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

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Abstract

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.

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 (Higuera-Ciapara et al. 2006, Ambati et al. 2014). Recent advances in metabolic engineering have enabled the efficient production of these bioactive compounds in microbes, including cyanobacteria (Sandmann 2015, Mao et al. 2017).

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 (Schafer et al. 2005, Graham and Bryant 2009, Breitenbach et al. 2013, Kusama et al. 2015). 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 (Schafer et al. 2005, Graham and Bryant 2009). 3'-Hydroxyechinenone is a chromophore of the orange carotenoid protein, which reversibly quenches excitation energy in the phycobilisome, the major photosynthetic antenna in 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, Choi et al. 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 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 (Univ 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*/*pds*/*dxs* 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

147 of DCMU and glucose, very few such suppressors were detected in short-term
148 maintenance but brown suppressors appeared slowly in long term maintenance. These
149 results suggest that mainly *crtZ* was toxic to the photosystem II photochemistry to
150 evolve oxygen, which was almost inhibited by DCMU. However, it is unlikely that the
151 combination of astaxanthin and oxygen evolution produces some toxic substances,
152 because astaxanthin is very active in scavenging oxygen. As a result of astaxanthin
153 production, essential β-carotene as well as zeaxanthin were missing in the cells. In this
154 context, it should be mentioned that the brown suppressors slowly grew in BG11
155 photoautotrophically, suggesting that *crtW* itself was not fatal compared with *crtZ* in the
156 *crtWZ* strain. The carotenoid content in the photosystem II complex may be different
157 between the crtWZ strain and the brown suppressors.

158 Recently, astaxanthin production in *Synechocystis* sp. PCC 6803 was reported
159 using heat-inducible expression of the *Brevundimonas crtW* and *crtZ* (Menin et al.
160 2019). The cellular pigmentation after two days of induction slightly changed from
161 green to yellowish green. Endogenous β-carotene and zeaxanthin were largely
162 converted to astaxanthin and canthaxanthin. However, almost no net increase in total
163 carotenoid content per chlorophyll was observed judging from the cellular absorption
164 spectra, although the cellular content of carotenoids and chlorophyll may have been
165 changed simultaneously. Transient induction of *crtW* and *crtZ* under photoautotrophic
166 condition might not be sufficient for full accumulation of astaxanthin and other
167 carotenoids, according to our observation of heterotrophic growth. They also made a
168 strain harboring only *crtW*. Two-day induction of *crtW* hardly changed the pigment
169 composition of the *crtW* strain. This finding contrasts with our brown suppressors. The
170 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.

Acknowledgements

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248

249 Figure legend

250 Fig.1 Astaxanthin biosynthesis pathway in *Synechocystis* in this study.

251 CrtO and CrtR are endogenous enzymes to produce echinenone and zeaxanthin. CrtW

252 and CrtZ are heterologously expressed enzymes to produce astaxanthin. Note that a part

253 of the carotenoid biosynthesis pathway and chemical structures are shown here.

254

255 Fig. 2 Typical culture of astaxanthin-producing *crtWZ* strain.

256 WT: wild type.

257

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

259 strain. Absorption was normalized to the cell density of OD_{730} = 1. ML, 20 μmol

260 photons m^{-2} s^{-1}. Glucose 5 mM and DCMU 10 μM.

261

262 Fig. 4 Absorption spectra of methanol extracts from astaxanthin-producing *crtWZ/dxs*

263 strain (upper) and wild type (lower). Absorption was normalized to the cell density of

264 OD_{730} = 1. ML, 20 μmol photons m^{-2} s^{-1}. Glucose 5 mM and DCMU 10 μM.

265

266 Fig. 5 Absorption spectra of methanol extracts from astaxanthin-producing

267 *crtWZ/pds/dxs* strain. Absorption was normalized to the cell density of OD_{730} = 1. ML,

268 20 μmol photons m^{-2} s^{-1}. Glucose 5 mM and DCMU 10 μM.

269

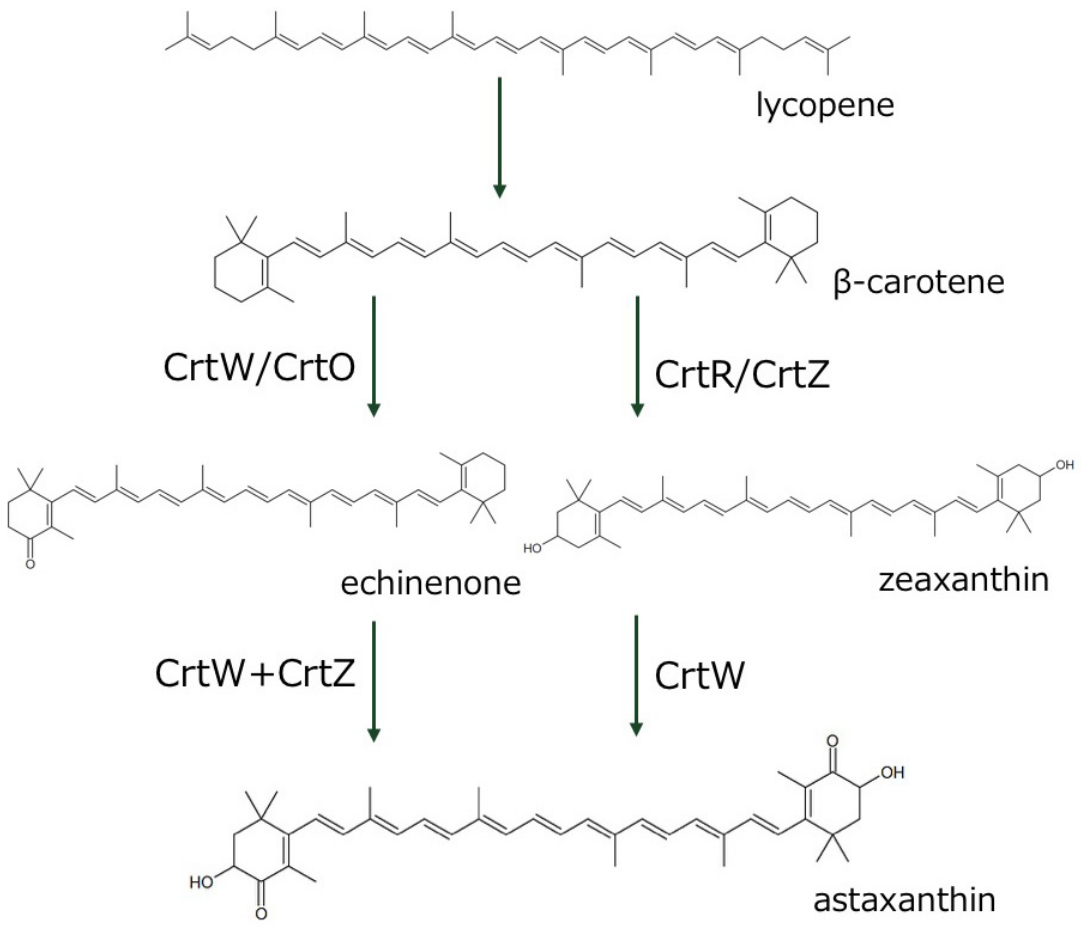


Fig.1 Astaxanthin biosynthesis pathway in *Synechocystis* in this study.
CrtO and CrtR are endogenous enzymes to produce echinenone and zeaxanthin. CrtW and CrtZ are heterologously expressed enzymes to produce astaxanthin. Note that a part of carotenoid biosynthesis pathway and chemical structures are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

Fig. 2 Typical culture of astaxanthin-producing *crtWZ* strain.

Fig.3

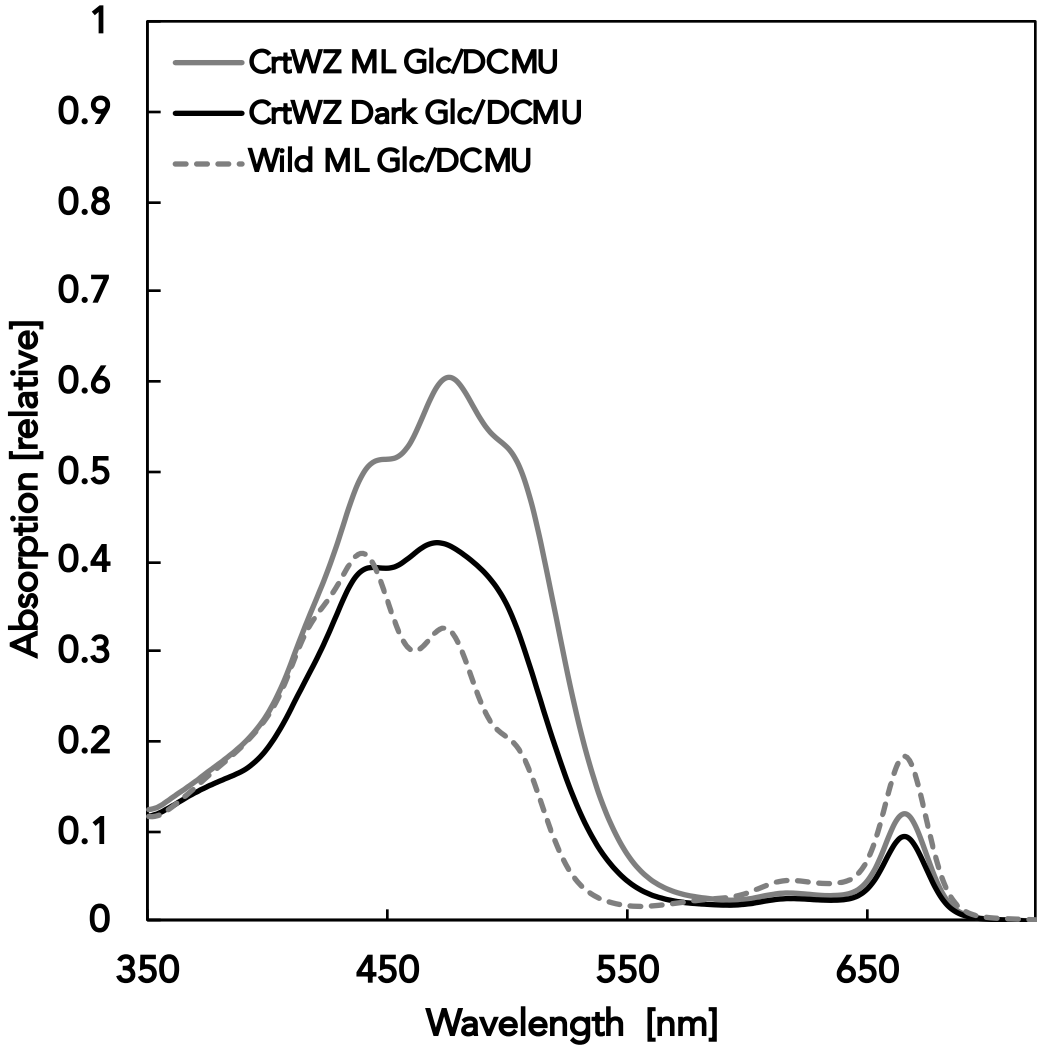
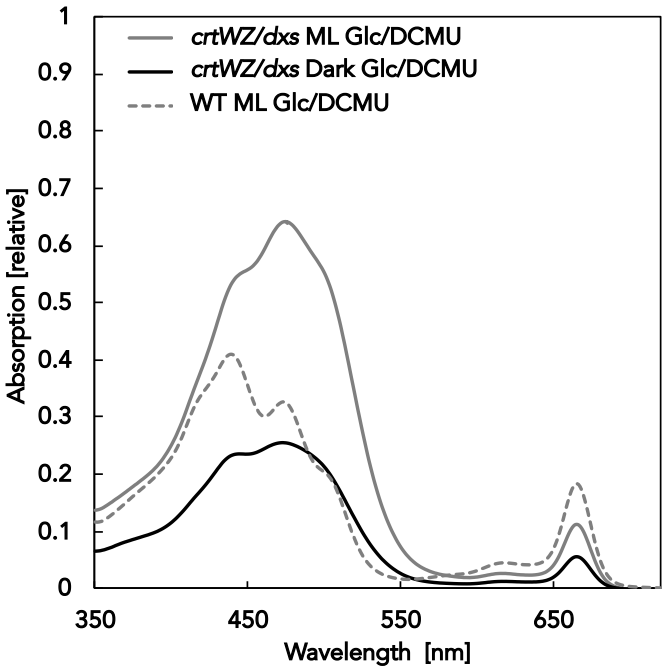

CrtWZ ML Glc/DCMU
CrtWZ Dark Glc/DCMU
Wild ML Glc/DCMU
Absorption [relative]
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
350
450
550
650
Wavelength [nm]

Fig4


crtWZ/dxs ML Glc/DCMU
crtWZ/dxs Dark Glc/DCMU
WT ML Glc/DCMU
Absorption [relative]
Wavelength [nm]
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
350
450
550
650

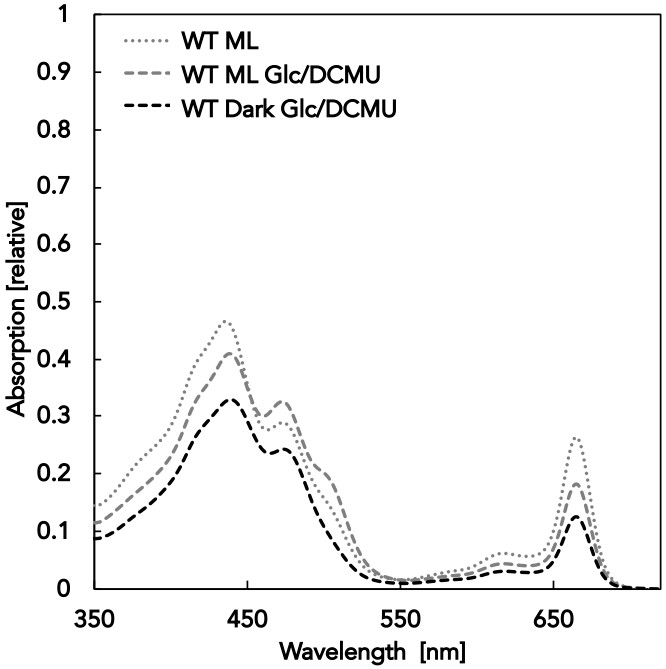

WT ML
WT ML Glc/DCMU
WT Dark Glc/DCMU
Absorption [relative]
Wavelength [nm]
0
0.1
0.2
0.3
0.4
0.5
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Fig5

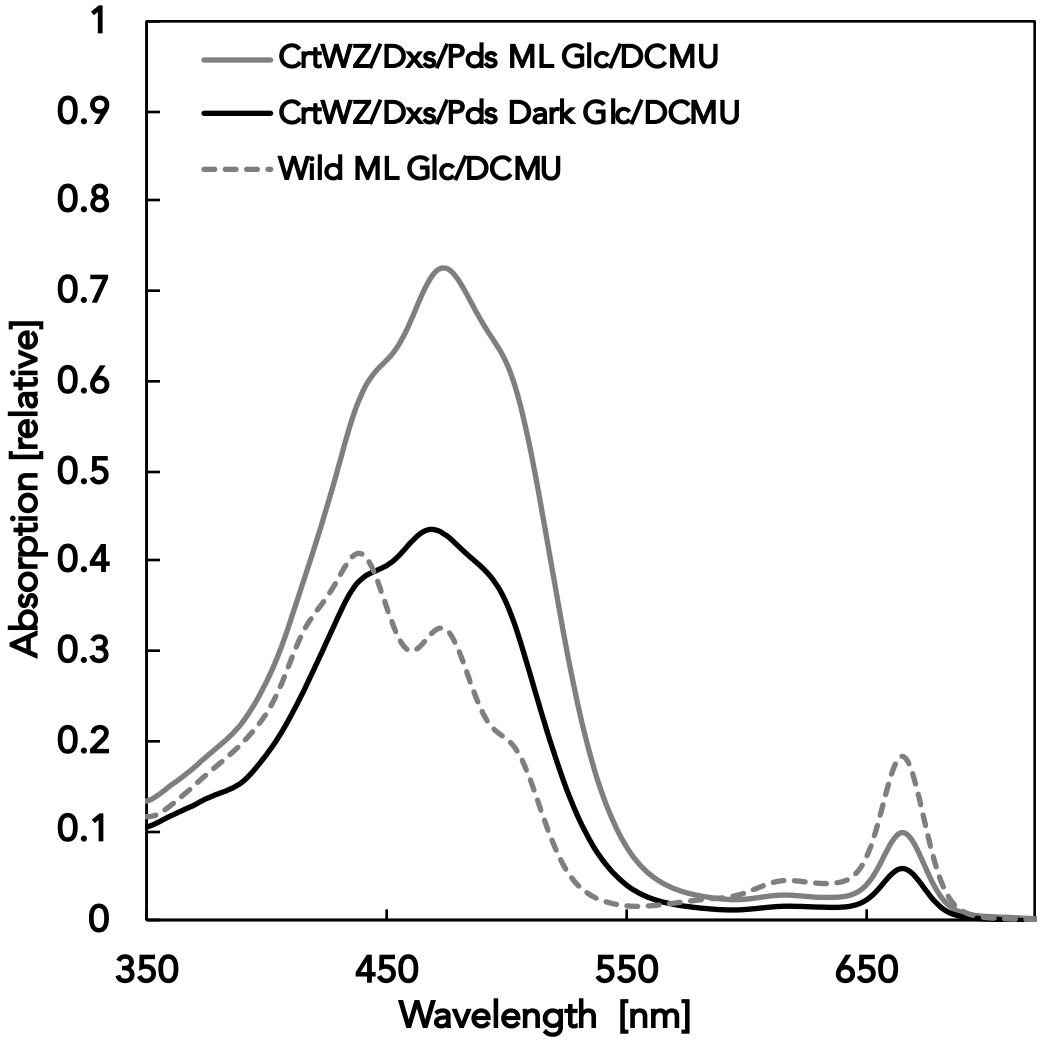
CrtWZ/Dxs/Pds ML Glc/DCMU
CrtWZ/Dxs/Pds Dark Glc/DCMU
Wild ML Glc/DCMU
Absorption [relative]
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
350
450
550
650
Wavelength [nm]

Table 1. Carotenoid content of cells

	medium	BG11	BG11+Glucose/DCMU*								
carotenoid	light	+	+	-	+	+	-	+	-	+	-
[μg/mL/OD730]	genes overexpressed	WT	WT	WT	*dxs*	*crtWZ*	*crtWZ*	*crtWZ/dxs*	*crtWZ/dxs*	*crtWZ/dxs/pds*	*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.