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ORIGINAL ARTICLE

Sll0396 regulates transcription of the phycocyanin genes in *Synechocystis* sp. PCC 6803

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Abstract An olive-green mutant was generated in *Synechocystis* sp. strain PCC 6803 by inactivation of the *sll0396* gene. Whole-cell absorption spectra of the mutant revealed the missing of phycocyanin peak. An investigation of the low-temperature fluorescence emission spectra revealed that the *sll0396*Ω mutant has a reduced amount of phycocyanin. Western blot analysis showed that the mutant contained less phycocyanin β- and α-subunits and lacked the 30- and 32-kDa linker polypeptides, and northern blot analysis revealed that the transcription of the 1.4-kb *cpcBA* gene encoding the phycocyanin β- and α-subunits was lower in the mutant. The Sll0396 protein has a DNA-binding motif and shares homology with known response regulators. Our results indicate that Sll0396 plays a regulatory role in the transcription of the phycocyanin genes during phycobilisome synthesis.

Keywords Interposon mutagenesis · Phycobilisomes · Phycocyanin · Response regulator · *Synechocystis* sp. PCC 6803 · Transcription regulator

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Introduction

Light-harvesting in cyanobacteria is mediated by phycobilisomes (PBS), which are complex protein structures on the surface of the photosynthetic membrane. The major structural components of the PBS are the phycobiliproteins (PBPs). Light energy in the 500- to 650-nm range can be absorbed by different classes of PBPs and is rapidly and efficiently transferred through the PBS to chlorophyll complexes in the photosynthetic membrane. A typical hemidiscoidal PBS of cyanobacteria has two domains: a core and six rods emanating from the core. Each core is composed of three cylindrical structures that are parallel to one another and to the plane of the thylakoid membrane. The three major elements of PBPs are distinguished by their spectral properties. The allophycocyanin (APC: λ_{max} 650–665 nm) is located in the core of the PBS, which is in direct contact with chlorophyll complexes in the membrane. Phycocyanin (PC: λ_{max} 620 nm) is found in the rod substructures that are attached to the PBS cores. A third major PBP, phycoerythrin (PE: λ_{max} 565 nm), is synthesized in some—but not all—cyanobacteria and is attached to PC at the periphery of the rod substructures. *Synechocycistis* sp. PCC 6803 (hereafter referred to as Syn6803) is a cyanobacteria which does not have PE (Silder 1994). The PBPs are composed of two subunits, α and β, which are organized into $(\alpha\beta)_6$ hexamers; APC hexamers are located in the core substructure of PBS while PC hexamers form in the rod substructures that radiate from the core. The PC hexamers are associated with non-pigmented linker polypeptides rods.

The structure of the PBS in wild-type *Synechocystis* sp. PCC 6803 was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopy (Elmorjani et al. 1986). The molecular

masses of the four colored polypeptides were reported to be 19,800 (β-PC), 17,600 (α-PC), 18,800 (β-APC), and 16,200 (α-APC), and those of the four colorless linker proteins 92,000, 35,000, 33,000, and 27,000. A comparison of the electrophoretic profiles identified the M_r 27,000 polypeptide as the PC (phycocyanin) linker at the core-proximal end of the rods and the M_r 35,000 and 33,000 polypeptides as those located in that order from the core outward. In particular, the highest molecular weight polypeptide (L_{CM}) plays a crucial role in maintaining the structure of the core and in attaching the PBS to the thylakoid membrane (Capuano et al. 1991; Capuano et al. 1993).

PBS are degraded in a specific and ordered manner during nitrogen starvation in cyanobacteria via a process that requires NblA, a small polypeptide of 59 amino acids. NblR has been identified as the response regulator that controls transcription of the *nblA* gene in *Synechococcus* sp. strain PCC 7942, and a high degree of similarity between the NblR and the Sll0396 proteins has been demonstrated (Schwarz and Grossman 1998). The *sll0396* gene was designated *rre*28 by Zinchenko (Cyanobase: http://www.bacteria.kazusa.or.jp) and was reported to encode an OmpR-type two-component response regulator. Richaud et al. (2001) reported that NblR controls PBS degradation via the induction of *nblA* and probably controls additional functions critical for cell survival and went on to further state that studies of the NblR equivalent (Sll0396) could provide new information on the pivotal role of NblR and the differences in the regulatory and signal transduction pathways between Syn6803 and *Synechococcus* strain PCC 7942.

We report here that disruption of the *sll0396* gene in Syn6803 by interposon mutagenesis produced a mutant that is olive-green—rather than the blue-green of the wild type—under both normal and nitrogen starvation conditions. We also discuss how Sll0396 functions as a transcription regulator for the synthesis of β- and α-subunits of PC for the assembly of PBS.

Materials and methods

Strains and culture conditions

Syn6803 was obtained from the Pasteur Culture Collection. The wild-type and mutant strains were grown in BG-11 medium at 30°C under white-fluorescent light provided at an intensity of 30 μmol photons $m^{-2}s^{-1}$. Glucose (5 mM) was supplied as indicated.

Targeted disruption of the *sll0396* gene

Genomic DNA was isolated from Syn6803 as described by Porter (1998). The full-length *sll0396* gene was amplified by PCR using Syn6803 chromosomal DNA and the primers 5′-CAACCGTAGCCAGGAAATGA-3′ and 5′-CTATGGGGAATGGGAAGGAA-3′. The resulting fragment (1.3 kb) was cloned into the *EcoR*I site of pCR II (Stratagene, La Jolla, CA). To disrupt *sll0396*, we inserted a polar spectinomycin-resistance (Sp^r) gene (the *Sma*I fragment from pHP45Ω-Sp) (Prentki and Krisch 1984) into the internal *Hinc*II site of *sll0396*. The plasmid containing the disrupted gene was then used to transform Syn6803, and Sp^r transformants were selected. Genomic DNA was isolated from individual transformants, and PCR was used to confirm that all chromosomal DNAs carried the disrupted gene.

Southern blotting

Southern blot analysis of DNA was performed using a standard procedure (Southern 1975). The probe used in DNA blot analysis, a 222-bp fragment of *sll0396* generated from the PCR product described above by using *Hinc*II and *Ban*I, was labeled with ^{32}P using a Bca BEST labeling kit (Takara Shuzo, Kyoto, Japan).

Absorption and fluorescence spectra

Whole-cell absorption spectra were generated at room temperature using a spectrophotometer (model UV 2401-PC; Shimadzu, Kyoto, Japan) according to the method of Bruns et al. (1989). Whole-cell fluorescence emission spectra were recorded at 77 K according to the method of Grossman and co-workers (Bruns et al. 1989) using a spectrofluorometer (model 4800; LM Aminco Instruments, Rochester, NY).

Biliprotein quantification

The amounts of PC, APC, and chlorophyll were measured in exponentially growing cells (Tandeau de Marsac and Houmard 1988).

SDS-PAGE/Western blotting

Whole-cell protein samples were prepared by first grinding the cells with zirconium beads and then centrifuging the cellular material at 10,000 *g* for 10 min at 4°C. The supernatant was then separated by SDS-PAGE (16%) using the Laemmli buffer system (Laemmli 1970). Western blotting was performed according to standard protocols. Antibodies were made against the anchor protein (antibody no. 1 in Fig. 1 of Melchers and co-workers; Melchers et al. 1986), linker proteins (antibodies nos. 6 and 7), and the β- and α-subunits of PC (antibodies nos. 9 and 11) in *Porphyridium aerugineum* (Egelhoff and Grossman 1983).

The molecular weights of the proteins (antibodies nos. 1, 6, 7, 9, and 11) were 94, 38, 23, 17, and 16 kDa, respectively.

Northern blotting

The isolation of total RNA from Syn6803 and Northern blotting was performed using standard procedures (Mohamed and Jansson 1989). A fragment of the PC gene was amplified from genomic DNA using the primers 5′-GC TGAAGGCAACAAACGG-3′ and 5′-GAGGTCAAAGG TGCGGTT-3′. The resulting 0.9-kb PCR product was ^{32}P-labeled using a Bca BEST labeling kit (Takara Shuzo) and used as the hybridization probe. The RNA was quantified using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Results

Color mutant generated by disruption of the *sll0396* gene

The cyanobacterium Syn6803 harbors 3,661 putative genes, of which 47 encode histidine kinases and 45 encode regulators (Cyanobase). As a first step to elucidate the function of each of the kinases and the regulators, we attempted to make a series of disrupted mutants of these genes by inserting a spectinomycin resistance gene from the plasmid pHP45Ω-Sp into the open reading frame (ORF) of the genes. The disruption of one of the response regulator genes, *sll0396*, produced an interesting result in that the phenotype of the resulting mutant, *sll0396*Ω, exhibited an olive-green color, which differs from the blue-green color of the wild-type strain. In order to confirm whether the mutation in the *sll0396* gene was responsible for the phenotype, we performed PCR and southern blot experiments on the mutant. The presence of the mutation in the *sll0396* gene was confirmed by PCR and also by Southern blotting (data not shown).

Spectroscopic measurements

The absorption spectrum of the wild-type strain contained three major peaks, representing light absorption by carotenoids at 450 nm, by PC at 620 nm, and by chlorophyll-*a* at 680 nm (Fig. 1a). In contrast, the absorption data from the *sll0396*Ω mutant included a higher peak at 450 nm and a small peak, if present at all, at 620 nm, suggesting that the mutant contained a low level of PC. The mutant and wild-type strains grew at almost the same rate in BG-11 medium with or without 5 mM glucose (not shown). The APC content of the mutant was roughly the same as that of the wild-type cells; however, PC content in extracts prepared from mutant cells was approximately 20–30% of that in extracts prepared from wild-type cells, when normalized to the values for chlorophyll (Table 1).

Whole-cell 77-K fluorescence emission spectra

Figure 1b shows the 77-K low-temperature fluorescence emission spectral data for the wild-type and mutant cells, with excitation at 600 nm (PC). The different emission bands present in the spectra were assigned to PC (648–650 nm),

Table 1 Phycobiliprotein expression in wild-type Syn6803 and *sll0396*Ω mutant cells

Strain	A_{730}	Phycobiliprotein level (mg/ml)		Ratio ($\times 10^{-3}$)	
		PC	AP	PC/Ch	AP/Ch
Wild-type	0.52	0.112	0.060	13.9	7.5
*sll0396*Ω	0.52	0.029	0.063	3.1	6.6

AP Allophycocyanin, *PC* phycocyanin, *Ch* chlorophyll *a* (unit: mg/l)

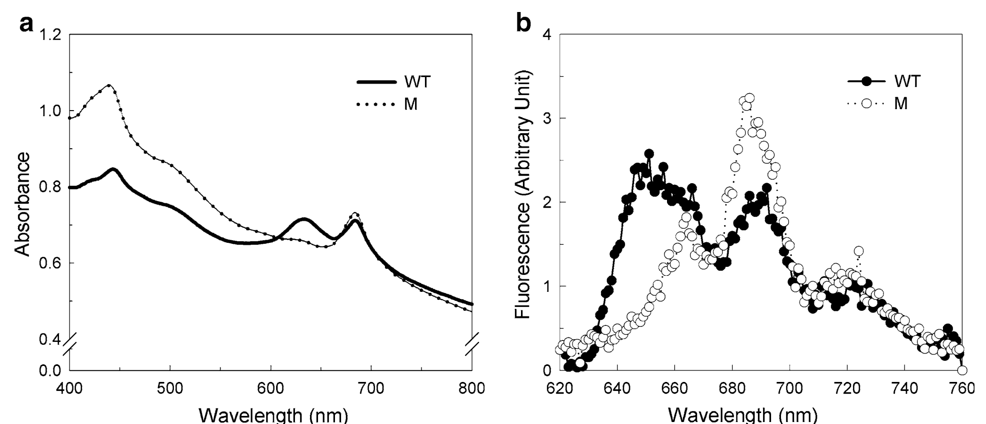

Fig. 1 Whole-cell absorption spectra (**a**) and 77-K fluorescence emission spectra (**b**) of wild-type (*WT*) and mutant (*M*; *sll0396*Ω) cells. The excitation wavelengths were 600 nm (phycocyanin, *PC*). The fluorescence spectra were generated on a scanning SLM-Aminco 4800 spectrofluorometer, which makes it possible to obtain corrected spectra using rhodamine B as a quantum counter

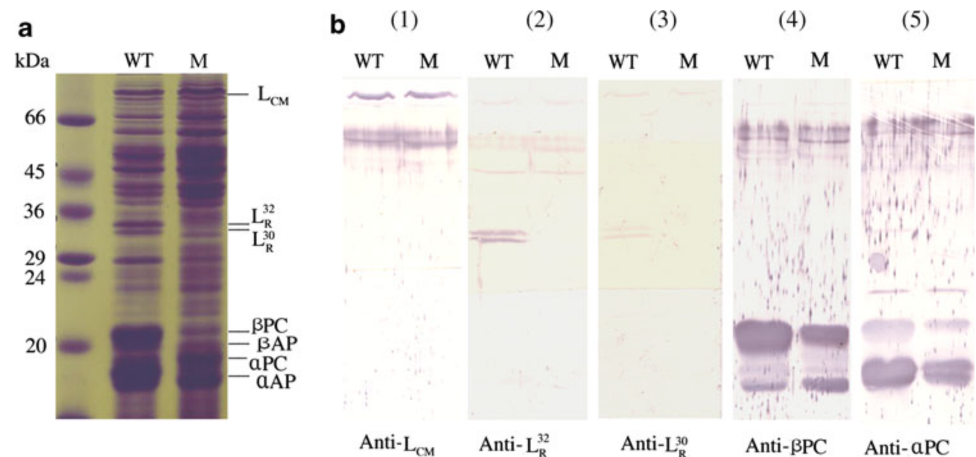

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole-cell proteins (**a**) and western blot analysis of linker protein and major phycobiliprotein expression in wild-type (*WT*) and *sll0396*Ω (*M*) cells (**b**). The antibodies were raised against anchor protein (*1*), linker proteins (*2*, *3*), and the *β*- (*4*) and α-subunits (*5*) of PC. The molecular weight markers (*X*) are 66, 45, 36, 29, 20, and 14 kDa. *Lanes*: *1* Molecular marker, *2* wild-type sample, *3* mutant sample

APC (664–665 nm), photosystem II (PSII)-connecting APC-B (685 nm), PSII (690–695 nm), and PSI (725–730 nm) (Elmorjani et al. 1986; Su et al. 1992).

When PC was excited at 600 nm, the wild-type cells exhibited three major peaks (PC, APC, and PSII) and a minor shoulder peak (PSI). In contrast, in the mutant, the PC peak was not detected at all, and the APC-B peak was higher than that in the wild-type. However, the peaks corresponding to APC, PSII, and PSI in the mutant were similar to those in wild-type (Fig. 1b). These results clearly demonstrate that the *sll0396*Ω mutant has a reduced emission at the PC wavelength.

Western blot analysis

Western blotting was used to determine whether PBP expression was also altered in the mutant. Whole-cell proteins of wild-type and mutant cells were prepared and analyzed by SDS-PAGE and immunoblotting (Fig. 2). The *sll0396*Ω mutant has normal amounts of APC, 27-kDa linker, and 99-kDa anchor polypeptides in its PBS (data not shown); however, it had reduced amounts of the *β*- and α-subunits of PC and did not express the 30- and 32-kDa linker polypeptides.

PC gene transcription

Northern blot analyses revealed the presence of a 1.4-kb transcript of the *cpcBA* (A and B subunit of cyanobacterial phycocyanin) gene in both the wild-type and *sll0396*Ω mutant strains, but the level of transcription in the mutant was reduced (Fig. 3).

Discussion

The mutant was produced by insertional inactivation of an ORF designated as *sll0396* in the Syn6803 sequence

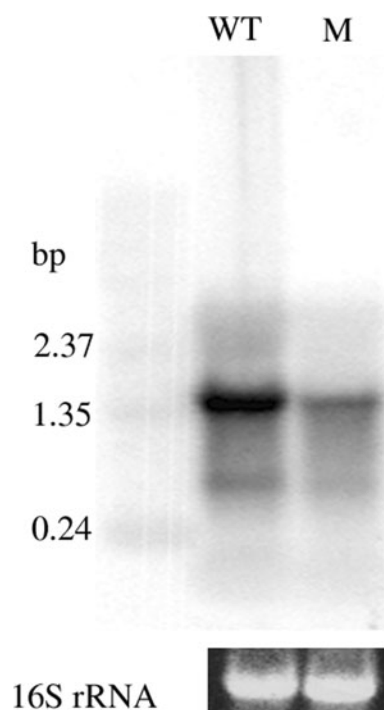

Fig. 3 Expression of *cpcAB* in *Synechocystis* 6803 cells. Total RNA from wild-type (*WT*) and *sll0396*Ω mutant (*M*) cells was hybridized with probes for the PC genes (*upper*) and 16S RNA (*lower*). Each *lane* was loaded with 5 μg of total RNA

(Kaneko et al. 1996). The *sll0396*Ω mutant has normal amounts of APC, 27-kDa linker, and 99-kDa anchor polypeptides in its PBS, but its PC content is reduced and it has no 32- and 30-kDa linker polypeptides.

Similar PC-less or pigment-deficient mutants have been isolated in Syn6803 (Elmorjani et al. 1986; Plank et al. 1995). A yellow-blue pigment mutant, PMB 2, retained only 20–30% of the PC present in wild-type cells (Elmorjani et al. 1986). SDS-PAGE experiments showed that this mutant had decreased levels of the PC *β*- and α-subunit, no 30- or 11.1-kDa linker polypeptides, and a

variable amount of the 32-kDa polypeptide. Electron micrographs of PMB 2 revealed that the PBS core was normal but was surrounded by up to six rods of either one or two—but never three—disks in length. Plank et al. (1995) reported a PC-minus mutant, 4R, that lacked the light-harvesting protein PC but had a normal level of PC transcription. Sequence analysis of the *cpcB* gene in 4R, which encodes β-PC, showed an insertion mutation that causes early termination of translation. The interruption of one subunit was accompanied by a complete absence of the unassembled partner subunit. Taken together, these data show that the formation of the PC monomer is critical for the assembly of biliproteins.

Our *sll0396*Ω mutant grew at almost the same rate as the wild-type strain on BG-11 medium with or without 5 mM glucose, even though the mutant has a low PC content, as also found in the PMB 2 mutant (Elmorjani et al. 1986). Our results show that in the *sll0396*Ω mutant, PC fluorescence is greatly reduced while PSII fluorescence is increased and PSI fluorescence is normal. The PC-deficient mutant PD-1 of *Synechocystis* PCC 6714 had only 30% of the PC normally present in wild-type cells; however, the maximum level of photosynthetic activity was almost equal between the two cell types at high cell density (Nakajima and Ueda 1997). The major pathway of excitation energy transfer is PC → APC → PSII in wild-type cells (Su et al. 1992). Thus, the maximum level of photosynthetic activity in this mutant may be the same as that in wild-type cells. Although *sll0396*Ω had about 20–30% of the wild-type level of PC, it was able to grow at an almost normal rate. One possible explanation for this (almost) normal growth rate may be that wild-type Syn6803 cells contain an excess amount of PC, which is not connected to PSII and which serves no function in photosynthesis (Elmorjani et al. 1986). A similar phenomenon has been observed in *Synechocystis* sp. PCC 6301 (Yamanaka et al. 1980).

The results from the immunoblot analyses indicate that PC subunit expression was reduced in the *sll0396*Ω mutant and that the 30- and 32-kDa linker polypeptides were not expressed. The distal and most distal rod linker polypeptides (32 and 30 kDa, respectively) were undetectable in the *sll0396*Ω mutant, probably because the unassembled proteins were degraded (Su et al. 1992). Most correctly folded proteins are stable because their proteolysis-sensitive domains are hidden; however, damaged, misfolded, or unassembled proteins may be rapidly degraded. Some classes of proteins have very short half-lives due to their N-terminal residue and/or their interactions with other proteins (Collier and Grossman 1994). Taken together with these previously reported results, our results suggest that a normal PBS core is present in *sll0396*Ω but that it is surrounded by six rods, each one disk in length.

We examined whether *sll0396* affects the biosynthesis of the PC subunits at the level of transcription. There was a high level of *cpcBA* transcription in wild-type cells, but in the mutant *cpcBA* transcription was <20–30% of that of the wild type. In Syn6803, the *cpc* operon contains five genes: *cpcB* and *cpcA*, which encode the β- and α-PC subunits, respectively, and *cpcC2*, *cpcC1*, and *cpcD*, which encode the rod linkers L_R^{30}, L_R^{32}, and L_R^{10}, respectively (Cyanobase). The wild-type operon specifies three differently sized mRNAs that begin upstream of the translation initiation codon of *cpcB* and terminate at three different sites (Ughy and Ajlani 2004). The smaller and most abundant transcript (1.4 kb), which contains *cpcB* and *cpcA*, is not affected by the deletion of *cpcC1*, *cpcC2*, and *cpcD* (Ughy and Ajlani 2004). Two larger and less abundant transcripts, which contain the linker-encoding genes *cpcC2*, *cpcC1*, and *cpcD*, were detected as 3.4- and 3.8-kb transcripts in wild-type cells; the latter two transcripts were not examined in our study. Our Northern blot results suggest that *sll0396* is essential for the normal expression of *cpcBA*.

A key difference between the wild-type and *sll0396*Ω cells lies within their PBS components. The mutant cells did not express L_R^{30} and L_R^{32}, which are the most and second-most distal linkers, suggesting that the mutant cells do not have two outer rods. PC assembly begins with the formation of an $\alpha\beta$ heterodimer (the monomer) and continues through higher-order trimeric and hexameric aggregates that associate with linker proteins to form the PBS rods. The PC-minus phenotype of 4R was due to a deletion mutation in the *cpcB* gene that removed the last 80% of CpcB by a premature termination of translation (Plank et al. 1995). Although the PC α subunit and the linker proteins encoded on the *cpc* transcripts are all functional in 4R, the defective PC β subunit results in the complete absence of the α subunit and linkers, indicating that monomer formation is a critical part of biliprotein assembly in Syn6803. Therefore, it is natural to assume that the low level of *cpcBA* transcription in the *sll0396*Ω mutant results in a low-level production of PC monomers and a complete loss of the distal and most distal linkers. We attempted but failed to measure the level of transcription of the linkers.

The predicted product of *sll0396* has a molecular weight of 26 kDa. Figure 4 shows that Sll0396 is homologous with several response regulators, including PhoB (Lee et al. 1989), VirG (Melchers et al. 1986), OmpR (Forst et al. 1989), and NblR (Schwarz and Grossman 1998). These response regulators activate the transcription of genes involved in the acclimation of cells to stressful conditions. For example, PhoB activates the transcription of the *pho* regulon, which is composed of several operons important for scavenging phosphorus from the environment. NblR plays a pivotal role in integrating different environmental

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sll0396 149 LIDLTAKEFELLDYLLSHARQVLTREQILERVWGYDFMGDSNIIEVYIRYLRLKLEA-AGEPRLIQTVRGVGYVLRD   Sim%  Ident%
NblR    152 QIDLTMKEFDLLRFLMEHPREVLTREQILENVWGYDFMGESNVIEVYIRYLRLKIEI-EGEKRLVQTVRGVGYVLRE    89    77
PhoB    152 PLDMGPTEFKLLHFFMTHPERVYSREQLLNHVWGTNVYVEDRTVDVHIRRLRKALEH-SGHDRMVQTVRGTGYRFSA    71    42
VirG    179 EVKLTAGEFNLLLAFLEKPRDVLSREQLLIASRVRDEEVYDRSIDVLILRLRRKLEADPSSPQLIKTARGAGYFFDA    74    45
OmpR    158 PMPLTSGEFAVLKALVSHPREPLSRDKLMNLARGREYSAMERSIDVQISRLRRMVEEDPAHPRYIQTVWGLGYVFVP    74    45
                H      T    H
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Fig. 4 The putative response regulator Sll0396 contains a helix–turn–helix (*HTH*) DNA-binding motif, which is very similar to those found in members of the receiver–output II subfamily of response regulators. Sequence homology was assessed using the Lipman–Pearson method (DNASIS). *Arrows HincII* insertion site in our mutant, *asterisks* residues that are conserved in all four sequences listed, *double dots* sequence identity, *single dots* higher and lower degrees of sequence similarity. The percentage similarity (*Sim %*) and percentage identity (*Ident %*) of each sequence to Sll0396 are shown to the *right*

signals that link the metabolism of the cell to the light-harvesting capabilities and activities of the photosynthetic apparatus. The predicted product of *sll0396* has a DNA-binding motif (helix–turn–helix) that is very similar to the DNA-binding domains of members of the receiver–output II subfamily of response regulators (Fig. 4). This gene, which was designated as *rre*28 in 2000 by Zinchenko (Cyanobase) is believed to encode an OmpR-type two-component response regulator.

Schwarz and Grossman (1998) described NblR as a response regulator that controls the transcription of *nblA* in *Synechococcus* sp. strain PCC 7942 and mentioned that *sll0396* may be an *nblR* equivalent (Richaud et al. 2001). However, the data presented in this paper demonstrate that this is not the case. First, the *nblR* mutant has a PC peak at 620 nm in its whole-cell absorption spectra and thus has a pronounced non-bleaching phenotype in complete medium (Schwarz and Grossman 1998), whereas the *sll0396*Ω mutant does not exhibit a PC peak under the same conditions. Second, the *nblR* mutant is blue-green and retains most of the absorbance peak at 620 nm under conditions of limited sulfur or nitrogen, whereas the mutant *sll0396*Ω is olive-green and retains no peak at 620 nm during nitrogen limitation (data not shown). Third, the *nblR* mutant dies quickly when deprived of sulfur and nitrogen, whereas the *sll0396*Ω mutant grows at the same rate as wild-type cells when it is deprived of nitrogen, albeit with a slight decrease in viability (data not shown).

The levels of PC, APC, and all three rod-linker polypeptides (27, 32, and 30 kDa) in the *sll0396* mutant were nearly identical to those reported earlier in wild-type cells (Zabulon et al. 2007). They also indicated that inactivation of *sll0396* affected neither the expression of *nblA* nor the sequential degradation of PBS after nitrogen deprivation, unlike the *nblR* mutation in *Synechococcus* 7942. In contrast, our mutant (*sll0396*Ω) had only 20–30% of the wild-type level of PC and no 30- and 32-kDa linker polypeptide expression. In addition, *cpcBA* transcription was reduced in our mutant.

At the present time, it is difficult to explain the difference in phenotype between the inactivation mutants described by Zabulon et al. (2007) and the mutant described in this paper. The mutant used by the former authors had an insertion in the *Bam*H1 site located at the 5′ end of *sll0396*, whereas the mutant we used has a spectinomycin-resistance gene inserted in the *Hinc*II site at the 3′ end of *sll0396*. Sll0396 has a receiver (REC) domain at its N-terminus and a trans-regulator (trans_reg-C) domain at its C-terminus, which is the DNA-binding domain of the protein. The REC domain is a cheY-homologous receiver domain. CheY regulates the clockwise rotation of *Escherichia coli* flagellar motors. This domain contains a phosphoacceptor site that is phosphorylated by histidine kinase homologs. In comparison, the trans_reg-C domain is a response regulator effector domain containing a DNA-binding site. Cyanobacterial mutants displaying different phenotypes for a 5′- versus 3′-end disruption in the same gene have been reported, namely, a 5′-terminal mutant of *taxAY1* in Syn 6803 showed a nonmotile phenotype, whereas a 3′-terminal mutant of the same gene exhibited inverted phototaxis (Bhaya et al. 2001). Distinct phenotypes of cyanobacterial strains with 5′ and 3′ disruptions of *rcaE*, which encodes a sensor that controls the response of filamentous cyanobacteria to light quality, have been reported (Kehoe and Grossman 1996).

In conclusion, our results imply that Sll0396 is responsible for PC gene transcription. Low-level PC gene transcription may result in the reduced production of the PC subunits and a lack of some linker polypeptides. Future studies will focus on how the product of *sll0396* regulates PC gene transcription; thus, this mutant will be very useful in analyzing how the synthesis of the constituents of PBS is regulated.

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