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NOTES

Functional characterization of *sll0659* from *Synechocystis* sp. PCC 6803

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Synechocystis sp. PCC 6803 lacks a gene for the any known types of lycopene cyclase. Recently, we reported that *Sll0659* (unknown for its function) from *Synechocystis* sp. PCC6803 shows similarity in sequence to a lycopene cyclase gene—*Cru*A from *Chlorobium tepidum*. To test, whether *sll0659* encoded protein serves as lycopene cyclase, in this study, we investigated the carotenoids of the wild types and mutants. In the *sll0659* deleted mutant, there is no blockage at the lycopene cyclization step. Our results demonstrate that *sll0659* does not affect lycopene cycilzation. However, the ultrastructure of mutants suggests the involvement or necessity of *sll0659* in the cell division.

Keywords*:* *Synechocystis* sp. PCC6803, *sll0659*, Lycopene cyclase, Cell division

Carotenoid pigments are widely distributed in photosynthetic organisms and play a crucial role in light-harvesting and photoprotection[1,2]. Similar to plants, the biosynthesis of carotenoids in cyanobacteria starts with the condensation of two geranylgeranyl pyrophosphate (GGPP) molecules[1] by phytoene synthase, followed by the formation of four double bonds by phytoene desaturase and zeta-carotein desaturase to yield lycopene. Toward the end of the biosynthetic pathway, lycopene cyclase shapes the acyclic ends of lycopene into α or β rings.

So far, several types of lycopene cyclase such as crtY[3,4], crtL[5-7] and CruA[8] have been identified in different groups of organisms. Although cyclic carotenoids have been detected in *Synechocystis* sp. 6803, *Thermosynechococcus elongatus*, *Trichodesmium erythraeum*, *Gloeobacter violaceus*, *Crocosphaera watsonii* WH8501, *Nostoc punctiforme* and *Anabaena* sp.[9-11], presence of lycopene cyclases have not been reported in these cyanobacterial genomes. Thus, it would be of interest to determine the enzymes converting lycopene to β-carotene in these cyanobacteria.

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Although *sll0254* from *Synechocystis* is involved the formation of β-carotene, but no evidence for sll0254 catalyzed β-carotene formation is found in *E. coli*[12]. Recently[13], we reported that *sll0659* from *Synechocystis* sp. PCC6803 shows sequence similarity to the *Cru*A gene from *Chlorobium tepidum*. In this paper, we deleted *sll0659* from the same strain to investigate whether *sll0659* encoded protein serves as lycopene cyclase.

Materials and Methods

Strains and conditions

Synechocystis sp. PCC 6803 was cultivated in BG-11 medium at 30°C[14], supplemented with 5 m*M* glucose. For growth on the plates, 1.5% (w/v) agar was added. Antibiotic kanamycin was added at the concentration of 50 μg/ml for the *Synechocystis* mutants.

Construction of deleted mutants

The *sll0659* of *Synechocystis* sp. PCC6803 was cloned based on the sequence information released from the Cyanobase database (http://www.kazusa.or.jp/cyanobase/). The forward primer (GAG*CTCGAG*ATGAACTATGCAACTACA) contained a *Xho*I restriction site, while the reverse primer (CTC*GGTACC*TGGTAACAAAGGCAATA) a *Bam*HI restriction site. The PCR product was double-digested with *Bam*H I and *Xho*I, and then inserted into *Xho*I-*Bam*HI pBluescript II SK+ (Stratagene, USA). A 1.3 kb *Hin*d III fragment containing kanamycin-resistant cassette was ligated into the plasmid containing a *Hin*dIII site in *sll0659 coding region*, resulting in an insertional disruption construct of *sll0659*. This created the plasmid psll0659-k, which was directly used for the transformation of the wild-type *Synechocystis* sp. PCC6803 cells. Transformants were selected by screening for resistance to 50 μg/ml kanamycin in BG11 medium supplemented with 10 m*M* glucose according to previously described method[15]. Insertion of kanamycin resistant gene was confirmed by PCR.

Carotenoid analysis

Synechocystis sp. PCC 6803 cell were harvested by centrifugation and freeze-dried. Pigments were extracted from the freeze-dried cells by an acetone/methanol mixture (8:2, v/v) and analyzed using high performance liquid chromatography

(HPLC) on a Nucleosil C-18 reversed-phase silica gel (125 × 4.6 mm) (Waters, USA) with a linear 25 min gradient of ethyl acetate (0-70%) in methanol-water (8:2, v/v) at a flow rate of 1 ml/min. Absorbance spectra were recorded in 450 nm with online photodiode array detector.

Transmission electron microscopy

Synechocystis cells were fixed in 2.5-3% glutaraldehyde in 0.1 *M* sodium phosphate buffer, post-fixed in 1% osmium tetroxide in the same buffer for 3-5 h, then finally dehydrated in acetone and embedded in epoxy resin. They were washed in phosphate buffer and stained with 1.5% uranyl acetate for 2-5 h at room temperature, before dehydration in acetone and epon embedding[16]. Sections, stained with uranyl acetate and lead citrate were observed in an electron microscope.

Results and Discussion

Sll0659 from *Synechocystis* sp. PCC6803 showed 30% similarity at the amino acid level to the *Cru*A gene, which was found to be a new type of lycopene cyclase gene[13]. *Sll0659* ORF orthologues were found in only selected cyanobacterial genomes that lacked lycopene cyclase homologues[13]. To investigate the function of the probable lycopene cyclase genes in *Synechocystis* PCC 6803, we constructed deletion mutants of *sll0659* by targeting disruptions (Fig. 1A). The *sll0659* deletion strain was constructed by replacement of *sll0659* with a kanamycin-resistant gene cassette using established procedures, and complete segregation of the mutant allele was verified by PCR analysis (Fig. 1B). The PCR fragments containing each target gene from the mutants were about 1.3 kb longer than that from the wild type,

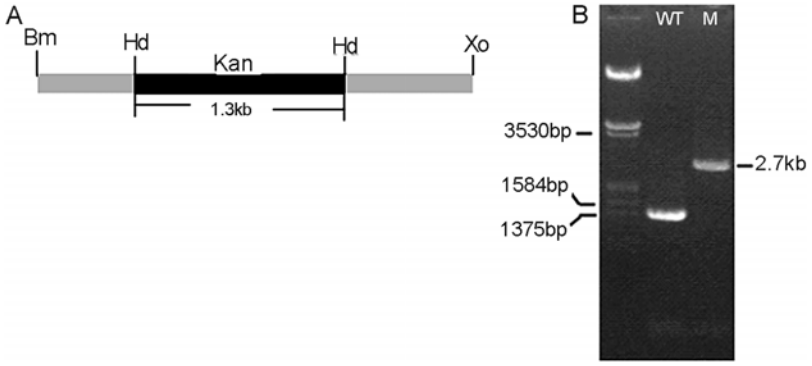

Fig. 1—Disruption of the *sll0659* gene [(A): Depiction of the constructs used to generate the *sll0659 deletion* strain. The *sll0659* gene shown in panel A was replaced by a 1.4 kb *sll0659* gene linked with a 1.3 kb kanamycin resistant cartridge (Kan); (B): PCR analysis of the *sll0659* gene in potential mutants in which the *sll0659* gene was insertionally inactivated with the *kan* gene. PCR verification was conducted using two primers that amplify the *sll0659* locus plus up and downstream flanks. Left lane, DNA molecular marker; WT, wild type (1.4 kb); M, mutant (2.7 kb). Bm, *Bam*H I; Xo, *Xho* I; Hd, *Hin*d III]

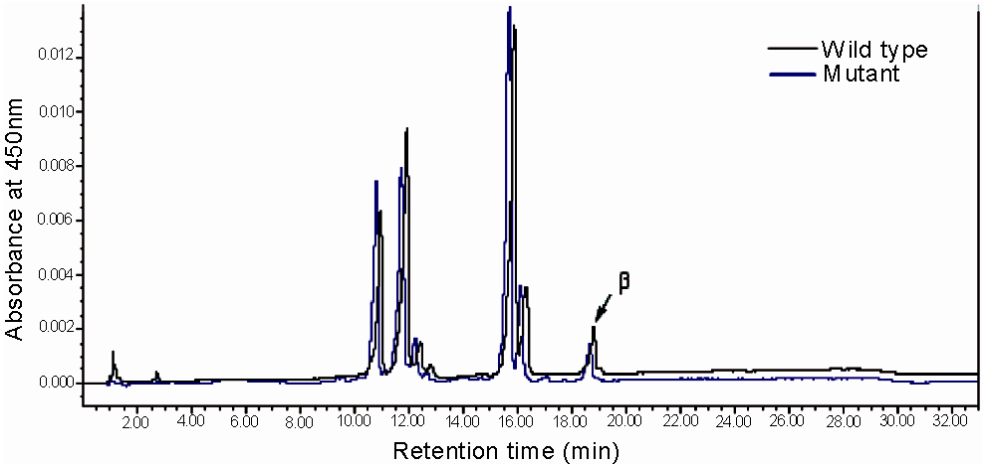

Fig. 2—HPLC fractionation of acetone/methanol extracts from transformants (blue line) and from wild-type (dark line) *Synechocystis* sp. PCC6803 [The absorption spectra of β-carotene was identified. B, β-carotene]

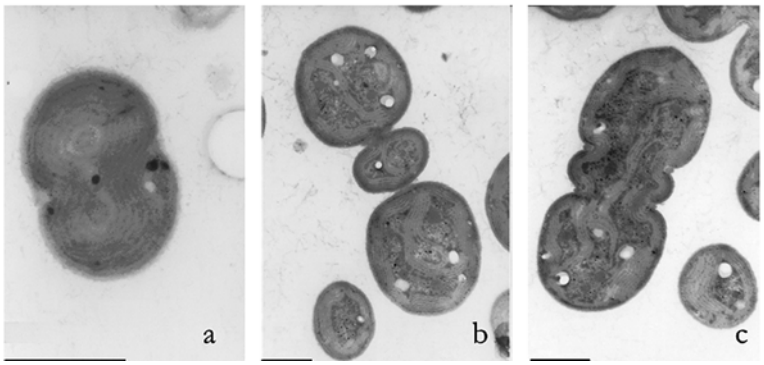

Fig. 3—Transmission electron micrographs of wild-type cells (a) and mutant cells (b, c) of *Synechocystis* PCC 6803 [The wild type cell dividing into two cells (a), three daughter cells formed from mutant cell (b), Scar bar, 1 μm]

indicating the insertion of kanamycin-resistant cassette. The correct insertion of the full-length cassette was verified by sequencing.

To test, if the carotenoids of the mutants had changed after the disruption of *sll0659*, pigments were analyzed by HPLC. Upon HPLC analysis, the spectrum profile in the mutant was almost the same as that of the wild type (Fig. 2). The presence of β-carotene peak in the mutant suggested that absence of *sll0659* did not affect the synthesis of β-carotene, indicating that cyclization of lycopene was not directly dependent on *sll0659*. Sll0659 might be inactive under normal conditions, a phenomenon observed earlier in cyanobacteria *Synechococcus* sp. strain PCC 7942[17], where glycosylated carotenoids were not detected under conditions of normal growth. Perhaps, sll0659 functions as cyclase, only in the specific growth states,such as the late growth phase, in which no difference of carotenoids was detectable. But, undoubtedly, a protein, other than sll0659 existing in the cell, is catalyzing β-carotene formation. Although sll0254 is reported to function as a cyclase/oxygenase, no evidence is found for sll0254 catalyzed β-carotene formation directly[12]. Thus, further studies are needed to determine which protein functions as cyclase in *Synechocystis* PCC 6803.

The ultrastructure of mutants and wild types was investigated by transmission electron microscopy. As shown in Fig. 3, the thylakiods were much distinct in mutant cells (Fig. 3b,c) and larger by 30-50% than wild type cells (Fig. 3a), as expected. But, the photoautotrophical growth of mutants was almost the same as that of the wild type (data not shown), indicating that *sll0659* gene had no significant effect on the growth. Additionally, a unique feature in the mutants was the abnormal phenotype of cell division. In some cells, there were four concavities and cells divided into three daughter cells — two larger cells and one smaller cell (Fig. 3b,c). This result indicated that *sll0659* was involved in the cell division either directly or indirectly.

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