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Full Paper

Astaxanthin production in a model cyanobacterium *Synechocystis* sp. PCC 6803

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Heterologous production of a useful carotenoid astaxanthin was achieved in a cyanobacterium *Synechocystis* sp. PCC 6803 with the aid of marine bacterial genes. Astaxanthin and its intermediates emerged at high levels, whereas β-carotene and zeaxanthin disappeared in the strain. Total carotenoid accumulation was nearly two fold compared with wild type. The astaxanthin-producing strain was capable of only growing heterotrophically, which was likely due to the absence of β-carotene. Further enhanced accumulation was pursued by gene overexpression for possible rate-limiting steps in the biosynthesis pathway.

Key Words: astaxanthin; cyanobacteria; metabolic engineering; phototrophic grow

Introduction

Carotenoids are terpenoid pigments that play important roles for light harvesting in photosynthesis and protection against reactive oxygen species and excess light energy in not only phototrophs but also some heterotrophic organisms. Generally, tetraterpenoid carotenoids (C40 compounds) are produced via the non-mevalonic acid pathway (namely, the methylerythritol phosphate (MEP) pathway), geranylgeranyl pyrophosphate (GGPP, C20), and phytoene (C40). Further modifications (desaturation, cyclization, ketolation, hydroxylation, glycosylation, etc.) produce a wide variety of carotenoid species (Cunningham and Gantt, 1998; Paniagua-Michel et al., 2012; Zhao et al., 2013). Among various carotenoids, astaxanthin is found not only in algae but also many animals such as shrimp, crab, some fish and birds, although it was taken from algae as their food (Maoka, 2011). It is also accepted that astaxanthin serves as a useful bioactive compound for human health due to its antioxidant properties (Ambati et al., 2014; Higuera-Ciapara et al., 2006). Recent advances in metabolic engineering have enabled the efficient production of these bioactive compounds in microbes, including cyanobacteria (Mao et al., 2017; Sandmann, 2015).

Cyanobacteria are the sole bacteria that perform oxygenic photosynthesis like plants. In evolution, photosynthetic chloroplasts of plants were derived from cyanobacterial endosymbionts. So, cyanobacteria have been extensively studied for photosynthesis and other aspects as unique bacteria and a model for plants. Genes for carotenoid biosynthesis in cyanobacteria have mostly been identified and many mutants have been constructed (Breitenbach et al., 2013; Graham and Bryant, 2009; Kusama et al., 2015; Schäfer et al., 2005). Common carotenoids in many cyanobacteria are β-carotene, zeaxanthin, echinenone, and myxoxanthophylls (Takaichi 2011). β-carotene is an essential component for photosynthetic complexes. Zeaxanthin and myxoxanthophylls serve as protection against excess light stresses (Graham and Bryant, 2009; Schäfer et al., 2005). 3′-Hydroxyechinenone is a chromophore of the orange carotenoid protein, which reversibly quenches excitation energy in the phycobilisome, the major photosynthetic antenna in

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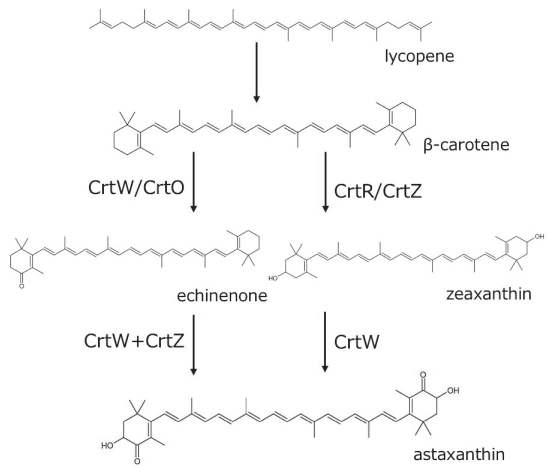

Fig. 1. Astaxanthin biosynthesis pathway in *Synechocystis* in this study. CrtO and CrtR are endogenous enzymes to produce echinenone and zeaxanthin. CrtW and CrtZ are heterologously expressed enzymes to produce astaxanthin. Note that a part of the carotenoid biosynthesis pathway and chemical structures are shown here.

Fig. 2. Typical culture of astaxanthin-producing *crtWZ* strain. WT: wild type.

cyanobacteria (Bao et al., 2017). These xanthophylls possess hydroxyl and/or keto groups but either on the same β-ionone rings, while astaxanthin possess both hydroxyl and keto groups on the β-ionone rings. This difference suggests that the endogenous hydroxylation and ketolation enzymes (CrtR and CrtO) is not capable for modifying the same β-ionone ring (Fig. 1). Therefore, we introduced *crtW* and *crtZ* from a marine bacterium *Brevundimonas* sp. SD212, because they are potent genes for ketolation and hydroxylation of β-carotene to produce astaxanthin (Choi et al., 2005, 2006). We also tried to enhance the astaxanthin production in cyanobacteria by overexpression of some genes upstream in the biosynthesis pathway.

Materials and Methods

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803

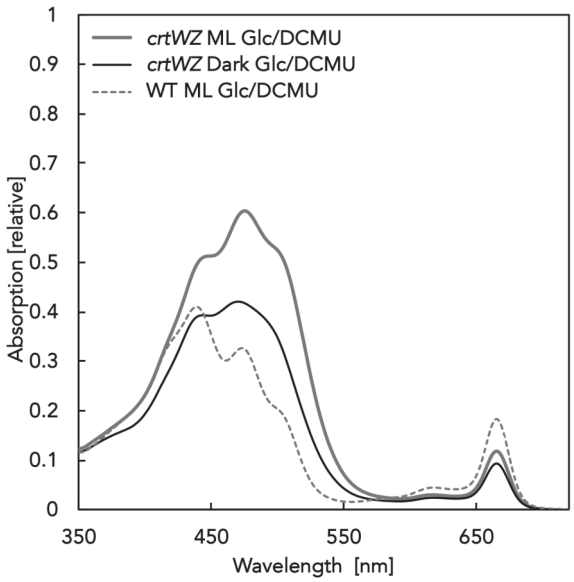

Fig. 3. Absorption spectra of methanol extracts from astaxanthin-producing *crtWZ* strain.

Absorption was normalized to the cell density of OD_{730} = 1. ML, 20 μmol photons m^{-2} s^{-1}. Glucose 5 mM and DCMU 10 μM.

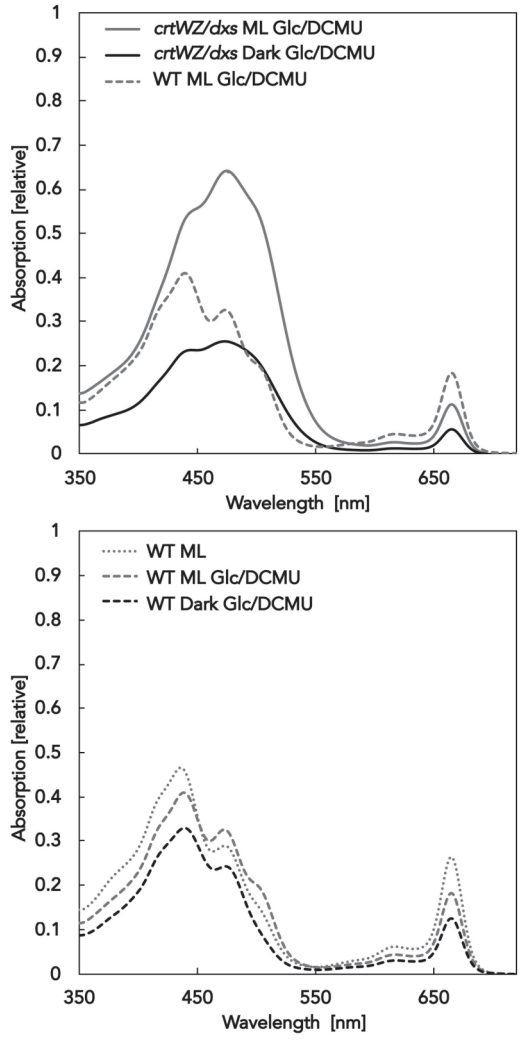

Fig. 4. Absorption spectra of methanol extracts from astaxanthin-producing *crtWZ*/*dxs* strain (upper) and wild type (lower).

Absorption was normalized to the cell density of OD_{730} = 1. ML, 20 μmol photons m^{-2} s^{-1}. Glucose 5 mM and DCMU 10 μM.

Table 1. Carotenoid content of cells.

carotenoid	medium	BG11	BG11+Glucose/DCMU*									
	light	+	+		-		+		-		+	
[μg/mL/OD730]	genes overexpressed	WT	WT		*dxs*	*crtWZ*	*crtWZ/dxs*		*crtWZ/dxs*		*crtWZ/dxs/pds*	
astaxanthin		-	-		-	1.075 ± 0.026	0.563 ± 0.048	1.089 ± 0.087	0.461 ± 0.036	1.070 ± 0.078	0.378 ± 0.041	
zeaxanthin		0.358 ± 0.024	0.359 ± 0.095	0.441 ± 0.019	0.312 ± 0.016	-	-	-	-	-	-	
canthaxanthin		-	-	-	-	0.026 ± 0.003	0.172 ± 0.016	0.077 ± 0.006	0.074 ± 0.003	0.119 ± 0.008	0.126 ± 0.011	
3-hydroxyechinenone		-	0.074 ± 0.027	-	-	0.153 ± 0.016	0.291 ± 0.019	0.377 ± 0.038	0.130 ± 0.010	0.400 ± 0.036	0.256 ± 0.023	
echinenone		0.135 ± 0.011	0.044 ± 0.077	0.111 ± 0.015	0.168 ± 0.006	0.112 ± 0.006	0.494 ± 0.020	0.410 ± 0.043	0.163 ± 0.010	0.477 ± 0.034	0.446 ± 0.039	
β-carotene		0.501 ± 0.039	0.448 ± 0.122	0.404 ± 0.015	0.267 ± 0.004	-	-	-	-	-	-	
lycopene		-	-	-	-	-	-	-	-	0.101 ± 0.009	0.174 ± 0.031	
γ-carotene		-	-	-	-	0.019 ± 0.003	0.099 ± 0.089	0.093 ± 0.012	0.031 ± 0.001	-	-	
synechoxanthin		0.030 ± 0.006	0.052 ± 0.004	0.045 ± 0.015	0.088 ± 0.012	-	-	-	-	-	-	
ketomyxol fucoside		-	-	-	-	1.236 ± 0.036	0.412 ± 0.034	0.867 ± 0.073	0.276 ± 0.016	0.946 ± 0.063	0.261 ± 0.027	
myxol fucoside		0.433 ± 0.074	0.758 ± 0.156	0.130 ± 0.009	0.842 ± 0.013	0.460 ± 0.019	0.303 ± 0.047	0.412 ± 0.087	0.148 ± 0.013	0.664 ± 0.087	0.229 ± 0.021	
ketodeoxymyxol fucoside		-	-	-	-	-	-	-	-	0.160 ± 0.002	-	
deoxymyxol fucoside		0.032 ± 0.004	0.045 ± 0.015	0.001 ± 0.002	0.032 ± 0.004	-	-	-	-	-	-	
total		1.491 ± 0.056	1.781 ± 0.380	1.132 ± 0.062	1.725 ± 0.035	3.081 ± 0.052	2.332 ± 0.058	3.326 ± 0.337	1.284 ± 0.087	3.937 ± 0.144	1.869 ± 0.190	

*Glucose (5 mM) and DCMU (10μM) were included. Light, 20 μmol photons/m^2/s. Values with standard error were obtained from three independent cultures.

Fig. 5. Absorption spectra of methanol extracts from astaxanthin-producing *crtWZ/dxs/pds* strain.

Absorption was normalized to the cell density of OD_{730} = 1. ML, 20 μmol photons m^{-2} s^{-1}. Glucose 5 mM and DCMU 10 μM.

was used in this study (Ikeuchi and Tabata, 2001). The strain that is capable of growing in the persistent dark was a kind gift from Prof. Hajime Wada (The Univ. of Tokyo). Cells were grown in BG11 plates or liquid with bubbling of 1% (v/v) CO_2 as described previously (Chin et al., 2018). Finally 5 mM glucose and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were added to support heterotrophic growth. Antibiotics (20 μg mL^{-1} erythromycin, 20 μg mL^{-1} spectinomycin, and 20 μg mL^{-1} chloramphenicol) were added for screening and maintenance. Mutants were grown in the light (~20 μmol photons m^{-2} s^{-1}) unless stated, but also grown in the dark with aluminum foil.

For overexpression, genes were expressed by a strong constitutive trc promoter at neutral sites, which were cloned in non-replicative plasmids (Supplementary Table 1). Plasmid constructs were made as described previously (Chin et al., 2018). Genes of *crtW* and *crtZ* from *Brevundimonas* sp. SD212 was chemically synthesized (Furubayashi et al., 2015) and integrated into the plasmid pBsgET, resulting in pBsgETcrtWZT. Genes of *dxs* (*sll1945*) and *pds* (*slr1254*) were amplified by PCR from the *Synechocystis* genomic DNA using primers for In-Fusion cloning as described previously (Supplementary Table 2 and Chin et al., 2018). The plasmid DNAs were introduced into cyanobacterial cells in the light by natural transformation and, thereby, the cassette and gene(s) were integrated into neutral sites on the chromosome by double homologous recombination (Supplementary Table 1 and Chin et al., 2018).

Carotenoids and chlorophylls were extracted with methanol from cells grown in the presence of glucose and DCMU in the light or dark for 3 to 4 days. Absorption spectra were recorded using a spectrometer (UV2600PC,

Shimadzu, Kyoto, Japan). Carotenoid composition was analyzed by HPLC as described previously (Kusama et al., 2015).

Results and Discussion

Overexpression of crtW and crtZ

When *crtW* and *crtZ* were introduced into wild type cells by natural transformation, both green and brown colonies emerged as transformants on the selecting BG11 plates. Because screening of the green colonies did not give any brown cells, these clones were discarded without further examination. Brown colonies were propagated on the glucose/DCMU plates in light until complete segregation was achieved. Such brown cells were transferred to a liquid culture, including glucose and DCMU, in the light (Fig. 2). Figure 3 shows the absorption spectra of cells after extraction with methanol, which were normalized to cell density (OD_{730} = 1). Overexpression of *crtW* and *crtZ* (hereafter *crtWZ*) enhanced accumulation of carotenoids peaking near 470 nm with a concomitant decrease in chlorophyll *a* peaking at 665 nm compared with the wild type. Culture in the light gave a higher accumulation (on the cell basis) of both carotenoids and chlorophyll than in the dark. The carotenoid/chlorophyll ratio tended to be lower in the light-grown cells than the dark-grown cells. The spectrum shape near 470 nm in the light was different from that in the dark and suggests that the carotenoid composition was also affected by light irradiation.

Carotenoid composition was analyzed by HPLC and normalized to cell density (Table 1). Wild type cells contained β-carotene, zeaxanthin, and myxol fucoside as major carotenoids as usual (Takaichi et al., 2001). The *crtWZ* strain that expressed *crtW* and *crtZ* gave a large accumulation of astaxanthin and ketomyxol fucoside. Light irradiation further increased the accumulation of astaxanthin and ketomyxol fucoside, but reduced canthaxanthin, 3′-hydroxyechinenone, and echinenone. In any case, the total carotenoid content per cell basis was nearly twofold in the *crtWZ* strain than in the wild type.

Overexpression of dxs and pds

First, endogenous *dxs* gene for 1-deoxy-d-xylulose-5-phosphate synthase was overexpressed according to our observations (Shimada et al., 2018). The Dxs catalyzes the first step of the MEP pathway and is known to be rate limiting in the isoprenoid biosynthesis in *Escherichia coli* (Harker and Bramley, 1999). But no positive effects were observed in the carotenoid accumulation in the light or even negative effects were observed in the dark (Fig. 4). Table 1 showed very little change in the composition between the *crtWZ* and *crtWZ*/*dxs* strains in the light, whereas accumulation of echinenone and ketomyxol fucoside was suppressed by *dxs* overexpression in the dark.

Second, endogenous *pds* gene for phytoene desaturase was overexpressed in combination with *dxs*, according to McQuinn et al. (2018). Pds catalyzes the desaturation of phytoene to generate ζ-carotene. Interestingly, the absorption peak normalized to the cell density in Fig. 5, and the total carotenoid content in Table 1, clearly demonstrated the increase in carotenoid accumulation in the light but not in the dark. Carotenoid composition revealed that a major increase was found in 3′-hydroxyechinenone and echinenone but not in astaxanthin. These results suggest that the accumulation level of astaxanthin was delimited by the activity of CrtW and/or CrtZ. Nevertheless, the total carotenoid content of the *crtWZ*/*dxs*/*pds* strain (3.937 μg/mL/OD_{730}) was 2.64 fold higher than that of wild type grown in BG11 with light.

Toxicity of astaxanthin production in cyanobacteria

The *crtWZ* strain and its derivatives could not grow on BG11, so that these strains were maintained under heterotrophic conditions with glucose and DCMU. When DCMU was omitted from the medium in the light, the *crtWZ* strain soon generated suppressor cells, which reacquired phototrophic growth with a concomitant loss of astaxanthin. We picked several clones form these suppressors and found that they are grouped into green clones and brown clones. When the *crtWZ* region was PCR amplified and sequenced, various deletion mutations were found in the green clones. On the other hand, a frame shift mutation or a large deletion was found in *crtZ* in the brown clones. In the presence of DCMU and glucose, very few such suppressors were detected in short-term maintenance but brown suppressors appeared slowly in long term maintenance. These results suggest that mainly *crtZ* was toxic to the photosystem II photochemistry to evolve oxygen, which was almost inhibited by DCMU. However, it is unlikely that the combination of astaxanthin and oxygen evolution produces some toxic substances, because astaxanthin is very active in scavenging oxygen. As a result of astaxanthin production, essential β-carotene as well as zeaxanthin were missing in the cells. In this context, it should be mentioned that the brown suppressors slowly grew in BG11 photoautotrophically, suggesting that *crtW* itself was not fatal compared with *crtZ* in the *crtWZ* strain. The carotenoid content in the photosystem II complex may be different between the *crtWZ* strain and the brown suppressors.

Recently, astaxanthin production in *Synechocystis* sp. PCC 6803 was reported using heat-inducible expression of the *Brevundimonas crtW* and *crtZ* (Menin et al., 2019). The cellular pigmentation after two days of induction slightly changed from green to yellowish green. Endogenous β-carotene and zeaxanthin were largely converted to astaxanthin and canthaxanthin. However, almost no net increase in total carotenoid content per chlorophyll was observed judging from the cellular absorption spectra, although the cellular content of carotenoids and chlorophyll may have been changed simultaneously. Transient induction of *crtW* and *crtZ* under photoautotrophic condition might not be sufficient for full accumulation of astaxanthin and other carotenoids, according to our observation of heterotrophic growth. They also made a strain harboring only *crtW*. Two-day induction of *crtW* hardly changed the pigment composition of the *crtW* strain. This finding contrasts with our brown suppressors. The induction period may also affect the full conversion of preexisting carotenoids depending on the induced gene.

Finally, it is important to cope with the toxicity of *crtZ* to achieve further improvement of astaxanthin production

in cyanobacteria. To this end, it would be essential to know the toxic point of the CrtZ in the photosynthetic complexes. We are now trying to isolate the photosystem I and II complexes from the *crtWZ* strain for future biochemical analysis.

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Supplementary Materials

Supplementary figure and tables are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

References

Ambati, R. R., Phang, S. M., Ravi, S., and Aswathanarayana, R. G. (2014) Astaxanthin: sources, extraction, stability, biological activities and its commercial applications—a review. *Mar. Drugs*, **12**, 128–152.

Bao, H., Melnicki, M. R., and Kerfeld, C. A. (2017) Structure and functions of Orange Carotenoid Protein homologs in cyanobacteria. *Curr. Opin. Plant Biol*., **37**, 1–9.

Breitenbach, J., Gerjets, T., and Sandmann, G. (2013) Catalytic properties and reaction mechanism of the CrtO carotenoid ketolase from the cyanobacterium *Synechocystis* sp. PCC 6803. *Arch. Biochem. Biophys*., **529**, 86–91.

Chin, T., Okuda, Y., and Ikeuchi, M. (2018) Sorbitol production and optimization of photosynthetic supply in the cyanobacterium *Synechocystis* PCC 6803. *J. Biotechnol*., **276–277**, 25–33.

Choi, S. K., Nishida, Y., Matsuda, S., Adachi, K., Kasai, H. et al. (2005) Characterization of β-carotene ketolases, CrtW, from marine bacteria by complementation analysis in *Escherichia coli*. *Mar. Biotechnol. (NY)*, **7**, 515–522.

Choi, S. K., Matsuda, S., Hoshino, T., Peng, X., and Misawa, N. (2006) Characterization of bacterial β-carotene 3,3′-hydroxylases, CrtZ, and P450 in astaxanthin biosynthetic pathway and adonirubin production by gene combination in *Escherichia coli*. *Appl. Microbiol. Biotechnol*., **72**, 1238–1246.

Cunningham, F. X. and Gantt, E. (1998) Genes and enzymes of carotenoid biosynthesis in plants. *Annu. Rev. Plant Physiol. Plant. Mol. Biol*., **49**, 557–583.

Furubayashi, M., Ikezumi, M., Takaichi, S., Maoka, T., Hemmi, H. et al. (2015) A highly selective biosynthetic pathway to non-natural C50 carotenoids assembled from moderately selective enzymes. *Nat. Commun*., **6**, 7534.

Graham, J. E. and Bryant, D. A. (2009) The biosynthetic pathway for myxol-2′ fucoside (myxoxanthophyll) in the cyanobacterium *Synechococcus* sp. strain PCC 7002. *J. Bacteriol*., **191**, 3292–3300.

Harker, M. and Bramley, P. M. (1999) Expression of prokaryotic 1-deoxy-D-xylulose-5-phosphatases in *Escherichia coli* increases carotenoid and ubiquinone biosynthesis. *FEBS Lett*., **448**, 115–119.

Higuera-Ciapara, I., Félix-Valenzuela, L., and Goycoolea, F. M. (2006) Astaxanthin: a review of its chemistry and applications. *Crit. Rev. Food Sci. Nutr*., **46**, 185–196.

Ikeuchi, M. and Tabata, S. (2001) *Synechocystis* sp. PCC 6803—a useful tool in the study of the genetics of cyanobacteria. *Photosynth. Res*., **70**, 73–83.

Kusama, Y., Inoue, S., Jimbo, H., Takaichi, S., Sonoike, K. et al. (2015) Zeaxanthin and echinenone protect the repair of photosystem II from inhibition by singlet oxygen in *Synechocystis* sp. PCC 6803. *Plant Cell Physiol*., **56**, 906–916.

Mao, X., Liu, Z., Sun, J., and Lee, S. Y. (2017) Metabolic engineering for the microbial production of marine bioactive compounds. *Biotechnol. Adv*., **35**, 1004–1021.

Maoka, T. (2011) Carotenoids in marine animals. *Mar. Drugs*, **9**, 278–293.

McQuinn, R. P., Wong, B., and Giovannoni, J. J. (2018) AtPDS overexpression in tomato: exposing unique patterns of carotenoid self-regulation and an alternative strategy for the enhancement of fruit carotenoid content. *Plant Biotechnol. J*., **16**, 482–494.

Menin, B., Santabarbara, S., Lami, A., Musazzi, S., Villafiorita Monteleone, F. et al. (2019) Non-endogenous ketocarotenoid accumulation in engineered *Synechocystis* sp. PCC 6803. *Physiol. Plant*., **166**, 403–412.

Paniagua-Michel, J., Olmos-Soto, J., and Ruiz, M. A. (2012) Pathways of carotenoid biosynthesis in bacteria and microalgae. *Methods Mol. Biol*., **892**, 1–12.

Sandmann, G. (2015) Carotenoids of biotechnological importance. *Adv. Biochem. Eng. Biotechnol*., **148**, 449–467.

Schäfer, L., Vioque, A., and Sandmann, G. (2005) Functional in situ evaluation of photosynthesis-protecting carotenoids in mutants of the cyanobacterium *Synechocystis* PCC6803. *J. Photochem. Photobiol. B*, **78**, 195–201.

Shimada, N., Maeda, K., and Ikeuchi, M. (2018) Biosynthesis and engineering of carotenoids in cyanobacteria. *BJS-Review*, **9**, 102.

Takaichi, S. (2011) Carotenoids in algae: distributions, biosyntheses and functions. *Mar. Drugs*, **9**, 1101–1118.

Takaichi, S., Maoka, T., and Masamoto, K. (2001) Myxoxanthophyll in *Synechocystis* sp. PCC 6803 is myxol 2′-dimethyl-fucoside, (3R,2′S)-myxol 2′-(2,4-di-*O*-methyl-alpha-L-fucoside), not rhamnoside. *Plant Cell Physiol*., **42**, 756–762.

Zhao, L., Chang, W. C., Xiao, Y., Liu, H. W., and Liu, P. (2013) Methylerythritol phosphate pathway of isoprenoid biosynthesis. *Annu. Rev. Biochem*., **82**, 497–530.