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Slr0151 in *Synechocystis* sp. PCC 6803 is required for efficient repair of photosystem II under high-light condition

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Abstract Cyanobacteria are ancient photosynthetic prokaryotes that have adapted successfully to adverse environments including high-light irradiation. Although it is known that the repair of photodamaged photosystem II (PSII) in the organisms is a highly regulated process, our knowledge of the molecular components that regulate each step of the process is limited. We have previously identified a hypothetical protein Slr0151 in the membrane fractions of cyanobacterium *Synechocystis* sp. PCC 6803. Here, we report that Slr0151 is involved in PSII repair of the organism. We generated a mutant strain (Δ slr0151) lacking the protein Slr0151 and analyzed its characteristics under normal and high-light conditions. Targeted deletion of *slr0151* resulted in decreased PSII activity in *Synechocystis*. Moreover, the mutant exhibited increased photoinhibition due to impairment of PSII repair under high-light condition. Further analysis using *in vivo* radioactive labeling and 2-D blue native/sodium dodecylsulfate polyacrylamide gel electrophoresis

indicated that the PSII repair cycle was hindered at the levels of D1 synthesis and disassembly and/or assembly of PSII in the mutant. Protein interaction assays demonstrated that Slr0151 interacts with D1 and CP43 proteins. Taken together, these results indicate that Slr0151 plays an important role in regulating PSII repair in the organism under high-light stress condition.

Keywords: Cyanobacteria; membrane protein; photosystem II repair; Slr0151; *Synechocystis*

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INTRODUCTION

Cyanobacteria are ancient and the only group of prokaryotes performing oxygenic photosynthesis. These organisms are believed to be the progenitors of chloroplasts in higher plants and have played a key role in the atmospheric transition from anaerobic to aerobic conditions on the Earth (Kasting 2001). During their long evolution, cyanobacteria have adapted successfully to all photic habitats and thrive in adverse environments. The widespread occurrence and remarkable acclimation capability have rendered cyanobacteria the largest group of photosynthetic prokaryotes on our planet (Golbeck 1994). It remains intriguing how these organisms have survived in a wide range of harsh environments and what adaptation mechanisms have evolved in cyanobacteria coping with various stress conditions.

High-light irradiation is one of the adverse conditions most frequently occurring to photosynthetic organisms. Under such condition, their photosynthetic activity is inhibited via a process known as photoinhibition or photodamage (Aro et al. 1993; Adir et al. 2003). Photoinhibition is considered to be a major cause limiting photosynthetic growth, especially when combined with other abiotic stresses (Murata et al. 2007). Thus, a comprehensive understanding of physiological

and molecular mechanisms of high-light acclimation becomes essential. During the past decades, considerable research has been conducted to examine the acclimatory responses to high-light stress as well as to study the molecular mechanisms of high-light acclimation evolved in eukaryotic and prokaryotic photosynthetic organisms (Li et al. 2009; Nixon et al. 2010; Muramatsu and Hihara 2012; Mulo et al. 2012). With regard to the latter, *Synechocystis* sp. strain PCC 6803 (henceforth referred to as *Synechocystis*) has been used as a popular model system virtually due to its privilege of being the first oxygenic photosynthetic organism with a known genome (Kaneko et al. 1996) and its characteristics (Shen and Vermaas 1994; Pakrasi 1995; Zak et al. 2001). Indeed, the genome information of *Synechocystis* deposited in the Cyanobase not only provides an easy access to a sequence of interest for individual analysis by reverse genetics but also supports investigations of global responses to high light by transcriptomic and proteomic tools. The expression profiles of known-function genes/proteins are in general correlated with the physiological activities of the cyanobacterial cells in response to high-light stress, such as acceleration of photosystem II (PSII) turnover and decrease in the content of light harvesting antenna (Muramatsu and Hihara 2012). However, the 'omics' studies also revealed a great number of hypothetical proteins differentially expressed

under high-light stress condition. Functional significance of those proteins is, in most cases, not experimentally determined. This situation substantially hinders the progress toward comprehensive understanding of the acclimation mechanism to high light in the organism.

Recent advances in structural biology have allowed high resolution of PSII structure in the cyanobacterium *Thermosynechococcus vulcanus* (Umena et al. 2011). In cyanobacteria, PSII contains approximately 20 different protein subunits. The core proteins are D1 and D2, which bind all the redox-active components involved in electron transfer of PSII. Under high-light condition, acceleration of D1 turnover is observed in all oxygenic organisms. Thus, it is widely accepted that the D1 protein is the primary target damaged during photoinhibition *in vivo* (Mulo et al. 2012). To maintain the function of PSII, a repair mechanism which is suggested to be similar in all photosynthetic organisms operates. This repair process involves partial disassembly of the damaged complex, the selective replacement of the damaged D1 subunit with a newly synthesized copy, and reassembly (Nixon et al. 2010). Based on analysis of photosynthetic mutants, numerous regulatory proteins that are either conserved or specific for cyanobacteria and chloroplasts have been identified (Nickelsen and Rengstl 2013), including Ycf48, CtpA, Psb27, and FtsH in *Synechocystis* (Shestakov et al. 1994; Inagaki et al. 2001; Silva et al. 2003; Komenda et al. 2008, 2012). However, results from these studies yet implicated that there are additional proteins involved in the regulation of the intricate processes. Indeed, earlier experiments carried out by Wegener et al. (2008) identified numerous known and hypothetical proteins that are associated with the PSII complex of *Synechocystis*. Six proteins (Slr0144–Slr0147, Slr0149, Slr0151) encoded in a gene cluster composed of nine genes (*slr0144–slr0152*) were identified. Further analysis showed that a deletion mutant lacking the gene cluster, which was previously proposed to be in an operon by Singh et al. (2004), displayed decreased activity of oxygen evolution in comparison with wild type and was designated Pap (Photosystem II Assembly Proteins) (Wegener et al. 2008). More recently, Kubota et al. (2010) reported that four of the proteins (Slr0146, Slr0147, Slr0149, Slr0151) were also associated with PSI monomer of *Synechocystis*. The function of the individual gene/protein remains unclear, such as how *slr0151* is involved in photosynthesis of the organism.

Using proteomic approaches, we have previously identified the hypothetical protein Slr0151 in plasma and thylakoid membranes of *Synechocystis* (Huang et al. 2002; Srivastava et al. 2005). Studies on the membrane protein complexes also revealed Slr0151 as a component of a protein complex with unknown function in *Synechocystis* (Herranen et al. 2004). Here, we have investigated its subcellular location in the cyanobacterium as well as the effects of *slr0151* gene mutation on its photosynthetic characters. We show that Slr0151 is an integral protein located in plasma and thylakoid membranes but not in soluble fraction and outer membrane of *Synechocystis*. Also, we show that the *slr0151*-knockout mutant displayed an increased photoinhibition phenotype. Moreover, we provide experimental evidence that the impaired repair of PSII in the mutant is the consequence of reduction in D1 synthesis and its integration into the PSII complex. We address that Slr0151 is required for PSII repair under high-light stress condition in *Synechocystis*.

RESULTS

Subcellular localization of Slr0151 in *Synechocystis*

We previously identified the hypothetical protein Slr0151 in membrane fractions of *Synechocystis* (Huang et al. 2002; Srivastava et al. 2005). To clarify its precise subcellular location and abundance in the cyanobacterium, antibody against Slr0151 recombinant protein was produced (Figure S1). As demonstrated in Figure S1B, a single band was detected (lane 2) and the position was at 37 kDa, which corresponds to the molecular mass calculated from the coding sequence of *slr0151* in *Synechocystis*. The antibody was therefore used for investigation of the subcellular location of Slr0151 in the organism. Cells were broken with glass beads followed by separation of soluble and membrane fractions via ultracentrifugation (Figure 1A).

Figure 1. Localization of Slr0151 in *Synechocystis*

(A) Immunoblot detection of Slr0151 in total protein extracts (Tp), soluble (Sp), and membrane (Mp) fractions. Proteins (5 µg, per lane) were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). (B) Immunoblot detection of Toc75, NrtA, CP43, D1, and Slr0151 in outer (OM), plasma (PM), and thylakoid (TM) membrane fractions. Proteins (5 µg, per lane) were separated by 12% SDS-PAGE. (C) Immunoblot detection of Slr0151 in pellet (M) and soluble (S) fractions of total membrane treated with ionic or non-ionic solubilizing agents. Ten micrograms of total membrane protein was treated with the indicated compounds followed by fractionation. Proteins were separated by 12% SDS-PAGE. D1 and PsbO were used as controls for integral and peripheral membrane proteins, respectively.

Three different membranes (outer, plasma, and thylakoid membranes) were then separated and purified from total membranes using a 2-D isolation method (Norling et al. 1998; Zhang et al. 2009). The purity of each membrane fraction was verified by immunoblot analysis using marker proteins specific to each membrane. The immunosignal for Toc75 (a marker protein for the outer membrane of cyanobacteria, Bölder et al. 1998), NrtA (a marker protein for the plasma membrane of cyanobacteria, Omata 1995), and CP43 (a marker protein for thylakoid membrane) could only be detected in outer, plasma, or thylakoid membranes, respectively. Slr0151 was detected in plasma and thylakoid membranes but not in soluble and outer membrane fractions of *Synechocystis* (Figure 1A, B), which is consistent with our proteomics data (Huang et al. 2002, 2004; Srivastava et al. 2005; Fulda et al. 2006).

To examine whether Slr0151 is an integral membrane protein, total membranes were treated with ionic or non-ionic solubilizing agents (Schottkowski et al. 2009) and separated into pellet (M) and soluble (S) fractions by centrifugation. These fractions were then analyzed by immunoblotting against the Slr0151 antibody. Obviously, treatment of the membranes with 0.25% Triton X-100 released the protein from the membranes, while that with 0.1 mol/L Na_2CO_3 , 2 mol/L urea, or 1 mol/L NaCl did not. The pattern was similar to the integral membrane protein D1 and different from the peripheral protein PsbO (Figure 1C). Therefore, we conclude that Slr0151 is an intrinsic protein located in the thylakoid and plasma membranes of *Synechocystis* probably through non-ionic interactions. TOPCONS, a consensus prediction server (<http://topcons.net/>) for membrane protein topology based

on five different methods (Bernsel et al. 2009), predicts at least one transmembrane helix in Slr0151.

Construction of *slr0151*-knockout mutant

To investigate function of Slr0151 in *Synechocystis*, we generated a *slr0151*-knockout mutant ($\Delta\text{slr0151}$) using the construct shown in Figure S2A. Complete segregation of the mutant was confirmed by polymerase chain reaction (PCR) using the primers amplifying the DNA fragments corresponding to the *slr0151* open reading frame (ORF) (1.0 kb) and kanamycin-resistance cartridge (1.1 kb), respectively. The size increase of the PCR products from 1.0 kb for the wild type to 2.1 kb for the mutant is ascribed to the insertion of a 1.1 kb kanamycin-resistance gene cassette (Figure 2A). Reverse transcription (RT)-PCR analysis using total RNA isolated from wild type and the mutant showed no transcript of *slr0151* in $\Delta\text{slr0151}$ (Figure 2B). This was further confirmed by immunoblot analysis using total proteins isolated from both wild-type and $\Delta\text{slr0151}$ cells. As shown in Figure 2C, the immunosignal of Slr0151 was detected in wild-type but not in $\Delta\text{slr0151}$ cells. To investigate whether the insertional mutation of *slr0151* affects the expression of the adjacent genes, we examined the transcripts of *slr0144*–*slr0150* in the upstream and *slr0152* in the downstream of *slr0151* using RT-PCR. The results showed that there is no significant difference in transcription for these genes between wild type and $\Delta\text{slr0151}$ (Figure 2B). Based on microarray analysis, a gene cluster *slr0144*–*slr0150* was downregulated in *Synechocystis* under low-iron condition as well as by H_2O_2 treatment. However, expression of *slr0151* and *slr0152* in the gene cluster was not

Figure 2. Disruption and complementation of *slr0151* in *Synechocystis*

(A) Polymerase chain reaction analysis of genomic DNA from wild type (WT, lanes 1 and 4), the mutant ($\Delta\text{slr0151}$, lanes 2 and 5), and *slr0151*-complementary (*reslr0151*, lanes 3 and 6) strains using the primers amplifying the open reading frame of *slr0151* (lanes 1, 2, and 3) or the fragment of Km^R (lanes 4, 5, and 6). (B) Reverse transcription polymerase chain reaction analysis of the expression of *slr0151* and adjacent genes in WT and $\Delta\text{slr0151}$. *rnpB* was used as a reference gene. (C) Immunoblotting of Slr0151 protein in WT, $\Delta\text{slr0151}$, and *reslr0151*. Proteins (5 μg per lane) were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis.

found regulated together with the seven genes above (Singh et al. 2004). Using a proteomic approach, six proteins encoded by the gene cluster (*slr0144*–*slr0147*, *slr0149*, *slr0151*) were identified among the proteins associated with the purified PSII and PSI monomer in *Synechocystis*, respectively (Wegener et al. 2008; Kubota et al. 2010). However, Slr0152 protein was not identified in the preparations used in the proteomic experiments. These observations may indicate that *slr0152* was not tightly regulated together with the other eight genes. Based on prediction by ARNold (<http://rna.igmors.u-psud.fr/toolbox/arnold/index.php>), a terminator between *slr0151* and *slr0152* exists. Together with our RT-PCR data showing no effect on adjacent genes including *slr0152*, we assume that transcription of *slr0152* could be independent of the operon.

Growth and photosynthetic characteristics of Δ *slr0151* mutant

Deletion of the gene cluster *slr0144*–*slr0152* caused approximately 20% reduction of PSII activity in *Synechocystis* (Wegener et al. 2008). To determine the potential contribution of *slr0151* deletion to this defect, we first compared the growth profiles and the rates of photosynthetic oxygen evolution between wild type and Δ *slr0151* under normal light condition

(50 $\mu\text{mol}/\text{m}^2 \text{ s}$). Both cell growth and oxygen evolution activity of the mutant was slightly repressed/reduced in comparison with wild type (Figure S3A, C), indicating that *slr0151* is required for maintaining maximal autotrophic growth of *Synechocystis*. To confirm this phenotype and gain insight into the functionality of photosystems in Δ *slr0151*, the rates of photosynthetic oxygen evolution and 77K fluorescence emission spectra of wild-type and the mutant cells were then compared under normal (Figure S3B) and high-light stress conditions (Figures 3–4). A differential decrease of chlorophyll (Chl) fluorescence emission peaks from PSII at 685 and 695 nm, corresponding to CP43 and CP47, respectively (Shen and Vermaas 1994), was observed in Δ *slr0151*. This indicates that the level of functional PSII was lower than in wild type. Measurements of *in situ* Chl *a* fluorescence with a modulated fluorometer also showed that the ratio of *Fv/Fm*, which is an indicator of the maximum efficiency of PSII photochemistry, the effective photochemical quantum yield of PSII, and the relative photosynthetic electron transfer rate seemed reduced in Δ *slr0151* (Figure 4B; Table 1). Because no decrease in Chl *a* content was detected (Table 1), and the light-induced redox kinetics of PSI (absorbance changes of P700 at 820 nm), characteristic of a functional PSI, was identical between wild type and Δ *slr0151* (Figure S3D), we conclude that function of

Figure 3. High-light sensitive phenotype of Δ *slr0151*

(A, C) Maximal photochemical efficiency of photosystem II (PSII) (*Fv/Fm*) in the (A) absence or (C) presence of chloramphenicol. (B, D) Rates of oxygen evolution in the (B) absence or (D) presence of chloramphenicol. Data are expressed as means ($\pm$ SD) of three independent experiments.

Figure 4. Restoration of the wild-type (WT) phenotype in the complementation strain of *Synechocystis*
(A) 77K low temperature fluorescence emission spectra of WT, *reslr0151*, and $\Delta slr0151$ under high light for 60 min. The spectra were normalized at 726 nm. All experiments were repeated twice with similar results. (B) Photoinhibition phenotype of WT, *reslr0151*, and $\Delta slr0151$.

PSII is significantly affected, due to the mutation of *slr0151*. To verify this, complementation experiments were performed. The *slr0151* gene was reintroduced into $\Delta slr0151$ mutant by transformation of the pFQ20-*slr0151* construct (Figure S2B). The pFQ20 plasmid was derived from pKW1188SL by integrat-

ing an expressing cassette consisting of the omega fragment, the RuBisCO promoter, the gene of interest (*slr0151*), and the RuBisCO terminator into the *slr0168* locus (Tan et al. 2011). The *slr0151* gene was amplified and ligated into pFQ20 plasmid at *Xba*I and *Sma*I sites. The transformants were selected by antibiotic resistance to spectinomycin and kanamycin. Integration of *slr0151* in the genome was confirmed by PCR (Figure 2A). Expression of Slr0151 was verified by immunoblotting (Figure 2C). In the resulting mutant (named *reslr0151*), the altered PSII phenotype was restored to the same extent under both normal (Figure S2C) and high-light stress (Figure 4) conditions based on 77K fluorescence emission spectra and Fv/Fm. These results indicate that the altered PSII activity in $\Delta slr0151$ cells was due to the absence of Slr0151 protein. The incomplete restoration of the wild-type phenotype is probably a result of the overexpression of Slr0151 protein in the complementation strain (Figure 2C).

Increased photoinhibition in $\Delta slr0151$ mutant

To investigate whether Slr0151 protein plays a role in the PSII repair in *Synechocystis*, we performed high-light-induced photoinhibition experiments. Wild-type and $\Delta slr0151$ cells were subjected to high-light treatment ($1,300 \mu\text{mol}/\text{m}^2 \text{ s}$) in the absence or presence of chloramphenicol, which inhibits the protein synthesis required for the repair process (Gombos et al. 1994). Photosystem II activity was monitored with Fv/Fm (Figure 3A, C) as well as the rate of oxygen evolution (Figure 3B, D) throughout the experiments. In the absence of chloramphenicol, PSII activity in the mutant was significantly lower than wild type, based on both Fv/Fm and rate of oxygen evolution measures. These data suggest that photoinhibition was increased in $\Delta slr0151$.

It is known that the rate of PSII damage is strictly proportional to the intensity of light (Allakhverdiev and Murata 2004). Because the light intensity used in the current photoinhibition experiment was identical for both types of cells, the greater decrease of PSII activity in $\Delta slr0151$ in the absence of chloramphenicol indicates that the efficiency of PSII repair was lower in $\Delta slr0151$ than wild type. In the presence of chloramphenicol, however, no difference in the reduction of PSII activity between wild type and the mutant was observed (Figure 3C, D). These results indicate that the decreased efficiency of PSII repair in $\Delta slr0151$ is mainly due to its impairment of protein synthesis rather than degradation of the damaged photosynthetic proteins.

Table 1. Chlorophyll *a* content and fluorescence parameters of wild type and $\Delta slr0151$ under normal and high-light conditions

Parameters	WT-NL	$\Delta slr0151$ -NL	WT-HL ^a	$\Delta slr0151$ -HL ^a	
Chl <i>a</i> ($\mu\text{g}/\text{OD}_{730}$)	4.15 $\pm$ 0.30	4.65 $\pm$ 0.53	3.97 $\pm$ 0.17	4.43 $\pm$ 0.18	Ave $\pm$ SD (n = 9)
		0.02		0.05	t-test (P-value)
Fv/Fm	0.52 $\pm$ 0.01	0.50 $\pm$ 0.01	0.33 $\pm$ 0.01	0.24 $\pm$ 0.01	Ave $\pm$ SD (n = 6)
		6.00E - 5*		1.32 E - 9*	t-test (P-value)
YII	0.31 $\pm$ 0.01	0.28 $\pm$ 0.01	0.11 $\pm$ 0.01	0.08 $\pm$ 0.01	Ave $\pm$ SD (n = 9)
		2.76E - 8*		1.18 E - 9*	t-test (P-value)
rETR	4.64 $\pm$ 0.10	4.42 $\pm$ 0.10	1.63 $\pm$ 0.07	1.17 $\pm$ 0.07	Ave $\pm$ SD (n = 9)
		2.19E - 4*		2.14E - 10*	t-test (P-value)

^aHigh-light treatment for 60 min. *Significantly different ($P < 0.01$, Student's t-test). Ave, average; Chl, chlorophyll; HL, high-light; NL, normal light; OD_{730} , optical density at 730 nm; rETR, the relative photosynthetic electron transfer rate; SD, standard deviation; YII, effective photochemical quantum yield of photosystem II; WT, wild type.

Decreased level of D1 protein in $\Delta slr0151$ mutant

Considering that the D1 subunit in PSII is the primary target damaged during photoinhibition *in vivo* (Mulo et al. 2012), we further monitored the amounts of D1 protein in wild type and $\Delta slr0151$ by immunoblot analysis (Figure 5). Membrane proteins isolated from both types of cells that had been exposed to high-light illumination for up to 120 min were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunodetection using the specific antibodies for D1, which was raised against a peptide conserved in the C-terminal of all known D1 proteins including cyanobacteria (Agrisera, Product no. AS05084, <http://www.agrisera.com/en/artiklar/psba-d1-protein-of-psii-c-terminal-pre-immune-serum.html>) and D2, respectively. In the absence of chloramphenicol, the amount of D1 protein was in most cases less in $\Delta slr0151$ than wild type during photoinhibition. By contrast, the level of D2 protein, another reaction center protein of PSII, remained relatively constant between the two types of cells (Figure 5). This is not surprising because it has been shown by Yao et al. (2012), using stable-isotope labeling combined with mass spectrometry, that in cyanobacteria D2 was more stable than D1 under normal and light stress conditions (Yao et al. 2012). Our finding that the decrease in D1 levels of the mutant was more pronounced than D2 in the absence of chloramphenicol (Figure 5) was consistent with their observations in *Synechocystis*. In the presence of

chloramphenicol, however, the distinction of D1 levels between wild type and the mutant was no longer observed (Figure 5). These results indicate that the decreased production of D1 protein could be one of the major causes for impairment of PSII repair in $\Delta slr0151$.

Synthesis of D1 protein is slowed in $\Delta slr0151$ mutant

To further investigate whether the rate of D1 synthesis was affected in $\Delta slr0151$, we performed radioactive pulse-chase labeling experiments using L-(35 S)-labeled methionine as described (Nixon et al. 1995). For the pulse, cell suspensions with radioactive-labeled methionine were exposed to white light ($100 \mu\text{mol}/\text{m}^2 \text{ s}$) for up to 20 min. For the chase, the labeled cells were washed and resuspended in the BG-11-S containing “cold” methionine. Cells were subjected to high-light irradiance ($1,100 \mu\text{mol}/\text{m}^2 \text{ s}$) for up to 90 min. Cell aliquots taken at different time intervals were subjected for membrane preparation followed by SDS-PAGE and autoradiographic detection (Figure 6A–C). The position of D1 was confirmed by immunoblot analysis (Figure S4). The data show that the synthesis of D1, based on the quantity of the radioactive-labeled D1 band in the pulse period, was apparently inhibited in $\Delta slr0151$ in comparison with that in wild type (Figure 6A). The rate of D1 turnover, as assessed by the intensity of the radioactive-labeled D1 band in the chase period, appeared similar in both types of cells under high light (Figure 6B, C).

Figure 5. Immunoblot detection of D1 and D2 proteins in membrane fraction of $\Delta slr0151$ under photoinhibition

(A) Protein levels were detected in the absence or presence of chloramphenicol. Proteins (equivalent to $0.2 \mu\text{g}$ chlorophyll per lane) were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis. (B) Relative levels of D1 in the absence or presence of chloramphenicol. Data are expressed as means ($\pm$ SD) of seven replicated experiments.

Figure 6. Synthesis and turnover of D1 protein in wild type (WT) and Δ slr0151

(A) Pulse-labeling analysis of membrane proteins in WT and Δ slr0151. Cells were pulse-labeled at 30 °C for the indicated time in the presence of (35 S)-Met. Membrane proteins (equivalent to 5 μ g chlorophyll per lane) were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue (Gel stain). Radioactive labeling of the proteins was visualized using a phosphor imager (Autorad). The radioactivity incorporated into the D1 of WT during 10 min pulse was taken as 100%. Values (bottom) show means of three measurements. (B, C) Chase analysis of the radio-labeled membrane proteins in (B) WT and (C) Δ slr0151. Radio-labeled cells were washed and incubated with BG-11-S containing “cold” Met for the indicated time under high light (1,100 μ mol/m 2 s). Membrane proteins (equivalent to 5 μ g chlorophyll, per lane) were separated by 12% SDS-PAGE and stained with Coomassie blue (Gel stain). Radioactive labeling of the proteins was visualized using a phosphor imager (Autorad). The radioactivity incorporated into the D1 after the pulse period (0 min) was taken as 100%. Values (bottom) show means of three measurements. (D) Real-time quantitative polymerase chain reaction analysis of *psbA* gene in WT and Δ slr0151 under high-light condition. Data are expressed as means ($\pm$ SD) of three independent experiments.

These experimental results provide additional indications that the decreased D1 levels in the mutant could be ascribed to its slowed synthesis. To test whether the slowed D1 synthesis in Δ slr0151 was due to its limited transcript, we then monitored the changes in the total amounts of *psbA* mRNA, which was the sum of the *psbA2* and *psbA3* mRNA (Mohamed and Jansson 1989), in wild type and Δ slr0151 via quantitative real-time PCR analysis. As shown in Figure 6D, the overall level of *psbA* transcript in Δ slr0151 was slightly higher than wild type under such conditions. Thus, a significant impact of *slr0151* at the transcriptional level could be ruled out.

Decreased efficiency of PSII disassembly/assembly in Δ slr0151

Considering that repair of PSII is a complex process that involves partial disassembly of the damaged complex, the

selective replacement of the damaged D1 subunit with a newly synthesized copy, and reassembly (Nixon et al. 2010), we further analyzed membrane complexes of wild type and Δ slr0151 by blue native (BN)/SDS-PAGE using the published protocol (Herranen et al. 2004). Total membranes were isolated from both wild-type and Δ slr0151 cells grown under normal condition. Solubilized membranes were separated using BN-PAGE in the first dimension in combination with denaturing SDS-PAGE in the second dimension followed by immunodetection with antibody against Slr0151. As shown in Figure S5, the overall 2-D gel pattern of membrane proteins from wild-type cells was consistent with the earlier publications (Herranen et al. 2004; Zhang et al. 2013), indicating that the technical reproducibility of membrane samples, including isolation of membrane fraction, protein solubilization, and separation by BN/SDS-PAGE from different experiments was

sufficiently high to yield essentially identical results. Using a proteomic approach, Slr0151 was earlier identified as a component of the membrane protein complex of unknown function in *Synechocystis* (Herranen et al. 2004). In this work, we verified the presence of Slr0151 in the membrane complex of wild type by immunoblot analysis. The immunosignal for Slr0151 could only be detected in wild-type *Synechocystis* cells (Figure S5).

We then compared BN/SDS-PAGE patterns of membrane proteins from wild-type and Δ slr0151 cells collected at different time points of high-light treatment, followed by immunodetection with antibodies against D1 and CP43 (Figure 7). Consistent with earlier observations (Komenda et al. 2004), D1 was found in the monomeric and dimeric PSII as well as the monomeric PSII complex lacking CP43 in both strains. Compared to wild type, the amount of D1 in the dimeric PSII complex, which is generally considered to be the functional form of PSII *in vivo* (Boekema et al. 1995; Umena et al. 2011), appeared lower in Δ slr0151 (Figure 7). This may explain the deficiency in PSII function of Δ slr0151 under normal growth condition (Figure S3B, C). Moreover, our data revealed major differences in the abundance of the CP43-less monomeric PSII complexes from wild-type and Δ slr0151 cells, based on

immunodetection of D1 protein. Under normal condition, the abundance of this subcomplex was low in wild-type *Synechocystis*. Upon exposure to high irradiance, however, the abundance of the subcomplex from wild type remarkably increased, whereas it appeared to slightly decrease in the mutant (Figure 7).

It is known that the CP43-less monomeric PSII complex is formed during de novo assembly as well as from disassembly of the dimeric PSII complex in the PSII repair cycle. Recent experiments by Boehm et al. (2012) revealed that newly synthesized D1 is preferentially incorporated into the subcomplex, and the subcomplex was suggested to be an appropriate substrate for selective D1 replacement (Boehm et al. 2012). Our finding of the limited amount of the subcomplex in the mutant under high irradiance (Figure 7) strongly indicates that efficiency of PSII disassembly/assembly was decreased in the mutant due to lack of slr0151. This was further evidenced by immunoblot analysis with antibody against CP43 protein. As observed earlier (Komenda et al. 2004; Rengstl et al. 2013) and in this work (Figure 7, WT), CP43 protein was mainly detected in the PSII monomer and the small precomplexes termed CP43a and CP43b in wild-type *Synechocystis*. Weak immunosignals for CP43 in the position of dimeric

Figure 7. 2-D blue native (BN)/sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of membrane complexes in wild-type (WT) and Δ slr0151 under high-light irradiation

Membrane fractions (equivalent to 3 μ g of chlorophyll) isolated from the high-light treated cells (HL) for the indicated time were solubilized with 2% n-dodecyl- β -D-maltoside then subjected to 2-D BN/SDS-PAGE and immunoblot detection against antibodies specific to D1 (α -D1) and CP43 (α -CP43). Photosystem II (PSII) complexes and unassembled proteins (U.P.) were designated according to Herranen et al. (2004) and Komenda et al. (2004). CP43a and CP43b, two forms of the CP43 subcomplex.

PSII complexes could only be visualized in the overexposed blots or those with increased sample loading (equivalent to 6 μg of Chl, data not shown), meaning that the affinity of the CP43 antibody was sufficiently high to detect the protein in the complexes resolved by BN-PAGE. The diminished amount of CP43 may indicate that the majority of the protein was removed from the photodamaged dimeric PSII complexes during photoinhibition. Because the major difference between wild-type and $\Delta\text{slr0151}$ cells under light stress was observed in the abundance of the intermediate PSII complexes (CP43-less PSII monomer, CP43a, CP43b), which was revealed on the immunoblotting of D1 with optimized BN-PAGE (sample loading equivalent to 3 μg of Chl) yielding best resolution and highest reproducibility, the corresponding blots against CP43 under optimized exposure time (30 min) were of choice for comparison (Figure 7). In contrast to wild type, the precomplex CP43a appeared diminished in $\Delta\text{slr0151}$ (Figure 7, $\Delta\text{slr0151}$). Earlier experiments by Komenda et al. (2004) showed that assembly of PSII was preferential in the sequential of shifting CP43b into CP43a then into the PSII monomer. Thus, CP43a was suggested to be the direct precursor protein complex for assembly of the monomeric PSII complex in *Synechocystis* (Komenda et al. 2004). The diminished CP43a precomplex in the mutant (Figure 7, $\Delta\text{slr0151}$) may indicate that the shifting of CP43b into CP43a precomplex was hindered due to the mutation of the *slr0151* gene. Taken together, we conclude that the impaired PSII repair was largely due to both slowed D1 synthesis as well as decreased efficiency of PSII disassembly/assembly in $\Delta\text{slr0151}$.

Because Slr0151 is not a component of PSII (Umena et al. 2011), its requirement for PSII repair suggests that the protein may transiently interact with one or more subunits of the PSII complex. It is generally known that the yeast split-ubiquitin system is an appropriate method to investigate protein-protein interactions of membrane proteins, whereas the classical yeast two hybrid assay could be used for screening

of protein-protein interactions of soluble proteins or fragments such as the C-terminal extension of the D1 precursor (Klinkert et al. 2004). Because the screening using the former system failed due to self-activation of Slr0151 (data not shown), the latter assay was applied to examine the putative interactions of Slr0151 with PSII core proteins (D1, D2, CP43, CP47) as well as the soluble C-terminal segments of the mature D1 (D1 292–344) and the precursor D1 (D1 292–360). The results showed that Slr0151 could interact with D1, CP43, and both C-terminal segments of the D1 forms, but not with D2 and CP47 (Figure 8A). To confirm these preliminary observations, pull-down assays were performed as described (Sun et al. 2007) using the recombinant Slr0151 protein fused with His-tags at both N- and C-terminals. As demonstrated in Figure 8B, D1 and CP43 were detected when the His-Slr0151 fusion protein was included in the assay (left lane), whereas no immunosignal for the proteins was detected when only the solubilized thylakoid membrane and the resin were included (middle lane). CP47, a core protein of PSII, was not detected when the His-Slr0151 fusion protein was used (left lane). These experimental results suggest that the interactions of Slr0151 with D1 and CP43 exist.

DISCUSSION

Synechocystis is a model organism for studying photosynthesis as well as cell adaptation to adverse conditions including high light. Recent advances in the study of transcriptomic and proteomic profiles revealed a large number of genes/proteins involved in the high-light acclimation process of *Synechocystis* (Muramatsu and Hihara 2012). However, compared to the known genes/proteins, functional significance of many hypothetical proteins involved in the process is not determined, which substantially hinders current understanding of cell acclimation mechanisms to high-light stress in cyanobacteria at

Figure 8. Slr0151-interacting protein assays

(A) Yeast two-hybrid analysis. Interactions were monitored by growth on SD/-Leu/-Trp/-His/-Ade selection medium containing 40 $\mu\text{g}/\text{mL}$ X- α -Gal. (B) Pull-down assays of Slr0151-interacting proteins. Thylakoid membranes (100 μg) isolated from wild-type *Synechocystis* cells were solubilized with 1% n-dodecyl- β -D-maltoside then incubated with His-tagged fusion proteins (10 μg) coupled to Ni-NTA resin. Bound proteins were eluted and then subjected to 12% sodium dodecylsulfate polyacrylamide gel electrophoresis and immunodetected with the antibodies specific to D1 (α -D1), CP43 (α -CP43), and CP47 (α -CP47). PsaB was used as a control protein. Similar results were obtained in two additional independent experiments.

a molecular level. In this work, we have investigated the function of hypothetical protein Slr0151 in *Synechocystis*. We have shown that Slr0151 is an integral protein located in thylakoid and plasma membranes of the cyanobacterial cells (Figure 1). We also have shown that disruption of the *slr0151* gene leads to the impaired function of PSII in *Synechocystis* (Figure S3). Furthermore, we provided several lines of experimental evidence demonstrating that Slr0151 is involved in the repair of PSII under high-light condition (Figures 3–7). Therefore, we suggest that Slr0151 is one of the important proteins required for efficient repair of PSII in *Synechocystis*.

The hypothetical protein Slr0151 was identified in *Synechocystis* by proteomic approaches, but its function in the organism was thus far not clarified. In this work, we found that Slr0151 was present in both plasma and thylakoid membranes of *Synechocystis* (Figure 1). Our finding that Slr0151 was recovered in the pellet or supernatant after treatment of total membranes with urea or Triton X-100, respectively (Figure 1C), indicates further that the protein was intrinsically located in the membranes of *Synechocystis* probably through non-ionic interactions.

Under normal growth condition, deletion of *slr0151* resulted in slightly repressed cell growth and PSII activity of *Synechocystis*, based on both oxygen evolution activity and *in situ* Chl *a* fluorescence measures (Figure S3, Table 1). These observations indicate that *slr0151* is not essential but required for optimal function of PSII in the organism. Clearly, deletion of *slr0151* also leads to increased photoinhibition in *Synechocystis* based on PSII activity measures (Figure 3). These findings suggest that *slr0151* is involved in PSII repair in *Synechocystis*. Because no difference in the rate of PSII photoinhibition was observed in the presence of the translation inhibitor chloramphenicol (Figure 3C, D), we presume that the impairment of PSII repair in Δ *slr0151* is largely due to slowed protein synthesis rather than increased degradation of the damaged photosynthetic proteins. Our data showing that not only the steady-state level of D1 (Figure 5A) but also the rate of D1 synthesis, which was revealed from the pulse-labeling experiments, was lower in Δ *slr0151* than wild type (Figure 6A), strongly support the assumption above. It remains, however, unclear how deletion of *slr0151* hinders synthesis of D1 in the organism. Earlier experimental results obtained from a mutant lacking YCF48, an assembly factor for PSII in *Synechocystis*, indicated that expression of the *psbA* gene encoding D1 was eightfold higher than wild type (Komenda et al. 2008). This was presumed to be a response to the limited availability of D1 protein in the mutant. In this work, we found that the expression of *psbA* was stimulated to a greater extent in Δ *slr0151* than wild type under high-light stress (Figure 6D). These experimental results suggest that the possibility of its transcriptional limitation for D1 synthesis in the mutant could be excluded.

Not only synthesis but also the accumulation of D1 in the different forms of the PSII complex decreased in Δ *slr0151* (Figures 6, 7). Our results from 2-D BN/SDS-PAGE and immunoblot analysis revealed that the mutant accumulated significantly less D1 in all of the PSII complexes (Figure 7). Because the synthesis and accumulation of D1 are required for PSII repair and assembly (Rengstl et al. 2013), the increased photoinhibition observed in the mutant might be ascribed to its deficiency in both processes. It is known that assembly of

PSII occurs preferentially in the sequential manner, that is, shifting the precomplex CP43b into CP43a then into the PSII monomer, and CP43a was suggested to be the direct precursor complex for assembly of the monomeric PSII complex in *Synechocystis* (Komenda et al. 2004). The diminished CP43a precomplex in Δ *slr0151* (Figure 7) suggests that the reassembly of PSII during the repair cycle was likely hindered at the step of shifting CP43b-into CP43a-PSII precomplexes in the mutant.

It is intriguing how Slr0151 affects PSII repair in *Synechocystis*. In photosynthetic organisms, several proteins containing tetratricopeptide repeat (TPR) motifs have been reported to play important roles during assembly and/or repair of the photosynthetic complexes. Those include the PSI assembly factors such as Ycf3 (Boudreau et al. 1997), the PSII assembly factors such as PrtA and Pitt (Klinkert et al. 2004; Schottkowski et al. 2009), as well as REP27 that was mainly involved in the PSII repair process (Park et al. 2007). In this work, we found that Slr0151, which contains two TPR domains (Figure S6), was required for repair of PSII in *Synechocystis*. Because TPR is well known as a structural motif for mediating protein-protein interactions and the assembly of multisubunit complexes, we assume this effect of Slr0151 was through its interaction with one or more subunits of PSII probably via the TPR domains. The data from protein interaction assays showing that Slr0151 specifically interacts with the D1 and CP43 proteins but not with the CP47 protein (Figure 8) are of support to this assumption. Further analysis is initiated to verify these protein-protein interactions in *Synechocystis* cells as well as their dynamics in the organism coping with high-light stress.

MATERIALS AND METHODS

Strains, culture conditions, and high-light treatment

Synechocystis sp. PCC 6803 glucose-tolerant strain served as the wild-type strain. Wild-type and *slr0151*-knockout cells were grown in BG-11 media (Rippka et al. 1979) phototrophically at 30 °C under continuous light (50 μ mol/m² s) from cool white fluorescent lamps. Liquid cultures were grown with vigorous air-bubbling to an exponential phase (optical density at 730 nm (OD₇₃₀) = 0.8) and this was used for all experiments. Cell growth was monitored by measuring OD₇₃₀ of batch cultures rotary shaking at 60 rpm. High-light treatment was performed according to Zhao et al. (2013) with modifications. Briefly, cells were collected by centrifuging as described (Zhang et al. 2009) and resuspended in BG-11 medium to a cell density at 15 μ g/mL Chl supplemented with or without chloramphenicol (100 μ g/mL). Cell suspensions were transferred to Petri dishes then incubated with gentle shaking (60 rpm) in a growth chamber (DHZ-D, Taicang, China) set at the desired high-light intensity (1,300 μ mol/m² s) and constant temperature (30 °C). Samples were taken at designated time points for photosynthetic or molecular analysis. Chlorophyll *a* content was determined according to Porra et al. (1989) using methanol extraction.

Production of antiserum against recombinant Slr0151 protein

Gene cloning and heterologous expression were performed as described (Zhou et al. 2008a) with modifications. Briefly, the coding region of the *slr0151* gene without N-terminal transmembrane sequence was amplified from genomic

Synechocystis DNA by PCR with PrimerSTAR HS DNA Polymerase (Takara, Ohtsu, Japan) and specific primers *slr0151*-F1 and *slr0151*-R1 containing restriction enzyme sites as listed in Table S1. The amplified PCR product was cloned into the pET-28a (+) vector (Merck, Darmstadt, Germany), which was then transformed into competent *Escherichia coli* Transetta (DE3) cells (Beijing TransGen Biotech, Beijing, China). Expression and purification of the recombinant Slr0151 protein were performed as described previously (Zhou et al. 2008b). A rabbit serum was produced by MBL (Nagoya, Japan) using the purified recombinant Slr0151 as immunogen. Specificity of the antibody was verified by immunoblotting using total proteins extracted from wild-type *Synechocystis*.

Membrane isolation and Slr0151 localization

Membrane and soluble fractions of *Synechocystis* were isolated as described (Norling et al. 1998; Huang et al. 2002, 2004) with modifications (Zhang et al. 2009). Frozen cells were broken with glass beads (diameter, 0.17–0.18 mm). Cell debris was removed by centrifugation at 3,500 g and 4 °C for 15 min. Total membranes were separated from soluble fractions by centrifugation at 203,000 g for 60 min. For further separation of plasma, thylakoid, and outer membranes, a total membrane pellet was resuspended in 5 mmol/L potassium phosphate buffer (pH 7.8) supplemented with 0.25 mol/L sucrose followed by a 2-D separation method using a combination of sucrose density centrifugation and aqueous polymer two phase partitioning. The final pellet of the purified membranes resulted after the centrifugation was resuspended in the buffer with 0.25 mmol/L sucrose and 5 mmol/L potassium phosphate (pH 7.8). To prevent protein degradation, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF; Amresco, Solon, OH, USA) was added during membrane preparations and subsequent storage of the membranes at –70 °C. The purity of each membrane was verified by immunoblot analysis as previously described (Huang et al. 2004; Zhang et al. 2009). Antibodies against photosynthetic proteins were purchased from Agrisera (Umeå, Sweden) and used by following the instructions. The dilution for the specific antibodies used in this study is as follows: anti-Slr0151, anti-D2 (1:10,000), anti-PsbO, anti-PsaB (1:5,000), anti-D1 (1:4,000), anti-CP43 (1:3,000), and anti-CP47 (1:2,000). The binding of the cross-reacting antibody was detected using the Pro-light HRP ECL detection system (Tiangen Biotech, Tiangen, China). The blots were scanned using a UMAX PowerLook 2100XL scanner (Willich, Germany) at a resolution of 600 dpi. Protein quantification was carried out using ImageJ software (<http://rsbweb.nih.gov/ij/>). Protein content was determined according to Peterson (1977) using bovine serum albumin as a standard.

Salt washes and detergent treatment of total membranes of *Synechocystis* were performed according to Schottkowski et al. (2009). Briefly, aliquots of 10 µg membrane proteins were suspended in 20 µL of 5 mmol/L HEPES buffer (pH 7.6) followed by addition of equal volume of 0.2 mol Na₂CO₃, 4 mol urea, 2 mol NaCl, and 0.5% Triton X-100, respectively. The mixture was incubated on ice for 30 min. After centrifugation at 18,000 g (4 °C) for 15 min, the supernatants were collected and the pellets were washed twice with the HEPES buffer above. All samples were analyzed by SDS-PAGE followed by immunoblotting using antibodies specific for Slr0151, D1, and PsbO, respectively.

Generation of *slr0151*-knockout and complementation mutants

The *slr0151*-knockout (Δ *slr0151*) and *slr0151*-complementation (*reslr0151*) mutants were generated according to Williams (1988) with slight modifications (Zhou et al. 2008). For knockout, the plasmids were constructed by inserting a fragment into ORF *slr0151* with kanamycin-resistance gene cassette (Km^R). The fragments upstream (83–482 bp from start codon) and downstream (82–481 bp from stop codon) of *slr0151* as well as the full-length kanamycin-resistance gene were amplified from genomic *Synechocystis* DNA and the bacterial transposon Tn 903 DNA by PCR, respectively. The three sets of PCR primers containing restriction enzyme sites for amplifying each of the fragments are listed in Table S2. These primers contained EcoRI and/or BamHI sites at the 5'- and/or 3'-end. The amplified fragments were digested with EcoRI and/or BamHI, respectively, followed by ligation into pMD19-T vector (Takara, Kyoto, Japan). The resulting plasmid was transformed into wild-type *Synechocystis* cells. Transformants were selected for growth on agar BG-11 plates supplemented with up to 50 µg/mL kanamycin. Complete segregation was verified by PCR analysis using the primer sets *slr0151*-F4 and *slr0151*-R4 (Table S2). Inactivation of *slr0151* expression was confirmed by RT-PCR using the primer sets *slr0151*RT-F and *slr0151*RT-R (Table S2). For complementation, the coding sequence of *slr0151* was amplified using the primer sets *reslr0151*-F and *reslr0151*-R (Table S2) and ligated into pFQ20 plasmid (Tan et al. 2011). The resulting plasmid was transformed into Δ *slr0151* mutant cells. The transformants were selected on agar BG-11 plates supplemented with 50 µg/mL kanamycin and 25 µg/mL spectinomycin.

Total RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated from *Synechocystis* wild-type and Δ *slr0151* cells using the published protocol (Mohamed and Jansson 1989). RNA purification and semiquantitative RT-PCR analysis were performed as previously described (Zhang et al. 2009) with minor modifications. Reverse transcription reactions using random primers were performed with a RevertAid M-MuLV Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA) suggested by the manufacturer. Real-time quantitative PCR was performed using the Rotor-Gene 3000 sequence detection system (Qiagen, Hilden, Germany) as described (Zhao et al. 2013). The *psbA* transcript was amplified using the *psbA*-F and *psbA*-R primers (Table S3). *rnpB* was used as internal control. The fold changes in gene expression level relative to control were calculated as described (Zhao et al. 2013).

Oxygen evolution and Chl *a* fluorescence measurements

Oxygen evolution rates of intact cells were measured with a Chlorolab-2 oxygen electrode (Hansatech, Norfolk, UK) at 30 °C by following the manufacturer's instructions. Measurements were taken for cell suspension with 10 µg/mL Chl in the presence of 1 mmol/L potassium ferricyanide (KFe(CN)₆) and 1 mmol/L 2,6-dichloro-*p*-benzoquinone as described (Gombos et al. 1994). Light intensity was adjusted by use of neutral density filters and determined using a quantum photometer (Hansatech). Chlorophyll *a* fluorescence from PSII was measured with PAM 101 or Maxi-Imaging PAM Chl

fluorometers (Heinz Walz, Effeltrich, Germany) by following the manufacturer's instructions. Samples were adjusted to a density of 15 µg/mL Chl in the growth medium and were dark-adapted for 2 min before each measurement. The minimal (F_0) and maximal fluorescence (F_m) were obtained after illumination of the dark-adapted state with low-intensity modulated light (5 µmol/m² s) and a single saturating pulse of white actinic light (1,200 µmol/m² s), respectively. The variable fluorescence (F_v) was calculated as $F_v = F_m - F_0$. Maximum quantum yield of PSII was estimated by F_v/F_m . Light-induced redox changes of P700 were monitored by measuring absorbance at 820 nm using the PAM101 fluorometer equipped with a dual-wavelength P700 unit (ED800T). Cell suspension with 30 µg/mL Chl was used. A far-red light illumination (FR, 720 nm, 24 µmol/m² s) was provided for 45 s to enable oxidation of P700⁺ to a steady state then turned off to monitor the initial rate of P700⁺ dark reduction as described (Klughammer and Schreiber 1998). 77K fluorescence emission spectra were measured using F-2500 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan). Cell suspensions with 15 µg/mL Chl were frozen in a measuring cuvette using liquid N₂. An excitation wavelength of 435 nm (bandwidth, 5 nm) was used to induce Chl *a* fluorescence. The emission spectra were recorded in the range of 650–800 nm. The spectra were normalized at 726 nm.

In vivo pulse-chase labeling of the membrane proteins

In vivo labeling of proteins using L-(³⁵S) methionine was performed by following the protocol described by Nixon et al. (1995). For the pulse (to assess the rate of D1 synthesis), cells were washed and resuspended in a sulfur-depleted BG-11 medium to the density of 25 µg/mL Chl. The cell suspensions were radio-labeled with 2 µCi/mL (³⁵S) methionine (>1,000 Ci/mmol/L, Perkin Elmer, Waltham, USA) at a light intensity of 100 µmol/m² s and 30 °C for up to 20 min. For the chase, the labeled cells were washed and resuspended in the BG-11-S containing "cold" methionine at a final concentration of 2 mmol/L. Cells were subjected to high irradiance (1,100 µmol/m² s) for up to 90 min. Aliquots were taken at different time intervals and were subjected to membrane preparation. The membrane proteins were separated by SDS-PAGE and immunoblot analysis as described above. For autoradiography, gels were stained with Coomassie blue R, dried, and exposed to phosphorimaging plates (Imaging Screen-K Kodak; Bio-Rad, Richmond, CA, USA). The radioactive signal was visualized with a Typhoon Trio scanner (Typhoon TRIO Variable Mode Imager, GE Healthcare, Pittsburgh, PA, USA). Quantification of radio-labeled D1 was performed using ImageJ software.

2-D BN/SDS-PAGE and protein detection

Two dimensional BN/SDS-PAGE of membrane protein complexes was performed according to Herranen et al. (2004) with minor modifications. For silver and immunoblot detection, 6 and 3 µg Chl of total membranes were used, respectively. The membranes were washed and resuspended in a buffer containing 25 mmol/L Bis-Tris/HCl (pH 7.0), 10 mmol/L MgCl₂, and 20% glycerol (w/v) at Chl *a* concentration of 1 mg/mL. An equal volume of resuspension buffer supplemented with 4% DM (Sigma-Aldrich, St Louis, MO, USA) was added under gentle mixing. The mixture was incubated on ice for 50 min

then at room temperature for 10 min in the presence of 1 mmol/L PMSF to allow the solubilization of membrane protein complexes. After centrifugation at 18,000 g for 15 min (4 °C), the supernatant was mixed with 0.1 volume of Coomassie blue solution consisting of 5% (w/v) Serva Blue G, 100 mmol/L Bis-Tris/HCl (pH 7.0), 500 mmol/L ε-amino-n-caproic acid, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), and 30% (w/v) sucrose. The samples were loaded onto 0.75 mm thick 5–12.5% acrylamide gradient gel (Hoefer SE250, GE Healthcare) containing 500 mmol/L ε-amino-n-caproic acid. Electrophoresis was performed at 4 °C and the running program was 50 V for 40 min followed by gradually increasing voltage from 50 up to 200 V. For resolving proteins in the second dimension, the lanes of BN gel were excised and incubated in SDS sample buffer containing 10% β-mercaptoethanol and 8 mol urea for 45 min at 30 °C. The lanes were then placed onto SDS-PAGE gel (Laemmli 1970) with 12.5% acrylamide and 6 M urea. The electrophoresis was carried out using Hoefer SE250 apparatus (GE Healthcare). Proteins were visualized using a silver staining kit (Sangon Biotech, Shanghai, China) or by immunoblot analysis as described above.

Protein-protein interaction assays

Yeast two hybrid analysis was performed using the Gal4 system (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. The coding sequence of *slr0151* was amplified and ligated into the bait vector pGBKT7. Coding sequences for the candidate proteins as well as for the fragments corresponding to the C-terminal of the mature D1 (D1 292–344) and the precursor D1 (D1 292–360) were amplified and ligated into the prey vector pGADT7, respectively. The primers used are listed in Table S4. The resulting respective construct was co-transformed into yeast strain AH109. Interactions were monitored by growth on SD/-Leu/-Trp/-His/-Ade selection medium containing 40 µg/mL X-α-Gal. Pull-down assays were performed as described (Sun et al. 2007) with minor modifications. Briefly, coding sequence of *slr0151* was amplified with specific primers *slr0151*-F5 and *slr0151*-R5 (Table S1) and cloned into the expression vector pET28a (+) (Merck) with His-tag sequences at both N- and C-terminal, yielding pET28a (+)-*slr0151*'. Expression and purification of the recombinant Slr0151' protein were performed as described above (Zhou et al. 2008). The purified proteins were desalted using a PD-10 Desalting Column (GE Healthcare). Ten micrograms of the desalted proteins were added to 100 µL of Ni-NTA suspension (agrose resin, equilibration buffer = 1:1, v/v) (GE Healthcare). The mixture was incubated for 1 h at 4 °C. The equilibration buffer contained 20 mmol/L Na₃PO₄ (pH 7.8) and 100 mmol/L NaCl. One hundred micrograms of Chl thylakoid membranes were solubilized in 500 µL of 25 mmol/L Bis-Tris/HCl buffer (pH 7.0) supplemented with 1% (w/v) DM (Sigma-Aldrich), 20% glycerol (w/v), and 1 mmol/L PMSF for 30 min at 4 °C. After centrifugation at 10,000 g for 10 min, the supernatant was added to the above mixture and incubated at 4 °C for 1 h. The beads were then washed fivefold with ice-cold washing buffer containing 50 mmol/L Tris/HCl (pH 7.5), 100 mmol/L NaCl, and 1 mmol/L EDTA and then resuspended and incubated in SDS-PAGE loading buffer for 30 min at 42 °C. The solubilized proteins were resolved via SDS-PAGE followed by immunoblot analysis as described above.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article at the publisher's web-site.

Figure S1. Heterologous expression of recombinant Slr0151 protein and specificity of Slr0151 antibody

(A) Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins extracted from *Escherichia coli* Transetta (DE3) cells. Proteins from the same number of bacteria were analyzed by 12% SDS-PAGE and stained with Coomassie brilliant blue. Lane 1, total proteins without isopropyl β-D-1-thiogalactopyranoside (IPTG) induction; lane 2, total proteins with IPTG induction; lane 3, recombinant Slr0151 purified using Ni-NTA column; M, protein molecular mass marker. **(B)** Specificity detection of Slr0151 antibody by immunoblotting. Lane 1, 0.02 μg purified recombinant Slr0151 protein; lane 2, proteins (20 μg) extracted from *Synechocystis*.

Figure S2. Schematic representation of the constructs used for (A) disruption and (B) complementation of *slr0151*

Figure S3. Growth and photosynthetic characteristics of Δ*Slr0151* mutant

(A) Growth of wild-type (WT) and $\Delta slr0151$ cells under normal light conditions. Photograph showing the cell suspension cultured for 72 h. Data are expressed as means ($\pm$ SD) of three independent experiments. (B) 77K low temperature fluorescence emission spectra of WT, $\Delta slr0151$ and $reslr0151$. The spectra were normalized at 726 nm. (C) Comparison of photosynthetic O₂ evolution between WT and $\Delta slr0151$. Data are expressed as means ($\pm$ SD) of three independent experiments. (D) P700 redox kinetics of WT and $\Delta slr0151$ cells at 820 nm induced by far-red light (720 nm). All experiments were repeated twice with similar results.

Figure S4. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detection of (³⁵S)-Met-labeled D1 protein in wild-type (WT) and $\Delta slr0151$

Membrane fractions isolated from 10 min radioactive-labeled cells were separated by 12% SDS-PAGE and visualized by Coomassie blue staining (stain) and autoradiography (autorad). The position of D1 was confirmed by immunoblotting (α -D1).

Figure S5. 2-D blue native (BN)/sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of membrane complexes in wild-type (WT) and $\Delta slr0151$ under normal growth condition

Membrane proteins corresponding to 3 μ g (for immunoblotting) or 6 μ g (for silver stain) of chlorophyll were solubilized with 2% n-dodecyl- β -D-maltoside (DM) then subjected to 2-D

BN/SDS-PAGE. Membrane complexes were designated according to Herranen et al. (2004). Proteins were visualized by silver staining. The presence of Slr0151 in wild-type cells was confirmed by immunoblotting (α -Slr0151).

Figure S6. Sequence alignment of Slr0151 with homologs from other cyanobacteria

Amino acid sequences are: *Crocospaera* (accession no. WP007310361), *Cyanothece* (accession no. YP003885651), *Pleurocapsa* (accession no. YP007079840), *Synechococcus* (accession no. YP007062190), and *Thermosynechococcus* (accession no. NP683093). Identical residues are highlighted in black. Residues that conserved at least 33% are shown in gray. Gaps introduced to optimize sequence alignment are indicated by dots. The sequences were aligned using the Clustal omega program (<http://www.ebi.ac.uk/Tools/msa/clustalo>). tetratricopeptide repeat (TPR) repeats were calculated with TPRpred software and marked by converging arrows. The transmembrane domain predicated by TOPCONS is underlined.

Table S1. Primers used for cloning and heterologous expression of *slr0151* gene

Table S2. Primers used for construction of $\Delta slr0151$ and complementation mutants

Table S3. Primers used for quantitative reverse transcription polymerase chain reaction analysis of *psbA* gene

Table S4. Primers used for yeast two hybrid analysis