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Effect of growth temperature on biosynthesis and accumulation of carotenoids in cyanobacterium *Anabaena* sp. PCC 7120 under diazotrophic conditions

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ABSTRACT

Carotenoid composition has been studied in mesophilic, nitrogen-fixing cyanobacterium *Anabaena* sp. PCC7120 grown photoautotrophically, under diazotrophic conditions at four different temperatures (15 °C, 23 °C, 30 °C and 37 °C). The relative accumulation of chlorophyll, carotenoids and proteins was the highest at temperature of 23 °C. At a suboptimal temperature (15 °C) β-carotene was the dominant carotenoid compound, whereas the increase in temperature caused ketocarotenoids (echinenone, canthaxanthin, keto-myxoxanthophyll) to accumulate. A significant increase in the accumulation of phytoene synthase (CrtB) transcript was observed at both extreme growth temperatures (15 °C and 37 °C). The relative amount of β-carotene ketolase (CrtW) transcript directly corresponded to the accumulation of its product (keto-myxoxanthophyll) with a maximum at 30 °C and a profound decrease at 37 °C, whereas the transcription level of β-carotene ketolase (CrtO) was significantly decreased only at a suboptimal temperature (15 °C). These results show that temperature affects the functioning of the carotenoid biosynthesis pathway in *Anabaena* cells under photoautotrophic growth. Specifically, the balance between β-carotene and ketocarotenoids is altered according to temperature conditions. The transcriptional regulation of genes encoding enzymes active both at the early (CrtB) and the final steps (CrtO, CrtW) of the carotenoid biosynthetic pathway may participate in the acclimation mechanism of cyanobacteria to low and high temperatures.

1. Introduction

Carotenoids, an important class of natural pigments, are widespread among both prokaryotic and eukaryotic organisms. In photoautotrophs they play an accessory role in light harvesting and serve as protectants of cells against photo-oxidative damage (Frank, 1999). Protection against reactive oxygen species, by quenching singlet oxygen and excited states of photosensitizing molecules, as well as by scavenging free radicals, is one of the main biological functions of carotenoids (reviewed by Domonkos et al., 2013).

Anabaena (*Nostoc*) sp. PCC7120 (further: *Anabaena* 7120), a filamentous, mesophilic, nitrogen-fixing cyanobacterium from paddy fields is a model organism commonly used in biochemical and genetic studies. The *Anabaena* genus has recently been recognized as a potential source of secondary metabolites of pharmacological and biotechnological importance (Prasanna et al., 2006).

The carotenoid biosynthesis pathway in *Anabaena* 7120 has been extensively studied (Takaichi et al., 2005; Mochimaru et al., 2008). Genetic and omics-based approaches have made it easier to identify those genes responsible for the key enzymes involved in the biosynthesis of carotenoids (Liang et al., 2006; Takaichi and Mochimaru, 2007). As in other cyanobacteria, the initial step in carotenoid biosynthesis includes a condensation of farnesyl pyrophosphate (FPP) into geranylgeranyl pyrophosphate (GGPP) catalyzed by geranylgeranyl pyrophosphate synthase (crtE). Subsequently, the first colorless carotene – phytoene (C40) is synthesized by condensing of two molecules of geranylgeranyl pyrophosphate (GGPP, C20). This process is catalyzed by phytoene synthase encoded by the *crtB* gene (Chamovitz et al., 1992;

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Sandmann and Misawa, 1992; Hoshino et al., 1993; Martínez-Férez et al., 1994). Further, the formation of all-trans-lycopene from phytoene requires four sequential desaturation steps and isomerisations, catalyzed by phytoene desaturase (crtP), zeta-carotene desaturase (crtQ) and cis-to-trans carotenoid isomerase (crtH). In contrast to other oxygenic phototrophs, *Anabaena* 7120 contains a ζ-carotene desaturase (CrtQa) that can utilize both all-trans- and 9-9′-di-cis- ζ -carotene as substrates and produce all-trans-lycopene (Linden et al., 1994). The subsequent cyclization of isoprene groups of lycopene and formation of ionone rings is catalyzed by lycopene cyclase(s). As homologs of lycopene cyclases known from *Synechococcus* 7002 (CruA/CruP) showed no lycopene cyclase activities in both *Anabaena* 7120 and *Synechocystis* PCC6803, it is assumed that these species may possess a different type of lycopene cyclase (see: Domonkos et al., 2013).

Lycopene cyclization is a branching point that leads to differentiation of hydrocarbons with cyclic ionone rings at one (γ-carotene) and at two ends (β-carotene). These compounds are further enzymatically modified to generate xantophylls - oxygenated carotenes with ionone rings complemented by epoxy, keto or hydroxy functional groups. In particular, β-carotene hydroxylase (CrtR) catalyzes the synthesis of myxol, which is a precursor of carotenoid glycosides – myxoxanthophylls formed after fucose binding by 2′-O-glycosyltransferase (Takaichi and Mochimaru, 2007). Unlike the vast majority of cyanobacteria, *Anabaena* has two distinct β-carotene ketolases: CrtW and CrtO with different substrate specificities (Mochimaru et al., 2005). CrtO is responsible for the synthesis of echinenone and canthaxanthin from β-carotene, whereas CrtW mainly catalyzes the synthesis of keto-myxoxanthophyll from myxoxanthophyll (Mochimaru et al., 2005; Makino et al., 2008; Breitenbach et al., 2013). A simplified scheme of the carotenoid biosynthesis pathway in *Anabaena* 7120 is presented in supplementary Fig. S1.

During photoautotrophic growth under diazotrophic conditions, the carotenoid pool of *Anabaena* 7120 consists of five main species of pigments: β-carotene, echinenone, canthaxanthin, myxoxanthophyll ((3R,20S)-myxol 20-fucoside) and keto-myxoxanthophyll ((3S,20S)-4-ketomyxol 20-fucoside) (Takaichi et al., 2006). Whereas β-carotene is an essential structural component of photosynthetic complexes in thylakoid membranes, xanthophylls play mainly protective function. In particular, hydroxy derivatives could inactivate peroxy radicals, while keto derivatives are effective quenchers and show higher resistance against peroxy radicals and photooxidation (reviewed by Domonkos et al., 2013).

Glycosylated derivatives (myxoxanthophylls) are deemed to participate in the structural stabilization of cyanobacterial membranes (Várkonyi et al., 2002; Mohamed et al., 2005).

It was shown that in cyanobacteria the activity of the carotenoid biosynthesis pathway and accumulation of specific carotenoids can be modulated by biotic and abiotic factors. Particularly, in several nitrogen-fixing, heterocystous cyanobacteria species, the analysis of the action spectra for N_2 fixation demonstrated the presence of echinenone and β-carotene in heterocysts (Staal et al., 2003). Based on the relative increase of echinenone/β-carotene ratio in *Anabaena* 7120 as observed upon a shift to diazotrophic conditions, Kosourov et al. (2016) postulated a role of this ketocarotenoid in nitrogen fixation. Xanthophylls, including echinenone, canthaxanthin and myxoxanthophyll were shown to be involved in photoprotection during acclimation to high light intensities (Muramatsu and Hihara, 2012). Enhanced accumulation of myxoxanthophyll and echinenone has been observed in *Synechocystis* PCC 6803 upon suppression of photosynthetic activity as generated upon phosphatidylglycerol depletion (Domonkos et al., 2009). Myxoxanthophyll was found to be preferentially synthesized and accumulated in *Synechocystis* at high light intensities (Steiger et al., 1999; Mohamed and Vermaas, 2004). It plays a protective role against both light and low temperature stress (Steiger et al., 1999; Várkonyi et al., 2002). Recently, overaccumulation of myxoxanthophyll and echinenone has been found to compensate stress effects as induced in *Synechocystis* cells by photosystem I monomerization at elevated temperatures (Kłodawska et al., 2015).

Temperature is one of the major abiotic factors that limit the growth of cyanobacteria in a natural environment (Tandeau de Marsac and Houmard, 1993). Temperature changes in the environment may induce damage in living organisms, therefore photoautotrophic species need to be able to acclimate to temperature conditions (Berry and Bjorkman, 1980). Under standardized conditions, *Anabaena* 7120 is cultivated at temperature around 27–30 °C. It tolerates a wide range of light quality with fluence rates not exceeding 160 µmol photons $m^{-2}s^{-1}$ (see e.g. Prasanna et al., 2006; Guoce et al., 2011; Katoh, 2012; Videau and Cozy, 2019).

Our preliminary data indicated that temperature changes may affect the content of ketocarotenoids in diazotrophically grown *Anabaena* 7120 (Kłodawska et al., 2016). In this study, we show that the carotenoid composition in *Anabaena* 7120 cells can be modulated by the growth temperature. At suboptimal (low) temperatures β-carotene is the dominant carotenoid species, whereas an increase in temperature causes ketocarotenoids to accumulate. Temperature-induced changes in the carotenoid composition may correlate, at least partially, with the expression levels of *crtB*, *crtO* and *crtW* genes which encode three key enzymes involved in the biosynthesis of carotenoids in *Anabaena*.

2. Materials and methods

2.1. Organism and growth conditions

Anabaena PCC 7120 (UTEX 2576) was grown photoautotrophically in BG11 (-N) medium (Rippka et al., 1979) supplemented with 5 mM HEPES-NaOH (pH 7.5) under constant illumination, supplied by daylight fluorescent lamps (Philips TL-D 18 W/33–640), with a fluence rate of 60 µmol photons $m^{-2}s^{-1}$. The cultures (usually 100 ml in 500 ml Erlenmayer flasks) were shaken on a rotatory shaker at 150 rpm. The growth of cyanobacteria at four temperatures (15 °C, 23 °C, 30 °C and 37 °C) was monitored over twelve days based on the OD_{750} as measured with Jasco-V650 spectrophotometer.

2.2. Analysis of pigment composition

For pigment analysis cyanobacteria were cultivated for 7 days (starting OD_{750} = 0.2; approx. 1×10^7 cells/ ml) and samples containing at least 5×10^8 cells were harvested by centrifugation (10000 g, 5 min) in late exponential phase. The pigment composition was analyzed essentially as described by (Domonkos et al., 2009; Kłodawska et al., 2015). The 1200 series chromatography system (Agilent Technologies) equipped with a diode array spectrophotometric detector G1315D and an Eclipse Plus C18 reversed phase column (4.6 x 150 mm, 3.5 µm particle size, Agilent) was used. The pigments were extracted in acetone: methanol (7 : 2, v/v) solvent, evaporated under gaseous nitrogen and loaded onto a column equilibrated with Solvent A (acetonitrile : water : triethylamine, 9 : 1 : 0.01, v/v/v.) in HPLC-grade ethanol. The samples were eluted from the column with Solvent B (ethyl acetate, 100%) by a three-step gradient (0–40% B for 10 min, 40–60% B for 10 min, 60–100% B for 3 min, followed by a 2 min isocratic hold at 100% B), with a constant flow rate of 1.5 ml min^{-1}. The absorption spectra of the eluate (380–800 nm) were recorded every 0.2 s. Carotenoids were identified on the basis of the absorption spectra and retention times. The relative content of each pigment was estimated by comparing the peak areas on chromatograms recorded at 440 and 460 nm. The concentrations of carotenoid species were calculated according to Beer–Lambert's Law using specific extinction coefficients at 440 nm (Mantoura and Llewellyn, 1983) and 460 nm for echinenone and canthaxanthin (Britton, 1995). The values are means ± SD calculated from at least three independent experiments.

2.3. Determination of pigment concentrations

The total Chl and carotenoid contents were measured after extraction in a 90% (v/v) methanol extract from cells, using Jasco-V650 spectrophotometer, according to (Lichtenthaler, 1987). Measurements were performed in triplicates on the seventh day after inoculation.

2.4. Protein determination

Protein concentrations in whole cells were measured in accordance with the micro Lowry method using bovine serum albumin as a standard, according to (Yeet Yeang et al., 1998). Measurements were performed in triplicates on the seventh day after inoculation.

2.5. Steady-state RNA level analysis

For RNA analysis 100 ml *Anabaena* cultures were inoculated in triplicates. OD_{750} was adjusted to about 0.05 (approx. 2.5×10^6 cells per mL). RNA from two-day-old cultures (at least 2.7×10^8 cells per sample) was isolated with a Spectrum Plant Total Kit (Sigma-Aldrich) and digested by DNase I (Thermo Scientific) during purification on the column. The RNA concentration was determined using a NanoDrop Lite spectrophotometer (Thermo Scientific). The RNA integrity was checked using electrophoresis on 2% agarose gel. Reverse transcription was prepared with the RevertAid Reverse Transcriptase Kit (Thermo Scientific) using random hexamer primers. Quantitative reverse-transcription PCR was carried out with Real Time PCR Mix A (A&A Biotechnology, Gdynia, Poland) and a thermal cycler (Eco Real-Time PCR System, Illumina). cDNA corresponding to 50 ng of RNA was used in a single reaction and all reactions were run in duplicate. The primer sequences for *crtB*, *crtO* and *crtW* were designed based on known *Anabaena* coding sequences (*alr1833*, *all3744* and *alr3189*, respectively), using Primer- BLAST (Ye et al., 2012). The primer sequences were as follows: crtB_Forward: ATCCGCCATTACTACGCCAG, crtB_Reverse: CCCGAAAGGGCTGAATGTCT, crtO_Forward: CTGCACCACCAG GAACAGAA, crtO_Reverse: GTGCAGGACATAACGGGCTA, crtW_Forward: CGCGCAGTATCCCATTACCT and crtW_Reverse: CCACCAAGGA AGTTGAGGGT. The primers for reference genes (*rrn16Sa.b.c.d.*, *secA* and *rnpA*) were as previously described in (Pinto et al., 2012). The results were calculated using a procedure based on geometric mean of expression of multiple selected reference genes as a normalization factor, according to (Vandesompele et al., 2002). The PCR conditions were as follows: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 10 s at 95 °C, 30 s at 60 °C. The specificity of these products was verified on a dissociation curve at the end of each run. At least three biological repetitions for each growth temperature and three technical repetitions for each sample were applied.

2.6. Statistical analysis

All experiments were conducted at least in three biological repetitions. Each sample was usually measured in three technical repetitions. All statistical analyses were carried out using GraphPad InStat. A one-way ANOVA test was followed by a Tukey-Kramer Multiple Comparisons Test when $P < 0.05$. The asterisks in the figures refer to: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3. Results

3.1. Culture characterization at selected temperatures

Cyanobacteria were grown at four different temperatures: 15 °C, 23 °C, 30 °C and 37 °C starting at the same optical density of 750 nm. The doubling times obtained are presented in Table 1. The cells divided most frequently at 23 °C and 30 °C whereas cell divisions was greatly slowed down at 15 °C and 37 °C. At these temperatures the lag phase was prolonged and the growth rates were significantly decreased (see. Suppl. Fig. S2). The chlorophyll *a* and total carotenoid accumulation per milliliter of culture was measured using the spectrophotometric method (Table 1). Both chlorophyll and carotenoids were present in their highest concentrations in cultures grown at 23 °C (Fig. 1). Cells cultivated at 15 °C showed the lowest concentration of chlorophyll *a* and carotenoids. The protein concentration was highest in cultures cultivated at 23 °C and lowest in cultures cultivated at 37 °C, which gives a higher chlorophyll *a* to protein ratio in cells grown at 37 °C than at 15 °C (Fig. 1).

3.2. Carotenoid composition in Anabaena cells at different growth temperatures

The carotenoid composition and concentration of particular compounds were analyzed using high performance liquid chromatography. The relative content of the individual species was estimated from their peak areas in the chromatograms (Domonkos et al., 2009). Five main carotenoid species in *Anabaena*: myxoxanthophyll, keto-myxoxanthophyll, β-carotene, echinenone and canthaxanthin were identified. In cells cultivated at 15 °C, 23 °C and 30 °C but not at 37 °C minute amounts of myxoxanthophyll forms that differ in terms of their retention times were found (not shown).

As measured using the HPLC method, the amount of total carotenoids per chlorophyll *a* was relatively speaking the highest and not significantly different in cells cultivated at mild growth temperatures, namely at 23 °C and 30 °C (0.481 and 0.469 mg per mg of chlorophyll respectively). Contrary to this, when *Anabaena* cells were cultivated in extreme temperature conditions (15 °C and 37 °C) the accumulation of total carotenoids decreased by more than 50% in comparison to that observed at mild conditions (Fig. 2A).

The accumulation of β-carotene and myxoxanthophyll was relatively high and showed no significant differences in cells grown at 23 °C and 30 °C. Both β-carotene and myxoxanthophyll were accumulated mostly at 23 °C (0.129 and 0.119 mg mg chl^{-1} respectively). The decreases in accumulation of these compounds at extreme temperatures reflected the general tendency as observed for total carotenoids (Fig. 2B, C).

In contrast, the accumulation of ketocarotenoids varied significantly according to temperature. The accumulation of keto-myxoxanthophyll increased according to temperature up to 30 °C (0.140 mg mg chl^{-1}) and dropped significantly in cells cultivated at a high temperature, 37 °C (Fig. 2D). Large quantities of echinenone accumulated in cells grown at 23 °C (0.154 mg mg chl^{-1}) and its relative concentration decreased at higher temperatures (30 °C and 37 °C), while the amount of canthaxanthin was highest in cells grown at 37 °C (0.038 mg mg chl^{-1}) (Fig. 2E, F).

Significant differences in the relative contribution of individual carotenoids to their total pool in cells grown at the temperatures selected were found. At 15 °C β-carotene was the dominant form (ca. 43%), whereas at 23 °C echinenone was the most abundant carotenoid compound (ca. 32%). At 30 °C the ketomyxoxanthophyll content reached almost 30% of cellular carotenoids, while at 37 °C about 70% of the carotenoid pool consisted of keto-derivatives due to high canthaxanthin accumulation (ca. 26%). The quantitative data are summarized in Table 2.

3.3. Gene expression

To evaluate the effect of temperature on the regulation of the activity of the carotenoid biosynthetic pathway, the accumulations of the transcripts of genes that encode the key enzyme in total carotenoid biosynthesis (*crtB*) and genes that encode enzymes directly involved in the biosynthesis of keto-derivatives (*crtO* and *crtW*) were analyzed using qRT-PCR. Fig. 3 shows the relative mRNA contents of *crtB*, *crtO* and *crtW* as normalized to the quantity of mRNAs present in cells at

Table 1
Biochemical characterization of *Anabaena* cultures at different growth temperatures. All measurements were taken in triplicate with three biological repetitions. Values ± SD are given.

Growth temperature	15 °C	23 °C	30 °C	37 °C
Doubling time [h]	46.3 ± 1.6	24.4 ± 1.2	24.8 ± 1.3	78.1 ± 2.1
Number of cells/mL after 7 days	$3.35 \times 10^7 \pm 0.10 \times 10^7$	$6.40 \times 10^7 \pm 0.20 \times 10^7$	$6.30 \times 10^7 \pm 0.35 \times 10^7$	$2.00 \times 10^7 \pm 0.05 \times 10^7$
Total protein in cell suspension [mg ml^{-1}]	3.821 ± 0.101	5.262 ± 0.172	3.921 ± 0.090	3.070 ± 0.105
Chlorophyll *a* per protein [μg mg^{-1}]	0.061 ± 0.002	0.822 ± 0.019	0.587 ± 0.024	0.244 ± 0.010
Carotenoids per protein [μg mg^{-1}]	0.013 ± 0.003	0.396 ± 0.012	0.275 ± 0.014	0.006 ± 0.001

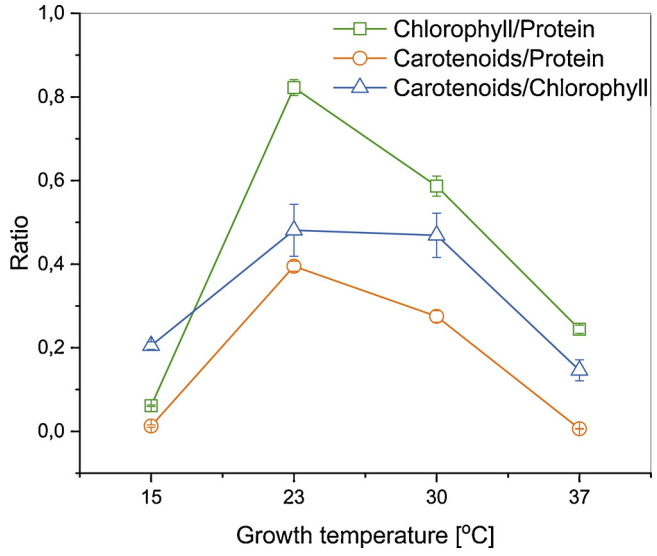

Fig. 1. Relative ratios of photosynthetic pigments to protein and chlorophyll to carotenoid in *Anabaena* cells cultivated at 15 °C, 23 °C, 30 °C and 37 °C for 7 days.

23 °C, assigned as 100%. As can be seen, the expression of *crtB* significantly increased both in cells grown at 37 °C (two-fold) and at 15 °C (ca. 25%) compared to cells grown at 23 °C and 30 °C. The expression of *crtO* gene was downregulated at 15 °C and slightly at 30 °C compared to 23 °C, while at 37 °C the *crtO* mRNA level was comparable to that found at 23 °C. The expression of *crtW* gene increased stepwise from 15 °C to 30 °C and dramatically decreased in cells grown at 37 °C.

4. Discussion and conclusions

Our data show that, among the temperatures investigated, 23 °C and 30 °C are the best ones for *Anabaena* PCC 7120 growth. The doubling time at around 24 h as observed in these temperatures is in agreement with earlier study performed on various *Anabaena* strains of different origin (Prasanna et al., 2006). Interestingly, under our experimental conditions, the cells showed the highest levels of protein and pigment accumulation at 23 °C, as compared to the other temperature conditions tested. This result suggests that *Anabaena* 7120 has a broad range of temperature tolerance (at least between 23–30 °C) and, in this temperature range, the accumulation of proteins, chlorophyll and carotenoids might be regulated, irrespective of cell division rate.

The relative accumulation of total carotenoids as calculated per mg of chlorophyll *a* was highest at 23 and 30 °C. On the contrary, in cells grown both at low (15 °C) and high (37 °C) temperature the total carotenoid content decreased. These results indicate that either the total carotenoid pool is reduced due to alterations caused by temperature or some carotenoid species could be over- and/or underproduced in response to the extreme temperature conditions, as it was observed for *Synechocystis* PCC 6803 (Kłodawska et al., 2015).

In cyanobacteria, the biosynthesis of carotenoids initiated by the formation of a common precursor – phytoene is catalyzed by phytoene synthase encoded by the *crtB* gene. In *Synechocystis*, the elimination of this gene resulted in a carotenoidless phenotype with impaired PS II formation and severe light sensitivity (Sozer et al., 2010). A significant increase in the accumulation of CrtB transcript both at low and high growth temperatures as observed in this study indicates that the activity of the initial steps of the carotenoid biosynthesis pathway might be critical for the functioning of *Anabaena* cells under extreme conditions. The transient induction of the promoter activities of the genes encoding some of carotenogenic enzymes, including *crtB* has also been observed in *Synechocystis* PCC6803 upon a shift to high intensity light (Fernández-González et al., 1998). The fact that overexpression of *crtB* is accompanied by a decrease in total carotenoid accumulation suggests a faster turnover of these compounds at suboptimal growth temperatures.

The composition of carotenoid pigments in cells grown at different temperatures varied significantly. The abundant accumulation of echinenone, as observed under diazotrophic conditions, agreed with earlier observations made for *Anabaena* 7120 (Kosourov et al., 2016). Apart from canthaxanthin all the other carotenoid species were present in cells in their highest amounts at 23 °C and 30 °C, as calculated per mg of chlorophyll *a*. In all photosynthetic organisms, including cyanobacteria, xanthophylls function as protective agents against damage caused by the reactive oxygen species (ROS), which are generated under stress conditions (Hirschberg and Chamovitz, 1994). In particular, in *Synechococcus*, a deficiency of xanthophylls (zeaxanthin, echinenone) led to an increase in ROS and the reactive nitrogen species (RNS) content (Zhu et al., 2010). An elevated accumulation of specific xanthophylls has also been demonstrated in *Synechocystis* PCC 6803 upon biotic stress accompanied by phosphatidylglycerol deficiency (Domonkos et al., 2009) and monomerization of PS I (Kłodawska et al., 2015). Thus, equilibrium shift observed towards an increased accumulation of xanthophylls and specifically ketocarotenoids with temperature elevation suggests that *Anabaena* cells grown at 30 °C and 37 °C are subjected to temperature-induced oxidative stress that requires the enhanced synthesis of protective compounds. Consequently, keto-derivatives accumulated at higher temperatures may act as antioxidants protecting thylakoid membranes from light- and radical-mediated oxidation (Domonkos et al., 2013).

In photosynthetic organisms, temperature down-shift during photoautotrophic growth results in an increase in photosystem II excitation pressure, which reflects the relative reduction state of the PS II (Huner et al., 1998). In consequence, the suppressed photosynthetic electron transport processes may generate deleterious substances such as ROS and other free radicals (Domonkos et al., 2009). Studies in model systems indicated that the initial rates of oxidation in carotenes such as lycopene and β-carotene with artificially generated radicals were significantly higher in comparison to xanthophylls including keto-derivatives (Miller et al., 1996; Woodall et al., 1997). In the filamentous cyanobacterium *Plectonema boryanum* grown at 15 °C and moderate light intensity, β-carotene was the most abundant carotenoid species (Miśkiewicz et al., 2000). Thus, the predominant accumulation of β-carotene in *Anabaena* cells grown at 15 °C suggests that this compound may play a role in photoprotection at low-temperature stress. On the other hand, the lower sensitivity of ketocarotenoids to autooxidation may increase the stability of these pigments at higher temperatures

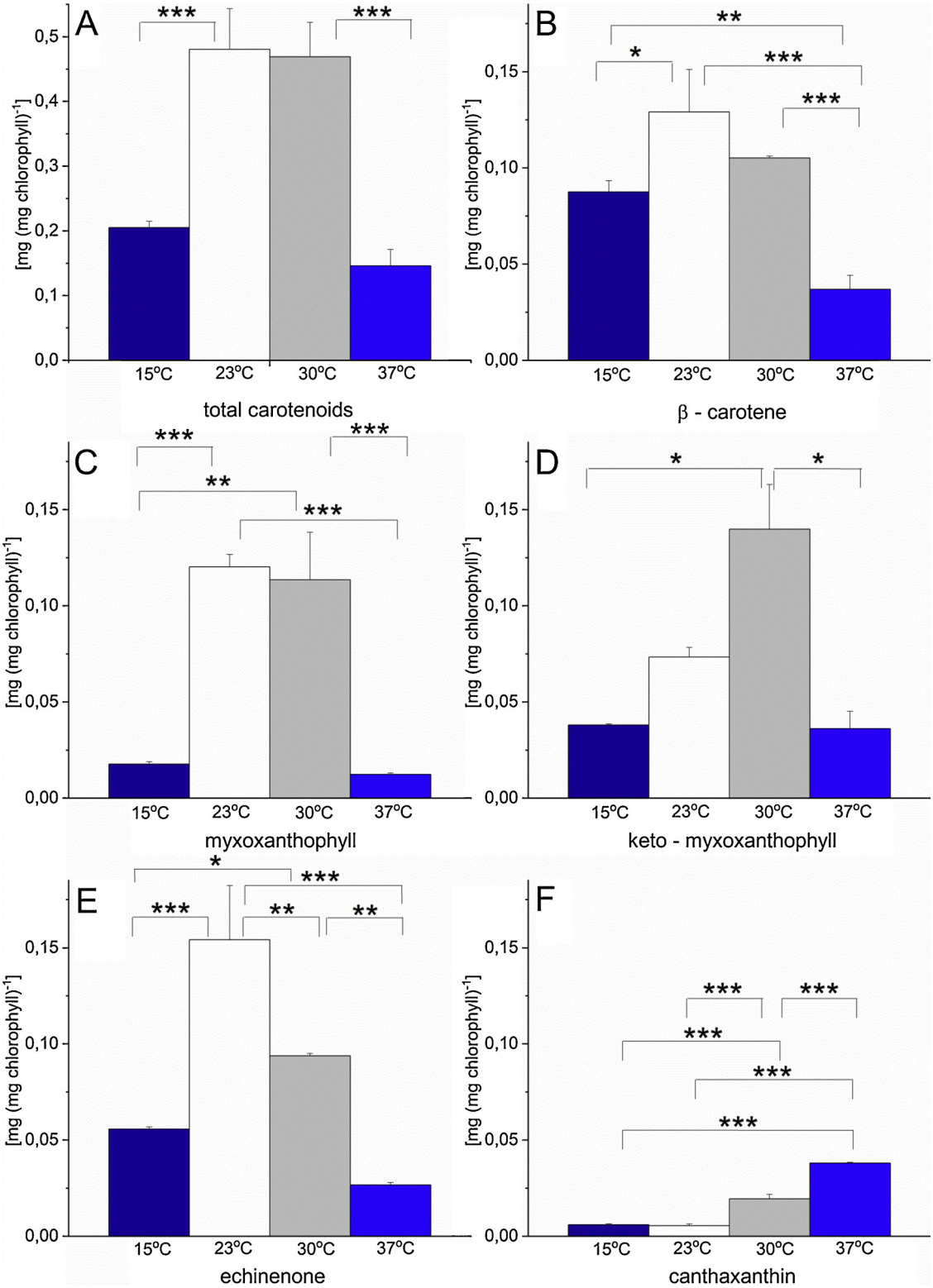

Fig. 2. Relative content of the main carotenoid species – echinenone, canthaxanthin, myxoxanthophyll, ketomyxoxanthophyll and β-carotene, and total carotenoids in *Anabaena* cells cultivated at 15 °C, 23 °C, 30 °C and 37 °C for 7 days. Error bars represent ± SD (n = 3). Note that the scale is different for total carotenoids.

(30 °C and 37 °C) (Pérez-Gálvez and Mínguez-Mosquera, 2001).

According to our data, cellular accumulation of individual keto-carotenoids correlated to some extent with the expression level of genes which encode enzymes that catalyze the synthesis of these pigments. In particular, the comparison of *crtW* mRNA accumulation and keto-myxoxanthophyll accumulation at different growth temperatures as measured by HPLC enables us to argue that the amount of CrtW enzyme transcript found in cells directly corresponds to the amount of pigment per chlorophyll *a*. In the case of the *crtO* gene the problem is more complex because this enzyme catalyzes the synthesis of both echinenone and then canthaxanthin. Echinenone can also be synthesized by CrtW ketolase but to a much smaller extent (Mochimaru et al., 2005).

Table 2
Distribution of main carotenoid species and their relative percentages in *Anabaena* 7120 cells, grown for 7 days at different temperatures, as calculated from HPLC analysis. The values represent the averages ± SD calculated from three independent experiments.

Carotenoid Species	15 °C		23 °C		30 °C		37 °C	
	[mg mg^{-1} of chlorophyll]	%	[mg mg^{-1} of chlorophyll]	%	[mg mg^{-1} of chlorophyll]	%	[mg mg^{-1} of chlorophyll]	%
Total carotenoids	0.205 ± 0.009	100	0.481 ± 0.062	100	0.469 ± 0.053	100	0.146 ± 0.025	100
β-carotene	0.087 ± 0.006	42.7	0.129 ± 0.022	26.86	0.105 ± 0.001	22.42	0.037 ± 0.007	25.26
Myxoxanthophyll	0.017 ± 0.002	8.66	0.119 ± 0.005	24.68	0.111 ± 0.016	23.60	0.009 ± 0.001	5.84
Keto-myxoxanthophyll	0.038 ± 0.001	18.54	0.073 ± 0.006	15.25	0.140 ± 0.023	29.82	0.036 ± 0.009	24.7
Canthaxanthin	0.006 ± 0.001	2.94	0.005 ± 0.001	1.14	0.019 ± 0.002	4.15	0.038 ± 0.001	25.99
Echinenone	0.055 ± 0.001	27.16	0.154 ± 0.028	32.07	0.094 ± 0.001	20.01	0.026 ± 0.001	18.21
Total ketocarotenoids	0.099	48.64	0.233	48.46	0.253	53.98	0.101	68.9

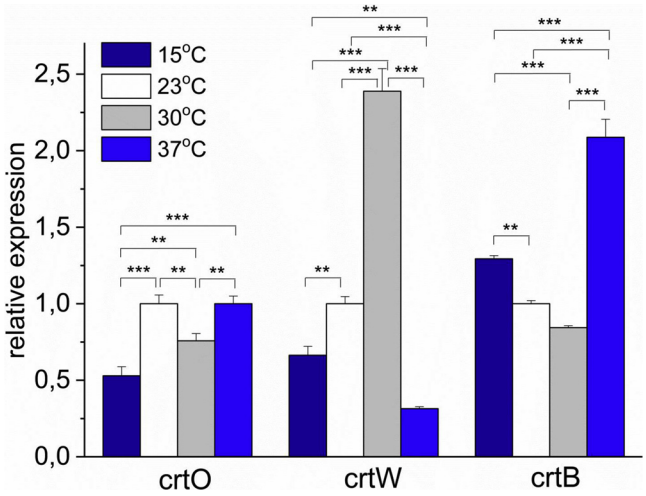

Fig. 3. Relative expression of selected genes encoding carotenoid biosynthesis enzymes in *Anabaena* cells cultivated at 15 °C, 23 °C, 30 °C and 37 °C. Error bars represent ± SD (n = 3).

This fact may explain the abundant accumulation of echinenone at optimal conditions, observed in this study, while both *crtO* and *crtW* were upregulated compared to extreme temperatures. In contrast, at higher growth temperatures i.e. at 30 and 37 °C, the production of diketo-pigment canthaxanthin could be favored over that of echinenone, thus the discrepancy between the level of *crtO* expression and the reduced echinenone content in cells grown at 37 °C, observed in this study, It has recently been shown that in *Anabaena* canthaxanthin is present in cells as bound to Orange Carotenoid Protein (OCP)-related, N-terminal domain-like proteins that confer various functions, including photoprotective phycobilisome fluorescence quenching and singlet oxygen quenching (López-Igual et al., 2016). In another study when heterologous ketolase was introduced to the *Synechococcus* strain resulting in the accumulation of canthaxanthin, the photosynthetic apparatus was better protected against photodamage by excess light and UV-B radiation than the native photosynthetic apparatus in the wild type strain (Albrecht et al., 2001). Our results suggest that stress induced by cultivation at 37 °C might redirect CrtO activity into diketocarotenoid formation as a way of enhancing photoprotective mechanisms. The CrtO expression level comparable to that observed at optimal temperature (23 °C) accompanied by a lower accumulation of products may be explained by the quicker turnover of pigment molecules as used in photoprotection. Additionally, the decrease in β –carotene content observed in cells grown at 37 °C indicates that ketocarotenoids are formed at the expense of this carotenoid, in a similar way to that observed in *Synechocystis* under other stress conditions (Domonkos et al., 2009; Kłodawska et al., 2015).

In conclusion, our results show that temperature is an important abiotic factor that affects the functioning of the carotenoid biosynthesis pathway and probably the turnover of carotenoids in *Anabaena* cells grown photoautotrophically, under diazotrophic conditions. Specifically, the balance between the early stable intermediate of carotenoid biosynthesis (β –carotene) and the final products of the oxidation of carotenes (keto-myxoxanthophyll, canthaxanthin) is fine-tuned according to temperature conditions. In consequence, profound changes in carotenoid composition are observed in *Anabaena* grown at different temperatures. The transcriptional regulation of genes encoding enzymes active both at the early (CrtB) and at the final steps (CrtO, CrtW) of the carotenoid biosynthetic pathway might contribute to the mechanism of the acclimation of carotenoid metabolism in cyanobacteria to low and high temperatures.

5. Declarations

Declarations of interest: none. The authors agree to authorship and submission of the manuscript for peer review.

6. Author contributions

PM conceptualized and designed the study. KK, AB and MT conducted experiments. PM, PŻ, KK performed analysis and interpretation of data. PM and KK wrote the original manuscript. PF and PM critically revised and finalized the article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.05.003.

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