Abstract The sll1961 gene was reported to encode a regulatory factor of photosystem stoichiometry in the cyanobacterium *Synechocystis* sp. PCC 6803. We here show that the sll1961 gene is also essential for the phycobilisome degradation during nitrogen starvation. The defect in phycobilisome degradation was observed in the sll1961 mutant despite the increased expression of nblA, a gene involved in phycobilisome degradation during nitrogen starvation. Photosystem stoichiometry is not affected by nitrogen starvation in the sll1961 mutant nor in the wild-type. The results indicate the presence of a novel pathway for phycobilisome degradation control independent of nblA expression.

Keywords: Cyanobacteria; Nutrient limitation; Photosystem stoichiometry; Phycobilisome degradation

1. Introduction

Photosynthetic organisms have developed various mechanisms that enable them to acclimate to ever-changing environmental conditions such as light or nutrient availability. These processes involve the coordination of different reactions in photosynthesis, or that of photosynthesis with other metabolic pathways [1,2]. For example, nitrogen assimilation requires reducing power produced by photosynthetic electron transfer, so that coordination between these two metabolic pathways is indispensable [3]. Modulation of photosystem stoichiometry, i.e. the ratio of two photosystems (PSI and PSII), is one of the regulatory mechanisms of photosynthesis and is important for the optimization of photosynthesis in response to changes in light intensity [4,5], light quality [6–8] and certain environmental stress conditions [9,10]. Under high light conditions, modulation of photosystem stoichiometry is essential to down-regulate photosynthetic electron transfer [11]. In our previous study, the sll1961 was identified as a gene involved in the modulation of photosystem stoichiometry in *Synechocystis* sp. PCC 6803 [12]. The phenotype of sll1961 disruptant is similar to that of pmgA mutant, pmgA being another gene that is involved in the regulation of photosystem stoichiometry under high light condition [5,11,13]. Both mutants lost the ability to suppress the level of PSI under high light condition [11,12]. Although several other genes were reported to be involved in the regulation of photosystem stoichiometry [14], the relationship among these components remains unknown.

An ortholog of the pmgA gene in *Synechococcus elongatus* PCC 7942 is necessary for the degradation of phycobilisome (PBS) upon nitrogen starvation, and was named nblC [15]. During nitrogen starvation, the degradation of PBS plays a critical role to meet cellular nitrogen demand [16]. The degradation of PBS results in a color change of the culture from blue-green to yellow, a process called bleaching [17,18]. During nitrogen starvation, NblC regulates transcriptional induction of nblA, whose product was reported to interact with some phycobiliprotein subunits [19,20] and to be involved in PBS degradation in *S. elongatus* PCC 7942 [15]. NblA orthologs are found in many cyanobacteria and red algae [21–23]. In *Synechocystis* sp. PCC 6803, there are two nblA genes, namely nblA1 (ssl0452) and nblA2 (ssl0453). Both genes are required for PBS degradation during nitrogen starvation [21].

The fact that the *Synechococcus* sp. PCC 7942 ortholog of pmgA, previously reported to be involved in the regulation of photosystem stoichiometry under high light conditions, is involved in the response to nitrogen starvation may suggest existence of a crosstalk between the two acclimatory responses. In the present study, we compare the effects of nitrogen starvation on pmgA and sll1961 mutants: while the pmgA mutant is able to degrade its PBS, the sll1961 mutant has a non-bleaching phenotype, demonstrating that the gene product of sll1961 participates in the degradation of PBS.

2. Materials and methods

A wild-type strain, the sll1961 mutant (0205-79) [12] and pmgA (sll1968) mutant disrupted by spectinomycin cassette [24] of *Synechocystis* sp. PCC 6803 were grown with bubbling of air (i.e. about 0.04% CO₂) as described previously [12], except for the growth light condition (50 μmol m⁻² s⁻¹). To confirm the non-bleaching phenotype, deletion mutant of sll1961 with spectinomycin cassette [12] was also used. For nitrogen starvation experiments, exponentially grown cells were harvested by centrifugation and washed with BG11 medium lacking nitrate (BG11₀). The washed cells were resuspended in BG11₀ and used for experiments.

Disruptant of nblA was generated by the replacement of a part of nblA genes (53rd nucleotide of nblA1 to 97th nucleotide of nblA2) with a spectinomycin-resistant cassette using SphI and Hpal sites. The nblA disruptant was grown in the presence of 20 μg/ml spectinomycin.

Absorption spectra of whole cells were measured using a spectrophotometer (Model 356, Hitachi, Tokyo, Japan) [15]. Chlorophyll (Chl) and phycocyanin (PC) contents were calculated using the equa-
tions of Arnon et al. [25]. The number of cells was determined by measuring OD730 (1.1 × 10^7 cells/ml at OD730 [14]) with a spectrophotometer (GeneSpec III, Hitachi, Tokyo, Japan).

Fluorescence emission spectra at 77 K were measured as described in [12] with a band-pass filter (CS 4-96, Corning, NY, USA) and a dichroic filter (DF Blue, Optical Coatings, Tokyo, Japan) for Chl excitation. Before fluorescence measurements, cells were dark adapted (>10 min) at room temperature to lock the cells to state II and frozen in the dark with liquid nitrogen.

For Northern blot analysis, total RNA was isolated from the cells as described in [32]. RNA was subjected to electrophoresis on 0.9% agarose gels (10 μg of total RNA was loaded per lane) and blotted onto nylon membranes (Pall Corporation Biodyne PLUS). DIG labeling and detection were performed according to the system application manual (Roche, Mannheim, Germany). To make a DIG-labeled DNA probe, nblA1–2 was amplified by PCR and then labeled with DIG (Roche, Mannheim, Germany). The following primers were used for the probe by PCR: nblA forward: 5'-TTGGAGGGGCGAAGCTATGAA-3', nblA reverse: 5'-GGGGAGGAGTGAATTTTTCATC-3'.

For RT-PCR, total RNA was isolated by the RNeasy Midi kit (Qiagen, Hilden, Germany) and treated with DNasel to eliminate genomic DNA. RT-PCR was performed by TaKaRa RNA PCR kit (TaKaRa, Tokyo, Japan) using 0.5 μg of total RNA. The following primers were used for amplification of sll1961 or rnpB: sll1961 forward: 5'-ATGCTACAGTTCCAAATTCA-3', sll1961 reverse: 5'-TTAAAGCGTGCCACCTTTTG-3', rnpB forward: 5'-CGCCCATGTGCGGCGAGCGTGAGGA-3', rnpB reverse: 5'-CTCTCGACCTTGCTTCCAACCGGG-3'. DNA was amplified using PTC-200 Peltier thermal cycler (MJ Research, USA).

### 3. Results and discussion

#### 3.1. sll1961 is involved in PBS degradation under nitrogen starvation

Wild-type (WT) cells grown in complete BG11 medium showed typical blue-green color (Fig. 1A-1), while those grown in medium lacking nitrogen source (BG110) showed bleaching after 48 h (Fig. 1A-4). Although the sll1961 disrupted mutant was indistinguishable from the WT grown in BG11 (Fig. 1A-2), the mutant grown in BG110 remained blue-green (Fig. 1A-5), suggesting that the reduction in the amount of PBS was impaired in the sll1961 mutant. In contrast, the pmgA mutant became chlorotic in BG110 (Fig. 1A-6). These changes in color could be confirmed by the change in the absorption spectra of the cell cultures. The absorption around 620 nm due to PC in PBS decreased during nitrogen starvation of the WT and the pmgA mutant cells but not of the sll1961 mutant cells (Fig. 1B). The levels of both Chl and PC decreased in all strains after the shift to BG110 (Fig. 1C and D), but the decrease in the PC content of the sll1961 mutant was less prominent, leading to the constant PC/Chl ratio upon nitrogen starvation (Fig. 1E, squares). Non-bleaching phenotype was also observed in the deletion mutant of sll1961 with spectinomycin cassette (data not shown). The results indicate that the sll1961 mutant has a defect in the reduction of PBS content upon nitrogen starvation, in addition to the defect in the regulation of photosystem stoichiometry under high light conditions that was previously reported [12]. The lack of decrease of PBS upon nitrogen starvation in the sll1961 mutant is not due to the enhanced synthesis of phycobiliproteins, since the suppression, rather than the enhancement, of the expression of the cpc genes encoding PBS components was observed during nitrogen starvation (Fig. 2). The pmgA mutant showed normal pigment reduction upon nitrogen starvation, unlike the nblC mutant in S. elongatus PCC 7942. In Synechocystis 6803, mutations in the orthologs of nblS (dspA) and nblR, which regulate PBS degradation in S. elongatus PCC 7942, result in normal PBS degradation and normal expression of nblA under nitrogen starvation [26,27]. Apparently, the mechanism of bleaching is different between S. elongatus PCC 7942 and Synechocystis sp. PCC 6803.

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**Fig. 1.** Pigment profiles during nitrogen starvation. (A) Color of liquid culture of WT (1 and 4), the sll1961 mutant (2 and 5) and pmgA mutant (3 and 6) grown in complete medium (1–3) or in nitrogen-deficient medium (BG110) for 48 h (4–6). (B) Absorbance spectra of whole cells of WT, the sll1961 mutant (Δsll1961) and the pmgA mutant (ΔpmgA) without (+N) and without (−N) nitrogen in BG11 medium. (C) The levels of phycocyanin (PC) on a per-cell basis in WT (circles), sll1961 mutant (squares) and pmgA mutant (triangles) during nitrogen starvation. (D) PC/Chl ratio. The 100% values correspond to the values before the shift to BG110. (E) rRNA was used as a loading control (lower panel).

**Fig. 2.** Expression of cpc genes encoding phycocyanin during nitrogen starvation. Northern blot analysis of cpcBA expression was performed in WT and the sll1961 mutant at 0, 3, 6, 12, 24 h following the shift to BG110. A DNA probe specific for cpcA was used for hybridization (upper panel). rRNA was used as a loading control (lower panel).
3.2. Effect of sll1961 disruption on the expression of nblA under nitrogen starvation

In all the nbl (non-bleaching) mutants reported so far, the level of nblA expression is lower than that in WT under nitrogen starvation [15,28–31], suggesting that nblA is a primary factor in PBS degradation. In WT cells, the transcript levels of nblA (nblA1 plus nblA2) increased after the shift to BG110 medium (Fig. 3). The increase of the transcript level of nblA during 12 h of incubation in BG110 medium was more prominent in the sll1961 mutant than in the WT. Thus, the inactivation of sll1961 leads to enhancement, rather than suppression, of the transcription of nblA under nitrogen starvation. In contrast, inactivation of nblA partially lowered the transcript level of nblA. The result suggests that pmgA may function in the positive regulation of nblA during nitrogen starvation, as previously reported for nblC in S. elongatus PCC 7942 [15]. In spite of less expression of nblA, normal bleaching was observed in the pmgA mutant, implying that mRNA level of nblA was sufficient for PBS degradation during nitrogen starvation in the pmgA mutant. In S. elongatus PCC 7942, disruptants of nblR, nblS and nblC all showed defects leading to a decreased nblA expression under nitrogen starvation [15,28–31]. Thus, it was assumed that the phenotype of the disruptants of nbl genes was ascribed to the decreased level of nblA in the mutants. The pmgA mutant, however, exhibits typical bleaching phenotype with lowered expression of nblA. This raises the possibility that there is some mechanism of PBS degradation independent of the mRNA accumulation of nblA.

3.3. mRNA level of sll1961 was lower in the nblA mutant under nitrogen starvation

The transcript level of sll1961 was low in WT cells grown in BG11 medium but increased after the shift to BG110 medium (Fig. 4). In contrast, the transcript level of sll1961 in an nblA1–nblA2 deletion mutant did not show significant increase even after the shift to BG110 medium (Fig. 4). The nblA mutant showed non-bleaching phenotype when grown in BG110 medium for 48 h (data not shown) as previously reported [26]. The result implies a decreased stability of sll1961 mRNA in the nblA mutant during nitrogen starvation, although one could not rule out the possibility that NblA acts upstream of sll1961 and up-regulates sll1961 to produce or activate other factor(s) such as protease during nitrogen starvation. Furthermore, the non-bleaching phenotype of the nblA mutant might be linked to the reduced level of the sll1961 transcript.

3.4. Nitrogen starvation does not cause a change in photosystem stoichiometry

We then tested whether the change in photosystem stoichiometry is brought about by nitrogen starvation and is regulated by sll1961. When chlorophyll fluorescence emission spectra is determined at 77 K, cyanobacterial cells show PSI fluorescence band at around 725 nm (F725) and PSII fluorescence band at around 695 nm and 685 nm (F695, F685). As a result, we can use F725/F695 as an index of photosystem stoichiometry. We determined fluorescence spectra with Chl excitation to avoid secondary effect of the change in PBS content on the fluorescence spectra through energy transfer from PBS to photosystems. Neither WT nor the sll1961 mutant showed any difference in photosystem stoichiometry after 48 h of nitrogen starvation (Table 1). The result demonstrates that photosystem stoichiometry is not different between WT and the sll1961 mutant irrespective of nitrogen availability.

Photosystem stoichiometry is modulated under various environmental conditions including different photon flux densities, different light quality and different salinity. The modulation was induced by the change in the redox level of electron transport [32], although little is known about the actual sensing mechanism. Since excess CO₂ fixation under high light condition can induce a high carbon/nitrogen (C/N) ratio in cellular metabolisms, the change in C/N balance might be sensed as a high light signal [33]. However, we showed that photosystem stoichiometry was not affected by nitrogen starvation, indicating that the signal for high light and the signal for nitrogen starvation are completely different. We propose that Sll1961 is a bi-functional protein that regulates both photosystem stoichiometry under high light condition and PBS degradation under nitrogen starvation. The gene product of sll1961 may act as a regulatory factor for PBS degradation independently of nblA induction under nitrogen starvation.

Table 1 Photosystem stoichiometry of WT and the sll1961 mutant before or after the shift to BG110 medium for 48 h

<table>
<thead>
<tr>
<th>Strain</th>
<th>F725/F695</th>
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<tr>
<td>WT</td>
<td>6.84 ± 0.40</td>
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<tr>
<td>Δsll1961</td>
<td>6.70 ± 0.44</td>
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The ratio of the peak height of PSI band (F725) and PSII band (F695) of the chlorophyll fluorescence spectra determined at 77 K was calculated for three different cultures and shown as means ± S.D.

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