The zeaxanthin biosynthesis enzyme β-carotene hydroxylase is involved in myxoxanthophyll synthesis in *Synechocystis* sp. PCC 6803

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Abstract **β-carotene hydroxylase is known to be involved in zeaxanthin synthesis. Disruption of the *crtR* gene encoding β-carotene hydroxylase in *Synechocystis* sp. PCC 6803 resulted in the absence of both zeaxanthin synthesis and myxoxanthophyll accumulation in the mutant strain. A new carotenoid was detected in this strain. Its chemical structure was close to that of myxoxanthophyll, but the hydroxyl group on the β-ring was lacking. This compound, deoxy-myxoxanthophyll, most likely is an intermediate in the myxoxanthophyll biosynthesis pathway. Therefore, β-carotene hydroxylase is involved not only in zeaxanthin synthesis but also in myxoxanthophyll synthesis in *Synechocystis*. Furthermore, myxoxanthophyll in *Synechocystis* was found to have a higher molecular mass than what was determined in other species. This difference is likely to be due to a difference in the sugar moiety.**

Key words: Carotenoid; Myxoxanthophyll; *Synechocystis* sp. PCC 6803

1. Introduction

Carotenoids are isoprenoid polyene pigments synthesized by photosynthetic and some non-photosynthetic organisms [1,2]. They are relatively hydrophobic molecules that are typically associated with membranes. In photosynthetic systems they are often found to be non-covalently bound to chlorophyll-binding proteins. In such systems, carotenoids may trap light energy and transfer it to chlorophyll, dissipate excess radiant energy and preserve the structural integrity of pigment-protein complexes [3,4], and/or play a protective role against the potentially damaging combination of oxygen, light and chlorophyll by quenching both the triplet excited states of chlorophyll and the highly reactive singlet oxygen [5]. To a lesser extent, carotenoids and their glycosides can be found in other membranes where they are thought to influence membrane fluidity [5,6].

The first committed step in carotenoid biosynthesis is phytoene synthesis by condensation of two molecules of geranylgeranyl pyrophosphate (GGPP). Phytoene is converted into various carotenes that are the substrates for a variety of xanthophylls (carotene derivatives containing one or more oxygen atoms incorporated into hydroxy-, methoxy-, oxo-, epoxy-, carboxy-, aldehydic and glycosidic functional groups). In *Synechocystis* sp. PCC 6803, only one carotene (β-carotene (β,β-carotene)) and three xanthophylls (zeaxanthin (β,β-carotene-3,3′-diol), echinenone (β,β-caroten-4-one) and myxoxanthophyll (2′-(β-1-rhamnopyranosyloxy)-3′,4′-didehydro-1′,2′-dihydro-β,Ψ-carotene-3,1′-diol)) accumulate significantly (Fig. 1).

Myxoxanthophyll is a xanthophyll glycoside found in cyanobacteria and in some non-photosynthetic bacteria but not in eukaryotic algae [2]. It was first isolated from the cyanobacterium *Oscillatoria rubrescens* [7]. Its structure was determined as a mixed carotenoid glycoside in which rhamnose is the dominant sugar moiety (90%) and a hexose is a minor component [8]. Although most of the genes involved in carotenoid biosynthesis have been identified in several organisms, including *Synechocystis* sp. PCC 6803 (Fig. 1), the myxoxanthophyll biosynthesis pathway remains unknown. This pigment is thought to be synthesized from γ-carotene [3]. This synthesis probably requires several steps including hydroxylation of the β-ring of γ-carotene. A carotene hydroxylase has been recently identified in *Synechocystis* [9]. This β-carotene hydroxylase, encoded by the *crtR* gene (*sll1468* according to CyanoBase (http://www.kazusa.or.jp/cyano/cyano.html)), has been shown to add hydroxyl groups to the β-rings of β-carotene, giving rise to zeaxanthin. In this paper we show that this β-carotene hydroxylase also appears to be involved in myxoxanthophyll biosynthesis.

2. Materials and methods

2.1. Strains and growth conditions

Synechocystis sp. PCC 6803 was cultivated at 30°C in modified BG-11 medium [10] buffered with 10 mM TES-NaOH (pH 8.0), at a light intensity of 50 μmol photons m^{-2} s^{-1}, unless indicated otherwise. The BG-11 modification consisted of partial substitution of $NaNO_3$ with an equal concentration of NH_4NO_3 (the final concentration of ammonia was 4.5 mM). For growth on plates, 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate were added. BG-11 was supplemented with 50 μg ml^{-1} kanamycin for kanamycin-resistant strains. For initial transformant selection, the DNA/cell mixture was plated on a BG-11 plate (50 ml), and the next day 2.5 mg kanamycin (dissolved in sterile water) was added to the bottom of the plate. This procedure allows a gentle exposure of transformants to increasing kanamycin concentration.

2.2. Cloning of the β-carotene hydroxylase gene from Synechocystis sp. PCC 6803

The *crtR* gene was cloned by PCR based on the sequence [11] available in CyanoBase (http://www.kazusa.or.jp/cyano/cyano.html). The forward primer (5′-GGGTCTGGGGAATTCCCATTTGAT-3′) contains an *Eco*RI site (underlined). Its relative position within the *Synechocystis* genome is 981590–981614. The reverse primer (5′-TTCTCCCTGGGATCCTACATGTTG-3′) contains a *Bam*HI site (underlined). Its relative position within the *Synechocystis* genome is 982709–982733. A PCR product of the expected 1.2 kb size was purified and introduced between the *Eco*RI and *Bam*HI sites in pUC18

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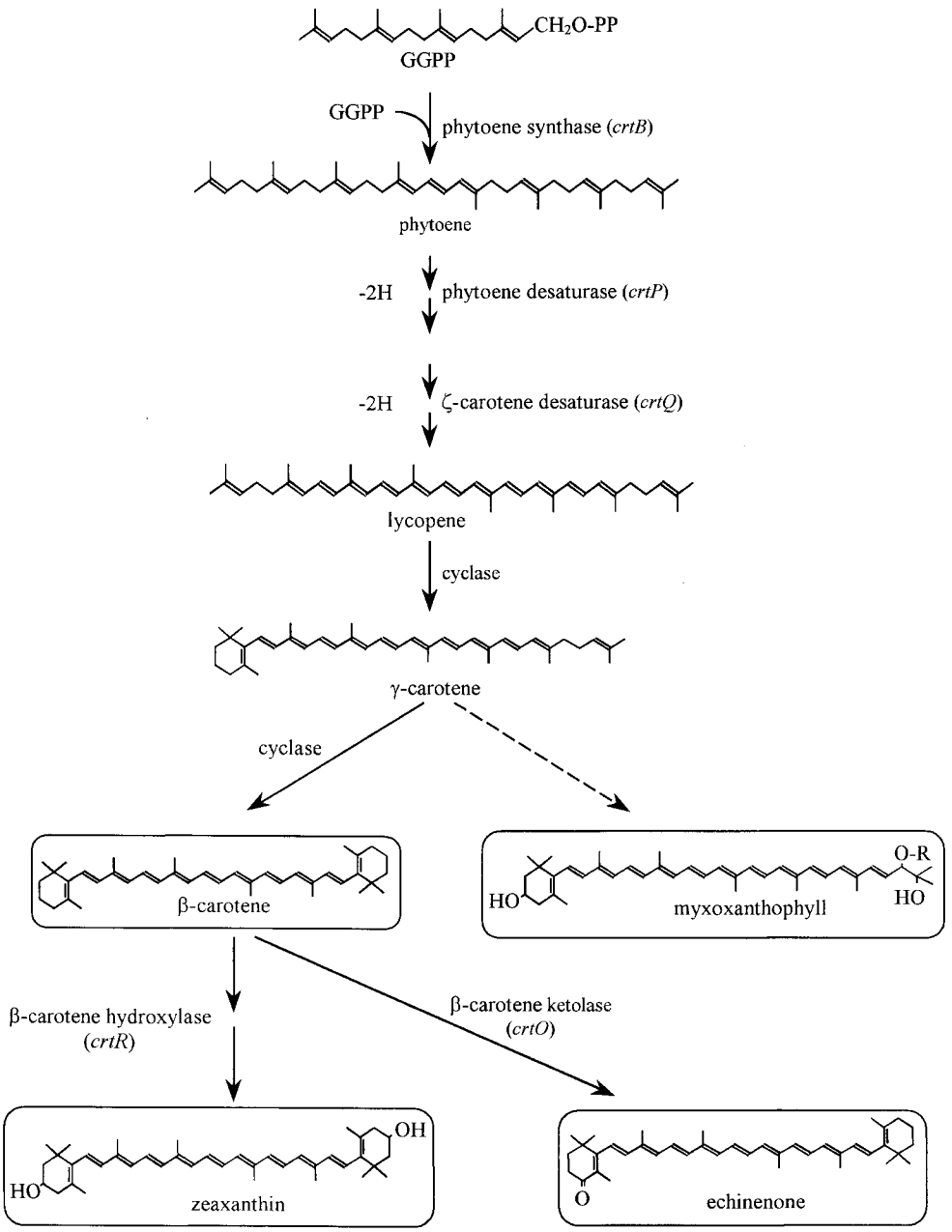

Fig. 1. Simplified carotenoid biosynthetic pathway in *Synechocystis* sp. PCC 6803. Carotenoids that have been boxed accumulate significantly in *Synechocystis* sp. PCC 6803. Gene names for biosynthetic enzymes that have been identified in the genome are in parentheses next to the enzyme that they encode: *crtB* [19], *crtP* [20], *crtQ* [21], *crtO* [22] and *crtR* [9]. Dotted arrow indicates pathways that remain hypothetical. R in the myxoxanthophyll structure indicates a glycosidic functional group.

(Pharmacia Biotech). To inactivate the *crtR* gene, a 3.1 kb blunt-end *Eco*RV fragment containing the kanamycin resistance gene (*aphX*) from pUC4K [12] and the *sacB* gene from pRL271 [13,14] was used to replace a 0.2 kb gene-internal *Msc*I-*Msc*I fragment in *crtR*. The plasmid generated was named pΔcrtR (Fig. 2A).

2.3. Carotenoid content analysis

Synechocystis sp. PCC 6803 cells were harvested from cultures in the logarithmic growth phase ($OD_{730} \approx 0.5$), and pigments were extracted with methanol at room temperature. Extracts were kept under nitrogen. Carotenoids were separated by HPLC on a Spherisorb ODS2 4.0 mm × 250 mm C18 column using a 15 min gradient of ethyl acetate (0 to 100%) in acetonitrile-water-triethylamine (9:1:0.01, v/v/v) at a flow rate of 1.5 ml min^{-1}. Absorption spectra for individual peaks were obtained with a photodiode array detector. Carotenoid species were identified by their absorption spectra and by their typical retention times. The content of each carotenoid was determined using the following equation:

$$C_{car} = C_{chl} \times \frac{\varepsilon_{chl} \times A_{car}}{\varepsilon_{car} \times A_{chl}}$$

where C_{chl} is the chlorophyll concentration in the pigment extract, and ε_{chl} and ε_{car} are the specific extinction coefficients at 440 nm of the chlorophyll and the carotenoid, respectively. The extinction coefficients of the chlorophyll and the carotenoid are the same in methanol, ethanol, and acetonitrile, as the absorbance values and the shape of the absorption spectra in these solvents are the same (data not shown). Therefore, the specific extinction coefficients used in this study were those reported previously in methanol or ethanol [15]. They were assumed to remain constant regardless of the ethyl acetate

concentration in the HPLC eluent. A_{chl} and A_{car} are the peak areas on the chromatogram (recorded at 440 nm) of the chlorophyll and carotenoid species, respectively.

2.4. Pigment purification for mass spectrometry analysis

Pigments were extracted from 1 l cultures of *Synechocystis* sp. PCC 6803 cells in the logarithmic growth phase ($OD_{730} \approx 0.5$). *Synechocystis* cells were centrifuged at $5000 \times g$ for 10 min. Extractions were performed on the pellet according to Kalen et al. [16]. Carotenoids were separated by HPLC on a Spherisorb ODS2 4.0 mm×250 mm C18 column using a 32 min gradient (0 to 100%) of methanol-isopropanol-hexane (2:1:1, v/v/v) in methanol-water (9:1, v/v) at a flow rate of 1 ml min^{-1}. Pigments of interest were collected after HPLC analysis of wild-type and mutant extracts. Solvents were evaporated under nitrogen and pigments were kept in the dark in a dry state. Mass spectra were obtained by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Before analysis, pigments (10 μg) were mixed with terthiophene (used as a matrix) dissolved in acetone.

3. Results and discussion

3.1. Inactivation of crtR in Synechocystis sp. PCC 6803

DNA from the plasmid pΔcrtR was used to transform *Synechocystis* sp. PCC 6803 (wild-type). Transformants (Δ*crtR*) were selected on BG-11 medium [10] in the presence of kanamycin. Complete segregation of the Δ*crtR* strain was confirmed by PCR using the set of primers used to amplify the *crtR* gene (Fig. 2B).

3.2. Carotenoid composition of the ΔcrtR strain

Carotenoid content analysis was performed on the wild-type and the Δ*crtR* strains. In the wild-type strain, four carotenoids, myxoxanthophyll, zeaxanthin, echinenone and β-carotene, were identified in the extract (Fig. 3A). As expected, in the Δ*crtR* strain zeaxanthin was absent. However, interestingly, myxoxanthophyll was absent as well. Instead, a new peak appeared on the chromatogram (indicated by 'X' in Fig. 3A). The spectrum of this new carotenoid is indistinguishable from that of myxoxanthophyll (Fig. 3C). This suggests that the new carotenoid is very closely related to myxoxanthophyll, even though it appears to be significantly more hydrophobic in view of its greatly increased retention time on the HPLC column. The carotenoid content of the wild-type and Δ*crtR* strains was quantified (Table 1). The β-carotene and echinenone contents were very similar in the wild-type and in the Δ*crtR* strains (Table 1). The absence of zeaxanthin synthesis in the Δ*crtR* strain did not lead to over-accumulation of β-carotene and/or echinenone. The amount of the new myxoxanthophyll-like compound in the Δ*crtR* strain was found to be comparable to the amount of myxoxanthophyll in the wild-type strain. As the absorption spectra of the new compound and of myxoxanthophyll were identical (Fig. 3C),

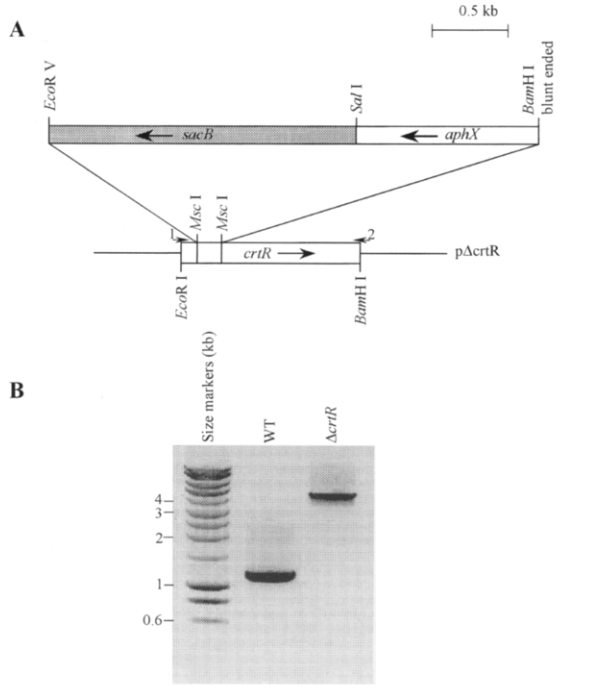

Fig. 2. A: Disruption of the *crtR* gene by insertion of the kanamycin resistance gene *aphX* and the *sacB* gene conferring sucrose sensitivity. Arrows indicate directions of the coding sequences. Numbers 1 and 2 stand for the primers used to amplify the *crtR* gene and to check for complete segregation of the Δ*crtR* strain. B: PCR products indicating complete segregation of the Δ*crtR* strain.

we assumed that these two pigments have the same extinction coefficient at 440 nm. With this assumption, the amount of myxoxanthophyll in the wild-type strain was virtually identical to that of the new compound in the Δ*crtR* strain. This observation suggests that the new myxoxanthophyll-like compound quantitatively replaces myxoxanthophyll. Therefore, in the mutant strain, zeaxanthin has disappeared without significant compensation by other carotenoids, and thus the total amount of carotenoids in the mutant strain is lower than in the wild-type one (Table 1).

3.3. Nature of the myxoxanthophyll-like compound in the ΔcrtR strain

An important question is what the chemical nature of the new carotenoid in the Δ*crtR* strain is. The spectral resemblance to myxoxanthophyll implies a close relationship to this xanthophyll, but the increased retention time on the hydrophobic HPLC column indicates a more hydrophobic nature than myxoxanthophyll. As the *crtR* gene codes for a

Table 1
Carotenoid content in the wild-type and the Δ*crtR* strains of *Synechocystis* sp. PCC 6803[a]

Strain	Carotenoid content (μg/OD_{730})				
	Myxoxanthophyll	Zeaxanthin	'X'[b]	Echinenone	β-carotene
Wild-type	0.45 ± 0.02	0.39 ± 0.02	–	0.25 ± 0.01	0.53 ± 0.02
Δ*crtR*	–	–	0.52 ± 0.04	0.31 ± 0.03	0.49 ± 0.03

[a]Values given are the averages from nine different determinations. Pigments were extracted from cells with methanol. Methanol extracts were analyzed by HPLC and the carotenoid content was derived from the area of the HPLC peaks on the chromatogram recorded at 440 nm and from the specific extinction coefficient (ε) of each pigment at 440 nm (see Section 2). The specific extinction coefficients of myxoxanthophyll and 'X' were assumed to be identical at 440 nm.
[b]See Fig. 3A.

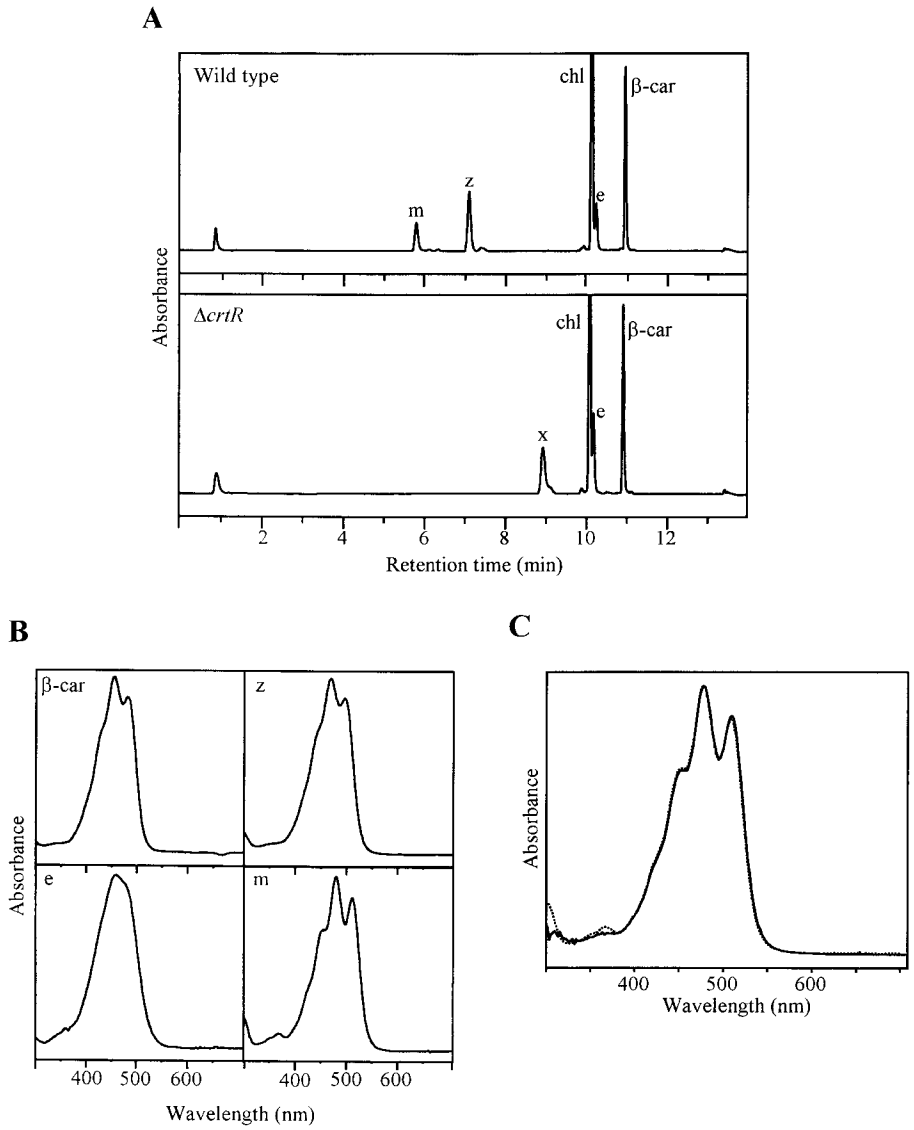

Fig. 3. HPLC analysis of pigments extracted from *Synechocystis* cells of the wild-type and the Δ*crtR* strains. A: Chromatograms were recorded as a function of absorbance at 440 nm. B: UV-visible absorption spectra in acetonitrile of carotenoids detected in the wild-type strain. C: Comparison between UV-visible absorption spectra of myxoxanthophyll (dashed line) [8], and the myxoxanthophyll-like compound from the Δ*crtR* strain (solid line), recorded with a photodiode array detector on the HPLC. m, myxoxanthophyll; z, zeaxanthin; chl, chlorophyll a; e, echinenone; β-car, β-carotene; and x, the myxoxanthophyll-like compound that is specific for the Δ*crtR* strain.

hydroxylase, a reasonable hypothesis is that the new compound that is found in the Δ*crtR* strain is a myxoxanthophyll without an OH group on the ring. Indeed, the absence of an hydroxyl group on the β-ring of myxoxanthophyll is not expected to affect its spectrum [17] but would render the compound to be less hydrophilic, which is in agreement with the increased retention time on the HPLC chromatogram. These results suggest that the myxoxanthophyll-like compound found in the Δ*crtR* strain may be myxoxanthophyll without an hydroxyl group on the β-ring and that in *Synechocystis* sp. PCC 6803, β-carotene hydroxylase is involved in the hydroxylation of this compound to form myxoxanthophyll.

To test this hypothesis, the molecular masses of myxoxanthophyll from the wild-type strain and the new carotenoid from the Δ*crtR* strain were compared using mass spectrometry. These pigments were extracted from wild-type and Δ*crtR* *Synechocystis* cells, respectively, and were separated from other pigments and collected after HPLC analysis as described in Section 2. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was used to compare the molecular masses. Pigments were mixed with terthiophene (serving as a matrix) dissolved in acetone before analysis. The mass spectra of myxoxanthophyll and the new compound from the Δ*crtR* strain are shown in Fig. 4. In the Δ*crtR* mutant, the myxoxanthophyll-like compound was found to have a mass of 742, 16 mass units smaller than that found for myxoxanthophyll in the wild-type strain. This indeed is indicative of the loss of an oxygen in the myxoxanthophyll-like compound of the Δ*crtR* strain as compared to the usual myxoxanthophyll of *Synechocystis* sp. PCC 6803. Therefore, the β-carotene hydroxylase encoded by *crtR* may also be involved in the synthesis of myxoxanthophyll and may introduce the hydroxyl group in the β-ring. The myxoxanthophyll-like compound in the Δ*crtR* strain therefore is expected to be deoxy-

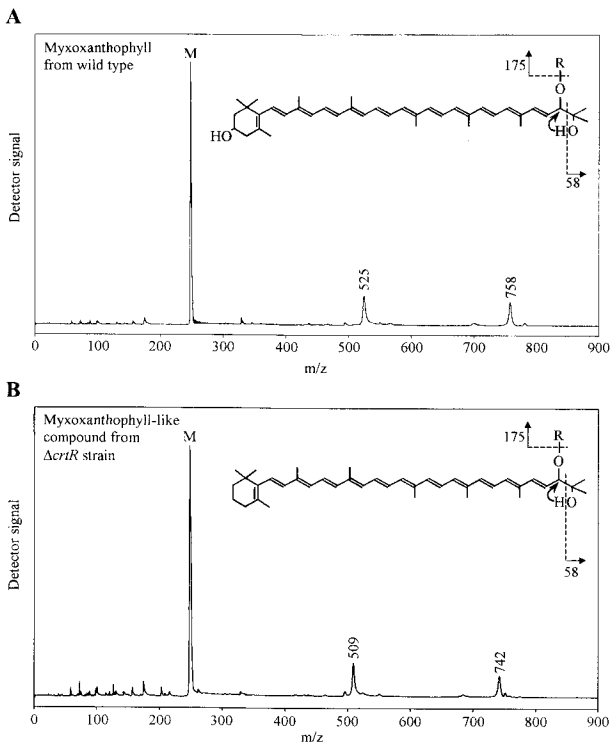

Fig. 4. Mass spectra of myxoxanthophyll from wild-type *Synechocystis* sp. PCC 6803 (A) and the myxoxanthophyll-like compound isolated from the Δ*crtR* strain (B) obtained by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. M, ion peak of the matrix (terthiophene, m/z 247.7).

myxoxanthophyll, even though an unequivocal structural elucidation of the compound will require NMR analysis.

The molecular mass of myxoxanthophyll ($C_{46}H_{66}O_7$) was expected to be 730 based on the determination of the structure of myxoxanthophyll isolated from *O. rubrescens* [8]. However, myxoxanthophyll isolated from *Synechocystis* sp. PCC 6803 had an ion peak at m/z 758. This result suggests that the *Synechocystis* sp. PCC 6803 myxoxanthophyll is 28 mass units heavier than the compound isolated from *O. rubrescens* [8]. As the absorption spectrum of myxoxanthophyll in *Synechocystis* is in agreement with the one that was previously published [8], the carotene backbone must be the same; however, the sugar moiety of this carotenoid glycoside may not be rhamnose. Instead, the apparent increase in molecular weight by 28 mass units suggests that the sugar moiety of the *Synechocystis* sp. PCC 6803 myxoxanthophyll has two additional CH_2 or an additional CO as compared to rhamnose.

Assuming that 758 represents the mass of myxoxanthophyll, the ion peak at m/z 525 (Fig. 4A) is expected to correspond to the carotenoid backbone resulting after cleavage of the carotenoid ether bond leading to a loss of a 175 mass unit (sugar moiety; $C_8H_{15}O_4$) and of a 58 mass unit (acetone; C_3H_6O) (Fig. 4A). This type of fragmentation had been previously observed [8,18]. By the same token, the two ion peaks m/z 742 and m/z 509 detected in the spectrum of the deoxy-myxoxanthophyll spectrum (Fig. 4B) corresponded to the molecular mass of deoxy-myxoxanthophyll and of the carotenoid lacking the sugar and the acetone group, respectively.

3.4. Light response of the ΔcrtR strain

The growth of the Δ*crtR* strain was studied at three different light intensities: 50, 100 and 200 μmol photons m^{-2} s^{-1}. Both the wild-type and the Δ*crtR* strains showed similar photoautotrophic growth rates at these three light intensities (doubling times about 13 h at all three light intensities); the highest intensity used is close to where in wild-type a slow down in growth is observed, presumably due to photodamage. These results suggest that the change in carotenoid content and composition does not affect the light sensitivity of the Δ*crtR* strain.

In conclusion, our study indicates that β-carotene hydroxylase appears to be involved not only in zeaxanthin synthesis but also in myxoxanthophyll biosynthesis in *Synechocystis* sp. PCC 6803. Furthermore, *Synechocystis* appears to have a different myxoxanthophyll than the one that had been first isolated from the cyanobacterium *O. rubrescens* [7,8]. These results are a first step towards the understanding of the myxoxanthophyll biosynthetic pathway.

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