinovoside

[b]myxol 2'-methylpentoside (fucoside, rhamnoside or chinovoside)

Table 3. Presumed genes and enzymes involved in carotenoid biosynthesis pathways in *Calothrix* sp. 336/3.

Gene	Enzyme	Accession number	Query sequence for BLASTP*	Identity, %	E-value
crtE	geranylgeranyl diphosphate synthase	WP_035157793.1	*Thermosynechococcus elongates,* BAA82613.1[1]	65	3e-151
crtB	phytoene synthase	WP_035154371.1	*Synechocystis* sp. PCC 6803, Slr1255[2]	70	6e-160
			Synechococcus sp. PCC 7942, Synpcc7942_1984[3]	67	5e-156
crtP	15-*cis*-phytoene desaturase	WP_035154373.1	*Synechococcus* sp. PCC 7942, Synpcc7942_1983[3]	69	0.0
			Synechocystis sp. PCC 6803, Slr1254[4]	73	0.0
crtQ	*β*-carotene desaturase	WP_035156290.1	*Synechocystis* sp. PCC 6803, Slr0940[5]	75	0.0
crtH	*cis*-carotene isomerase	WP_035155378.1	*Synechocystis* sp. PCC 6803, Sll0033[6,7]	74	0.0
cruA	lycopene cyclase**	WP_035155193.1	*Synechococcus* sp. PCC 7002, Synpcc7002_A2153[8]	64	0.0
cruP		WP_035157658.1	*Synechococcus* sp. PCC 7002, Synpcc7002_A0043[8]	55	0.0
cruF	1',2'-hydratase	WP_035148913.1	*Synechococcus* sp. PCC 7002, Synpcc7002_A2032[9,10]	58	5e-108
cruG	2'-O-glycosyltransferase	WP_035148914.1	*Synechococcus* sp. PCC 7002, Synpcc7002_A2031[9,10]	61	3e-154
crtD	3′,4′-desaturase	WP_035153491.1	*Synechocystis* sp. PCC 6803, Slr1293[11]	68	0.0
wcaG	GDP-fucose synthase	WP_035149078.1	*Anabaena* sp. PCC 7120, All4826[12]	86	0.0
			Synechocystis sp. PCC 6803, Sll1213[12,13]	79	0.0
crtR	*β*-carotene hydroxylase	WP_035154039.1	*Anabaena* sp. PCC 7120, Alr4009[12]	78	9e-163
			Synechocystis sp. PCC 6803, Sll1468[14]	71	1e-156
crtO	*β*-carotene ketolase	WP_035158015.1	*Anabaena* sp. PCC 7120, All3744[15]	84	0.0
			Synechocystis sp. PCC 6803, Slr0088[16]	64	0.0
			Gloeobacter violaceus PCC 7421, Gll0394[17]	83	0.0
crtG	2,2´-*β*-hydroxylase	WP_035157760.1	*Synechococcus* sp. PCC 7942, Synpcc7942_0680[9]	52	2e-84
			Thermosynechococcus elongatus BP-1, Tlr1917[18]	36	9e-47

*Only the enzymes with confirmed functions were selected for the query sequences.

**Orthologues have been found in *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803, but the lycopene cyclase activity has not been detected (Mochimaru et al. 2008).

References: 1, (Ohto et al. 1999); 2, (Martínez-Férez et al. 1994); 3, (Chamovitz et al. 1992); 4, (Martínez-Férez et al. 1992); 5, (Breitenbach et al. 1998); 6, (Masamoto et al. 2001); 7, (Breitenbach et al. 2001); 8, (Maresca et al. 2007); 9, (Graham 2008); 10, (Graham et al. 2009); 11, (Mohamed and Vermaas 2004); 12, (Mochimaru et al. 2008); 13, (Mohamed et al. 2005); 14, (Masamoto et al. 1998); 15, (Mochimaru et al. 2005); 16, (Fernández-González et al. 1997); 17, (Steiger et al. 2005); 18, (Iwai et al. 2008).

Figure legends

Fig. 1 HPLC elution profiles of the pigments extracted from *Anabaena* sp. PCC 7120 (A) and *Calothrix* sp. 336/3 (B) grown under diazotrophic conditions. The elution was performed with the solvent system 1 as described in Material and Methods. Carotenoids of *Anabaena* sp. PCC 7120: 1a – 4-ketomyxol 2'-fucoside, 1a' – *cis* form of ketomyxol 2'-fucoside, 2a – myxol 2'-fucoside, 2a' – *cis* form of myxol 2'-fucoside, 3a – 3'-hydroxyechinenone, 4a – canthaxanthin, 5a – echinenone, 6a – *β*-carotene, 6a' – 9-*cis*-*β*-carotene, 6a'' – 13-*cis*-*β*-carotene. Carotenoids of *Calothrix sp.* 336/3: 1b – 2-hydroxymyxol 2'-methylpentoside, 2b – myxol 2'-methylpentoside, 2b' – *cis* form of myxol 2'-methylpentoside, 3b – nostoxanthin, 3b' – *cis* form of nostoxanthin, 4b – adonixanthin, 5b – caloxanthin, 6b – zeaxanthin, 6b' – *cis* form of caloxanthin, 7b – 3'-hydroxyechinenone, 8b – echinenone, 9b – *β*-carotene, 9b' – 9-*cis*-*β*-carotene, 9b'' – 13-*cis*-*β*-carotene. Both strains also contained chlorophyll *a* (Chl *a*), two chlorophyll *a* allomers (Chl *a* allo), one chlorophyll *a* epimer (Chl *a'*) and a trace of pheophytine eluted at 21.2 min.

Fig. 2 (A) *In situ* absorption spectra of *Calothrix* sp. 336/3 and *Anabaena* sp. PCC 7120 cultures grown on full medium (Z8) and under diazotrophic condition (Z8x). The spectra were collected using the OLIS CLARiTY 17 spectrophotometer that allows correction for cellular scattering. The absorbance at 750 nm was subtracted and spectra were normalized to the 680 nm peak. PC – phycocyanin and PE – phycoerythrin peaks, respectively. (B) The average spectra of total carotenoids plus Chl *a* (green line) and total carotenoids (red line). The individual spectra of carotenoids and Chl *a* were collected by HPLC, combined and red-shifted by 15 nm (for convenience of comparison with *in situ* spectra).

Fig. 3 Proposed carotenogenesis pathways and responsible enzymes in *Calothrix* sp. 336/3. The major carotenoids accumulated in *Calothrix* sp. 336/3 are bolded.

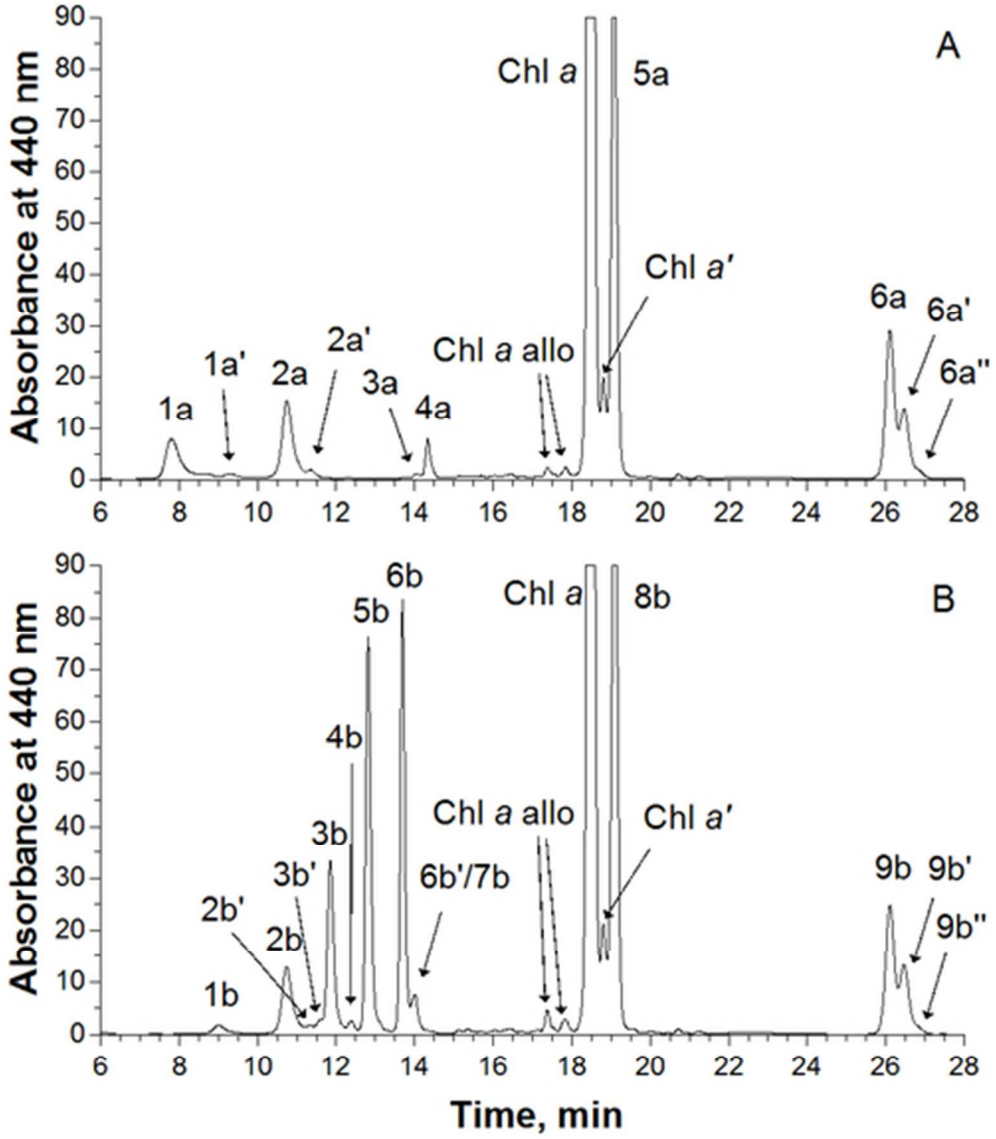

Fig. 1 HPLC elution profiles of the pigments extracted from Anabaena sp. PCC 7120 (A) and Calothrix sp. 336/3 (B) grown under diazotrophic conditions. The elution was performed with the solvent system 1 as described in Material and Methods. Carotenoids of Anabaena sp. PCC 7120: 1a – 4-ketomyxol 2'-fucoside, 1a´ – cis form of ketomyxol 2'-fucoside, 2a – myxol 2'-fucoside, 2a´ – cis form of myxol 2'-fucoside, 3a – 3'-hydroxyechinenone, 4a – canthaxanthin, 5a – echinenone, 6a – β-carotene, 6a´ – 9-cis-β-carotene, 6a´´ – 13-cis-β-carotene. Carotenoids of Calothrix sp. 336/3: 1b – 2-hydroxymyxol 2'-methylpentoside, 2b – myxol 2'-methylpentoside, 2b´ – cis form of myxol 2'-methylpentoside, 3b – nostoxanthin, 3b´ – cis form of nostoxanthin, 4b – adonixanthin, 5b – caloxanthin, 6b – zeaxanthin, 6b´ – cis form of caloxanthin, 7b – 3'-hydroxyechinenone, 8b – echinenone, 9b – β-carotene, 9b´ – 9-cis-β-carotene, 9b´´ – 13-cis-β-carotene. Both strains also contained chlorophyll a (Chl a), two chlorophyll a allomers (Chl a allo), one chlorophyll a epimer (Chl a´) and a trace of pheophytine eluted at 21.2 min.

42x50mm (300 x 300 DPI)

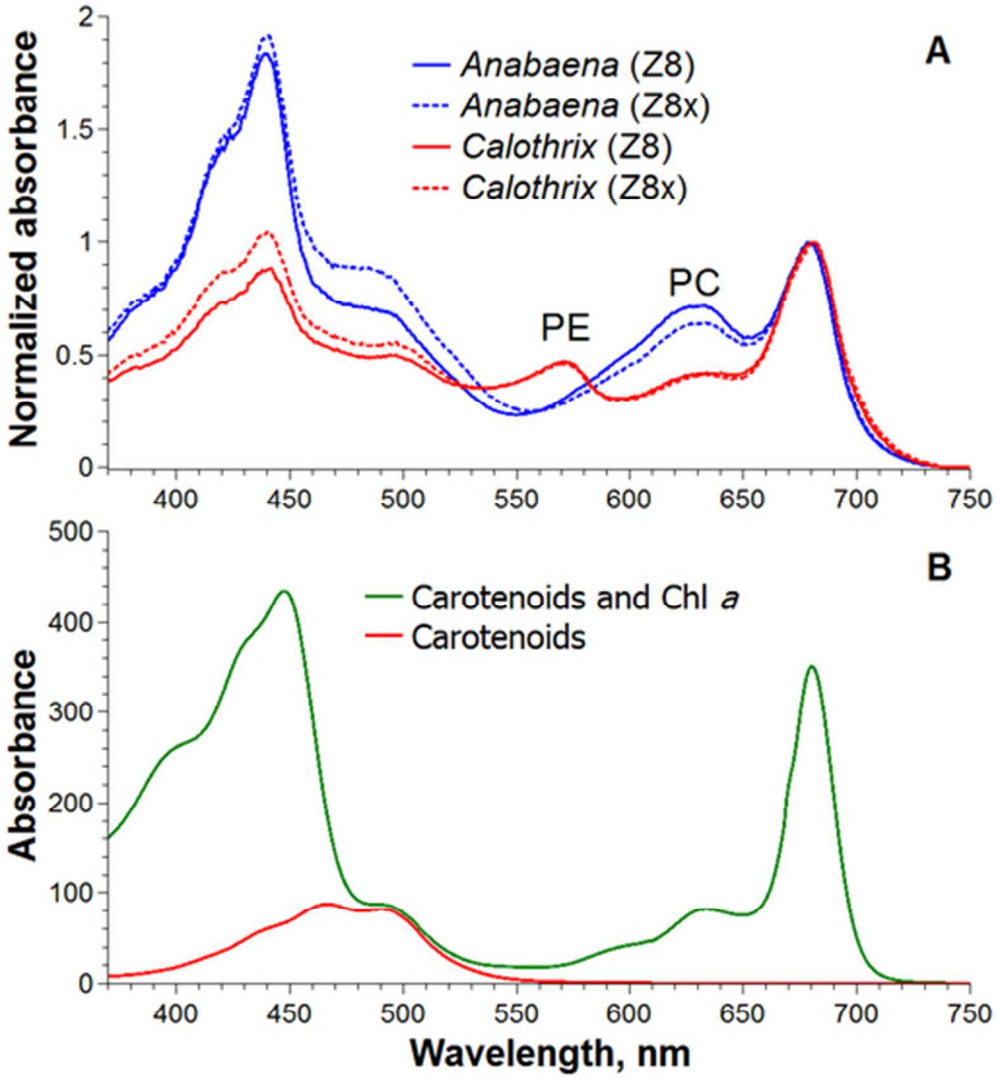

Fig. 2 (A) In situ absorption spectra of Calothrix sp. 336/3 and Anabaena sp. PCC 7120 cultures grown on full medium (Z8) and under diazotrophic condition (Z8x). The spectra were collected using the OLIS CLARiTY 17 spectrophotometer that allows correction for cellular scattering. The absorbance at 750 nm was subtracted and spectra were normalized to the 680 nm peak. PC – phycocyanin and PE – phycoerythrin peaks, respectively. (B) The average spectra of total carotenoids plus Chl a (green line) and total carotenoids (red line). The individual spectra of carotenoids and Chl a were collected by HPLC, combined and red-shifted by 15 nm (for convenience of comparison with in situ spectra).

43x48mm (300 x 300 DPI)

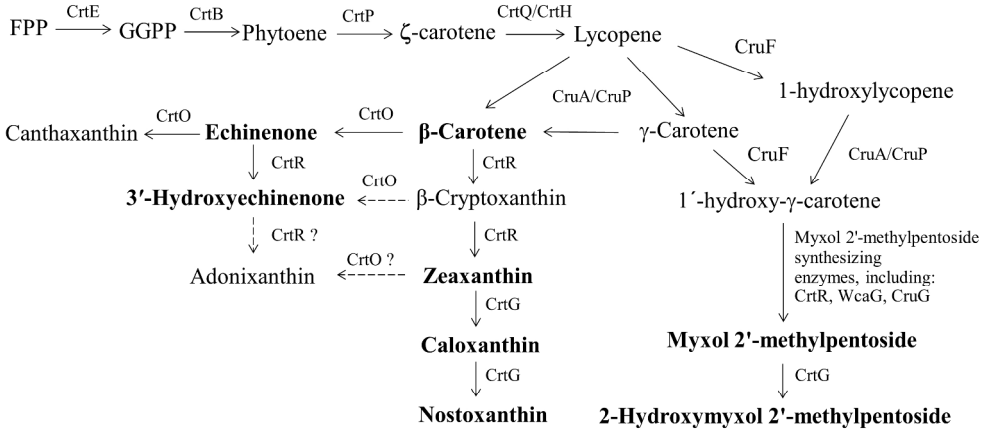

Fig. 3 Proposed carotenogenesis pathways and responsible enzymes in Calothrix sp. 336/3. The major carotenoids accumulated in Calothrix sp. 336/3 are bolded.

279x209mm (300 x 300 DPI)