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A Feed-Forward Loop Consisting of the Response Regulator RpaB and the Small RNA PsrR1 Controls Light Acclimation of Photosystem I Gene Expression in the Cyanobacterium *Synechocystis* sp. PCC 6803

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Since cyanobacteria need to decrease PSI content to avoid absorption of excess light energy, down-regulation of PSI gene expression is one of the key characteristics of the high-light (HL) acclimation response. The transcriptional regulator RpaB and the small RNA PsrR1 (photosynthesis regulatory RNA1) have been suggested to be the two most critical factors for this response in *Synechocystis* sp. PCC 6803. In this study, we found that the HLR1 DNA-binding motif, the recognition sequence for RpaB, is highly conserved in the core promoter region of the *psrR1* gene among cyanobacterial species. Gel mobility shift assay revealed that RpaB binds to the HLR1 sequence of *psrR1* in vitro. RNA gel blot analysis together with chromatin affinity purification (ChAP) analysis suggested that PSI genes are activated and the *psrR1* gene is repressed by the binding of RpaB under low-light (LL) conditions. A decrease in DNA binding affinity of RpaB occurs within 5 min after the shift from LL to HL conditions, leading to the prompt decrease in PSI promoter activity together with derepression of *psrR1* gene expression. Accumulating PsrR1 molecules then prevent translation from pre-existing PSI transcripts. By this dual repression at transcriptional and post-transcriptional levels, rapid and strict down-regulation of PSI expression under HL is secured. Our findings suggest that RpaB and PsrR1 constitute a feed-forward loop for the regulation of PSI gene expression to achieve a rapid acclimation response to the damaging HL conditions.

Keywords: Cyanobacteria • High-light acclimation • Photosystem I • Post-transcriptional regulation • Small RNA • Transcriptional regulation.

Abbreviations: ChAP, chromatin affinity purification; DIG, digoxigenin; HL, high light; HLR1, high light regulatory 1; LL, low light; OD, optical density; PsrR1, photosynthesis regulatory RNA1; qPCR, quantitative PCR; S.6803, *Synechocystis* sp. PCC 6803; sRNA, small RNA; 5′-UTR, 5′-untranslated region; WT, wild type.

Introduction

Photosynthetic organisms have to maintain a balance between the energy supply by the photosynthetic electron transport chain and its consumption by cellular metabolism. Regulation of these processes in response to changes in environmental factors, such as light intensity and nutrient availability, is particularly important for controlling the redox balance of the cell (Oelze et al. 2008). A decrease in the amount of photosynthetic pigments and photosystems, especially that of PSI, to avoid the absorption of excess light energy is one of the main strategies of high-light (HL) acclimation in cyanobacteria (Kawamura et al. 1979, Murakami and Fujita 1991, Muramatsu and Hihara 2012). Two mutants of *Synechocystis* sp. PCC 6803 (S.6803), disruptants of *pmgA* (sll1968) and *sll1961*, both of which are not able to maintain their PSI content at a low level, exhibit severe growth inhibition under prolonged HL conditions (Hihara et al. 1998, Fujimori et al. 2005). This suggests physiological significance of the selective decrease in PSI content during HL acclimation.

A whole-genome DNA microarray analysis suggested that a decrease in PSI content during acclimation to HL is regulated at the level of transcription (Hihara et al. 2001). Genes encoding subunits of PSI are actively transcribed under LL conditions, whereas their transcription is co-ordinately and greatly down-regulated immediately after a shift to HL conditions (Muramatsu and Hihara 2003). RpaB, a DNA-binding response regulator which is highly conserved in cyanobacteria and even in red algae plastid genomes, constitutes, together with the histidine kinase Hik33 (also named as NblS, DspA), a key regulatory system for HL acclimation (reviewed in Wilde and Hihara 2016). It has been proposed that RpaB is phosphorylated by Hik33 under LL and its phosphorylation level is lowered upon exposure to HL in *Synechococcus elongatus* PCC 7942 (Moronta-Barrios et al. 2012). We found that binding of RpaB to the high light regulatory 1 (HLR1) sequence located upstream of the core promoter region (position −70 to −46, relative to the transcription start site) enables the co-ordinated

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HL response of PSI genes dispersed throughout the S.6803 genome (Seino et al. 2009). Binding of RpaB to the HLR1 sequence under LL conditions results in the activation of PSI genes, and simultaneously results in the repression of HL-inducible genes such as *hliB* in S.6803 (Kappell and van Waasbergen 2007), and *hliA* and *rpoD3* in *S. elongatus* PCC 7942 (Seki et al. 2007).

However, the regulatory network that controls photosynthetic gene expression must integrate various environmental signals, such as the availability of inorganic carbon and other nutrients, and, most importantly, differences in ambient light which may change in sudden and unpredictable ways under natural conditions. This may involve non-coding small RNAs (sRNAs) that control gene expression at the post-transcriptional level. Although usually only 80–140 nucleotides in length, these sRNAs can repress or activate gene expression and frequently control multiple mRNAs (Wright et al. 2013). In enterobacteria, sRNAs are known to modulate almost every aspect of bacterial physiology and behavior (Storz et al. 2011). Cyanobacteria were also shown to harbor many potential regulatory RNA molecules, which control different cellular functions (Hess et al. 2014, Kopf and Hess 2015). Indeed, the HL response of PSI genes in S.6803 has been found recently to be under additional control of the sRNA PsrR1 (photosynthesis regulatory RNA1) (Georg et al. 2014). PsrR1, widely conserved within the cyanobacterial phylum, is induced under HL conditions and interacts with the ribosome-binding site of transcripts of photosynthetic genes such as *psaLI*, *psaJ*, *chlN* and *cpcA* to inhibit translation. Moreover, PsrR1 is likely to decrease the stability of target transcripts, judging from the observation that the *psaLI* mRNA was cleaved by the endonuclease RNase E upon the binding of PsrR1 to the 5′-untranslated region (UTR). The amounts of phycocyanin, Chl and the PSI complex all decrease upon overexpression of PsrR1, indicating that PsrR1 is critical for the post-transcriptional down-regulation of the photosynthetic apparatus under HL (Mitschke et al. 2011, Georg et al. 2014). In addition, overexpression of PsrR1 results in a decrease in the PSI trimer to monomer ratio (Georg et al. 2014). Trimeric PSI complexes were shown to have a larger cross-section than monomeric complexes and were suggested to be favored under LL (Baker et al. 2014). Under HL, a specific decrease in the amount of PSI trimer is observed (Kopečná et al. 2012). Thus, PsrR1 might be involved in balancing the excitation of both photosystems by down-regulation of *psaL* expression under HL, thereby inducing monomerization of PSI.

Accumulating data suggest that RpaB and PsrR1 are the two most important critical factors for the regulation of PSI gene expression during HL acclimation. Therefore, we addressed the possibility of an interplay between these two regulators. In this study, we found that RpaB binds to the HLR1 sequence located within the core promoter region of *psrR1* to repress its expression under LL conditions. Our findings suggest that RpaB and PsrR1 constitute a feed-forward loop for regulation of PSI gene expression to achieve rapid and strict repression upon the shift to HL conditions.

Results

HLR1 sequence is located in the core promoter region of the *psrR1* gene

First, we carried out an in-depth search for PsrR1 homologs in the cyanobacterial phylum. We were able to extend the number of potential homologs of PsrR1 and furthermore detected up to three homologs in individual organisms (**Supplementary Fig. S1**). PsrR1 is widely conserved in the cyanobacterial phylum, especially in the more complex filamentous cyanobacteria, whereas we could not identify homologs in marine picocyanobacteria (*Prochlorococcus* and *Synechococcus*), thermophilic *Synechococcus*, *S. elongatus*, *Gloeobacter* and some other strains. This distribution differs from that of RpaB, which is ubiquitously present in cyanobacteria.

PsrR1 expression was shown to be strongly up-regulated upon the shift to HL in S.6803 (Georg et al. 2014). To elucidate the mechanism of transcriptional regulation of *psrR1*, we searched the proposed promoter regions upstream of the *psrR1* homolog genes from several strains and carried out an initial motif search with MEME (Bailey et al. 2009). In a subset of the proposed *psrR1* promoters, we detected a motif which resembled the previously characterized HLR1 motif. A ClustalW multiple sequence alignment of the promoter regions of 14 PsrR1 homologs revealed that the HLR1 sequence was found in the core promoter region, overlapping with its −10 box (**Figs. 1A**, 2A). It should be noted that the promoter region including the HLR1 sequence is more highly conserved than the 5′ portion of the *psrR1* gene itself among different cyanobacteria (**Fig. 1B**).

RpaB binds to the HLR1 sequence of *psrR1* in vitro

In order to test whether RpaB binds to the promoter region of *psrR1* from S.6803, we overexpressed recombinant RpaB protein fused to a C-terminal 6× histidine-tag (His-RpaB) in *Escherichia coli*. His-RpaB was purified by nickel affinity chromatography from the soluble fraction (**Supplementary Fig. S2**). First, we examined DNA binding activity of the purified His-RpaB to the promoter fragment of *psaE* (−97 to +23, relative to the transcription start site, 120 bp) previously characterized as the target gene of RpaB (Seino et al. 2009) by gel mobility shift assay (**Supplementary Fig. S3**). In the previous study using N-terminal His-tagged RpaB purified from the insoluble fraction, the addition of 0.5 μg of His-RpaB was not sufficient for a band shift (Seino et al. 2009). In contrast, a distinct shifted band was observed with 0.5 μg of His-RpaB in the present study (**Supplementary Fig. S3**). When the *psrR1* promoter fragment (−84 to +16, relative to the transcription start site, 100 bp) was used as a probe, 0.5 μg of His-RpaB was also sufficient for a band shift (**Fig. 2B**).

In order to address whether RpaB binds to the HLR1 sequence, gel mobility shift experiments were performed with probes having a base substitution within the HLR1 sequence of *psrR1* (**Fig. 2**). RpaB could not bind to the 'sub1' fragment with four base substitutions within the half site of the HLR1

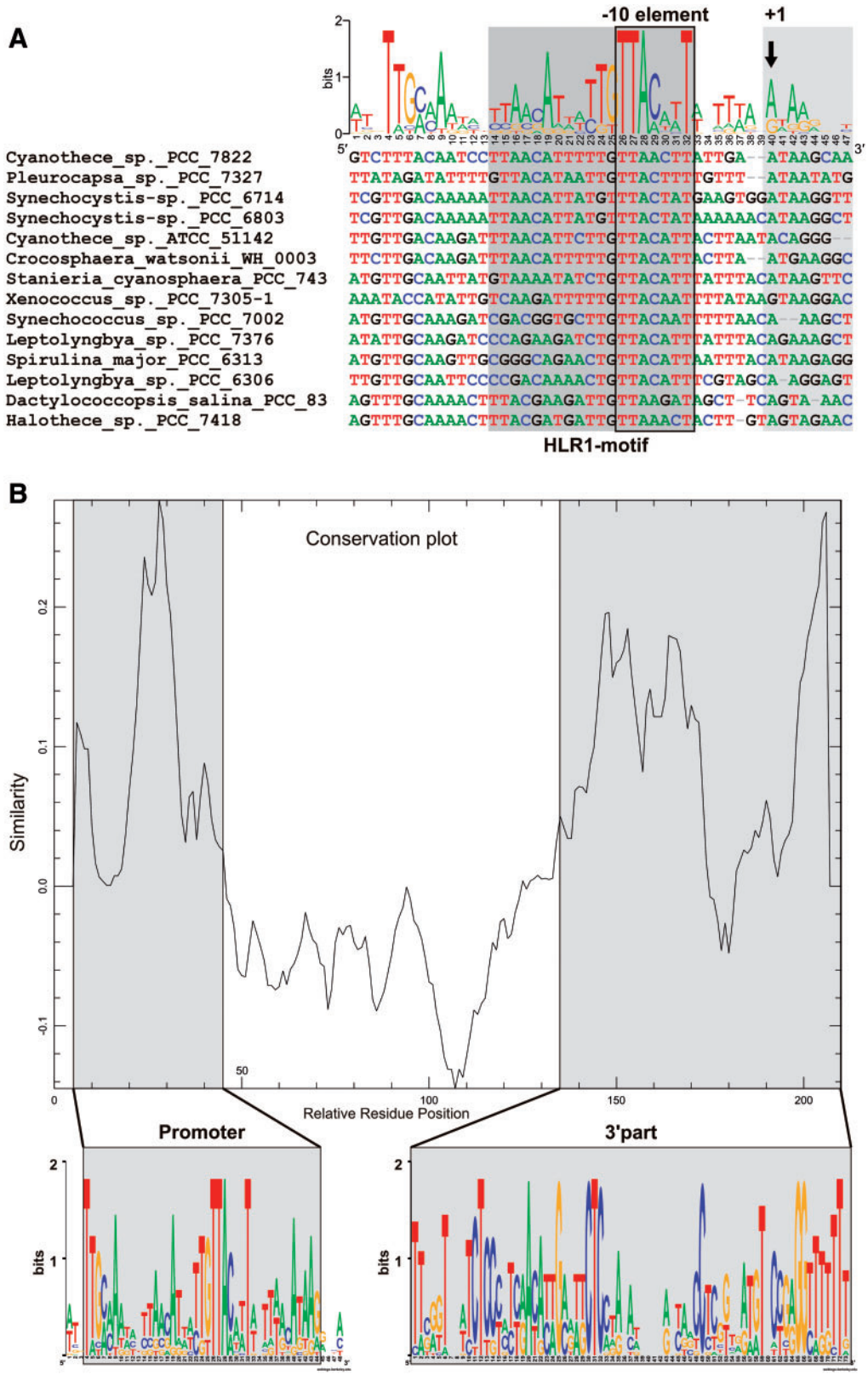

Fig. 1 (A) Multiple sequence alignment of the promoter regions of 14 *psrR1* homolog genes from selected organisms. Position-wise nucleotide conservation is visualized with a weblogo (Crooks et al. 2004). The proposed HLR1 motif and −10 elements are highlighted. The first transcribed nucleotide is corroborated by transcriptome data from S.6803, *Synechocystis* sp. PCC 6714 (Kopf et al. 2014, Kopf et al. 2015) and *Synechococcus* sp. PCC 7002 (Ludwig and Bryant 2012). (B) Conservation of the promoter sequences and the *psrR1* 3′ parts is more pronounced than that of the *psrR1* 5′ parts. The conservation plot was based on the full-length sequences of the 14 *psrR1* homologs including the promoter regions and drawn using the Emboss tool plotcon with a window size of 10. The sequence logos of the promoter regions and the 3′ parts are shown below the conservation plot.

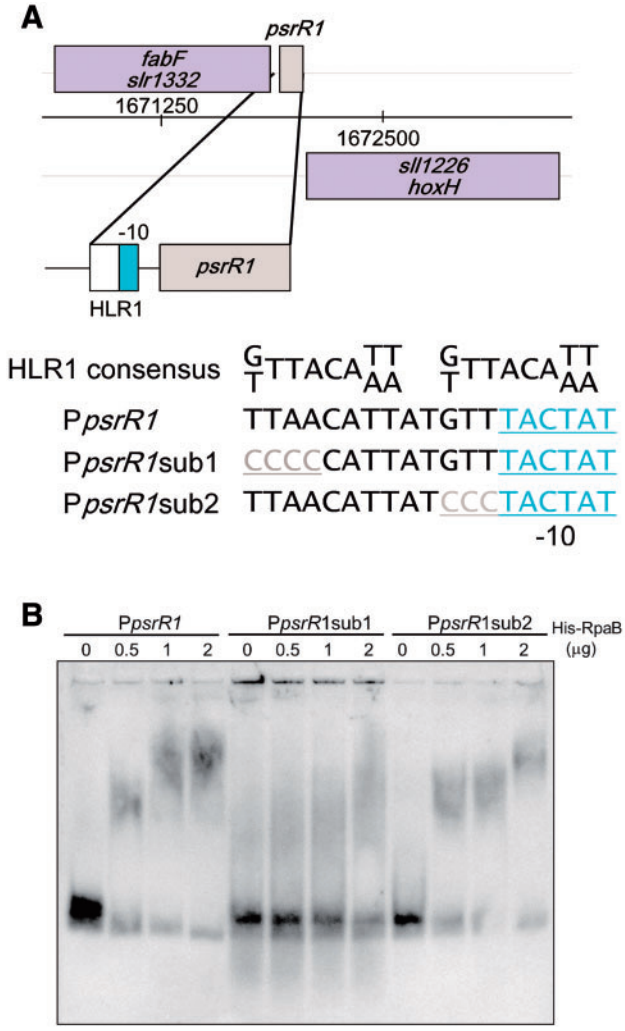

Fig. 2 Effect of base substitutions within the HLR1 sequence of the *psrR1* promoter. (A) Schematic representation of the location of the *psrR1* gene with its HLR1 sequence in the S.6803 genome and nucleotide sequences of the HLR1 sequences with or without base substitutions used for gel mobility shift assay. The sites of base substitution in P*psrR1*sub1 and P*psrR1*sub2 fragments are underlined. (B) Gel mobility shift assay of the *psrR1* promoter segments with or without base substitution. DIG-labeled *psrR1* promoter fragments (−84 to +16) with or without base substitution were incubated for 30 min with His-RpaB added at the indicated concentrations. Samples were separated on a 6% polyacrylamide gel.

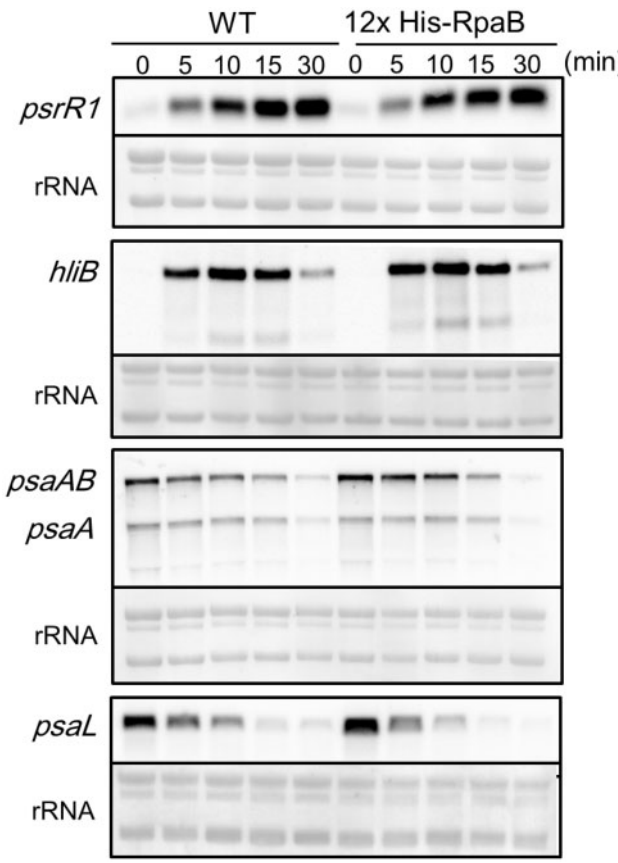

Fig. 3 Changes in transcript levels of the RpaB regulon genes, *psrR1*, *hliB*, *psaA* and *psaL*, upon the shift from LL to HL conditions in the wild-type (WT) and 12 × His-RpaB strains. Total RNA was isolated from cells incubated for the indicated time after the shift from LL (20 μmol photons m^{-2} s^{-1}) to HL (300 μmol photons m^{-2} s^{-1}). Transcripts were detected by RNA gel blot analysis using single-stranded riboprobes. The amount of total RNA loaded per lane was as follows: 2 μg for *psaA* and 4 μg for *psrR1*, *hliB* and *psaL*. rRNA was visualized with methylene blue staining.

sequence distal to the transcription start site, whereas it showed binding activity to the 'sub2' fragment with three base substitutions within the proximal half site of HLR1.

PsrR1 is induced within 5 min after the shift from LL to HL conditions

The HL response of *psrR1* and the established target genes of RpaB, *hliB*, *psaA* and *psaL*, was examined by RNA gel blot analysis in the wild-type (WT) strain (**Fig. 3**, left). Georg et al. (2014) reported that the transcript level of *psrR1* increased dramatically within 30 min after the shift to HL and then slowly decreased. Even after 24 h of incubation under HL, the accumulation level of PsrR1 was 4-fold higher than that under LL conditions. In this study, we examined changes in transcript levels of RpaB target genes within the first 30 min after the shift to HL, since RpaB is involved in transcriptional regulation just after the shift to HL. Accumulation of PsrR1 was induced within 5 min after the shift from LL (20 μmol photons m^{-2} s^{-1}) to HL (300 μmol photons m^{-2} s^{-1}) conditions and continued to increase during 30 min. The amount of *hliB* transcript also increased within 5 min, but began to decrease after 15 min of HL exposure. In the case of *psaA* and *psaL* genes encoding PSI subunits, transcript accumulation continued to decrease after the shift from LL to HL conditions as reported previously (Hihara et al. 2001, Muramatsu and Hihara 2003).

DNA binding activity of RpaB to the promoter regions of *psrR1* and other target genes decreased after the shift from LL to HL conditions

The in vivo DNA binding activity of RpaB to the upstream regions of *psrR1* and other target genes was examined by chromatin affinity purification (ChAP) analysis. For ChAP analysis, the 12 × His-RpaB strain was generated by disruption of the

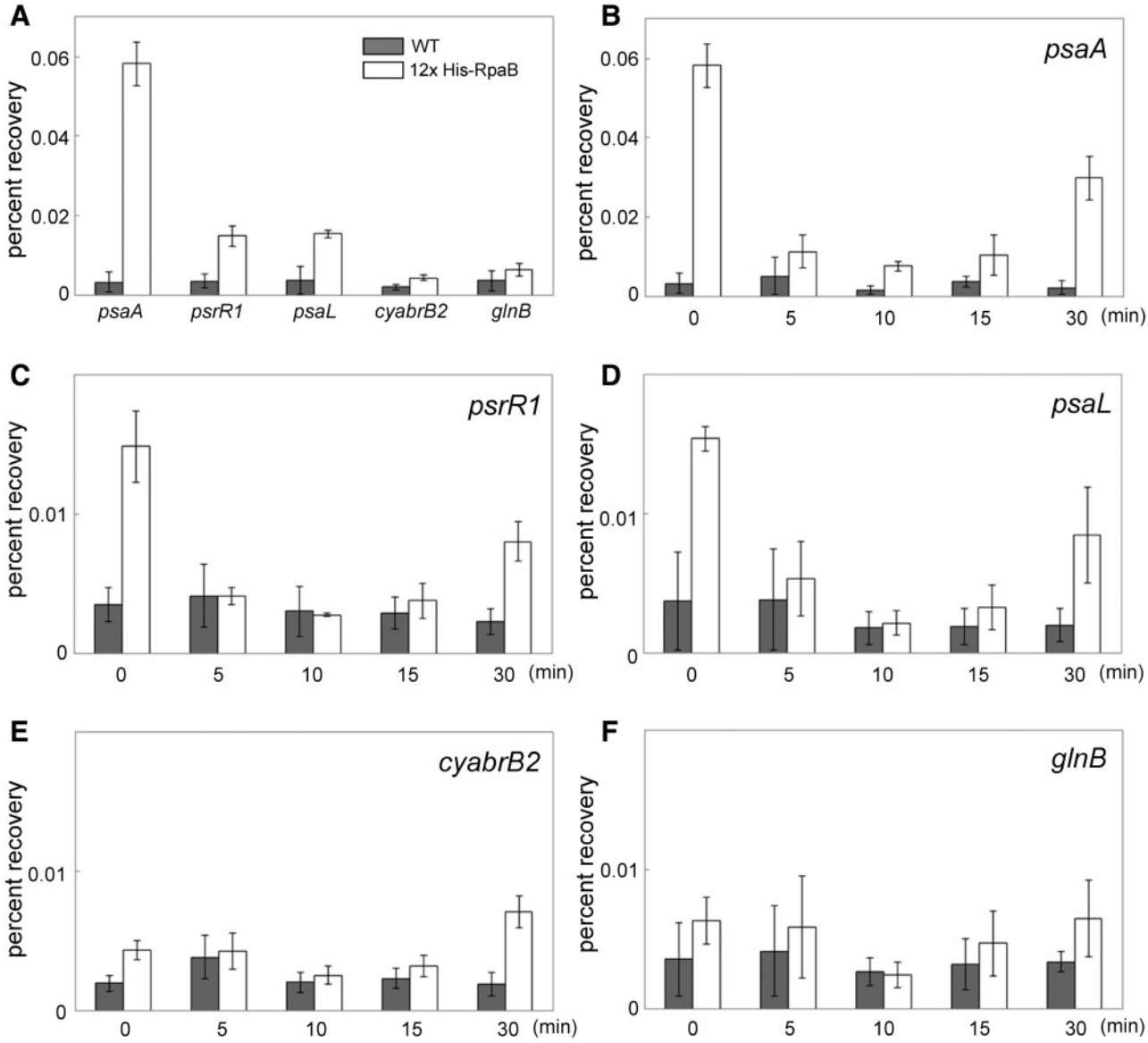

Fig. 4 Changes in DNA binding activity of RpaB to its target promoters upon the shift from LL to HL conditions in the wild-type (WT) and 12 × His-RpaB strains. ChAP was performed with whole-cell extracts of strains with or without expression of the 12 × His-tag RpaB protein grown under LL (20 μmol photons m^{-2} s^{-1}) or HL (300 μmol photons m^{-2} s^{-1}) conditions. The level of DNA co-purified by nickel chromatography was determined by qPCR analysis and is expressed as percentage recovery relative to the total input DNA. Data are means ± SD from three independent experiments. (A) Comparison of percentage recovery among *psaA*, *psrR1*, *psaL*, *cyabrB2* and *glnB* under LL conditions. Time course change of DNA binding activity of RpaB to the upstream region of (B) *psaA*, (C) *psrR1*, (D) *psaL*, (E) *cyabrB2* and (F) *glnB* after the shift to HL.

endogenous *rpaB* gene and insertion of the C-terminal 12 × His-tagged *rpaB* gene with its own promoter into a neutral site within the genome (**Supplementary Fig. S4A**). By using this strain, specific binding of RpaB to target promoters in vivo can be examined by affinity purification of cross-linked DNA–protein complexes. We confirmed the complete disruption of the endogenous *rpaB* and introduction of 12 × His-*rpaB* in the mutant cells by PCR (**Supplementary Fig. S4B**). Immunoblot analysis using anti-RpaB and anti-His-tag antisera revealed that only the His-tagged RpaB protein was expressed in the 12 × His-RpaB strain (**Supplementary Fig S4C**). We also confirmed that the HL response of the RpaB regulon was similar between WT and 12 × His-RpaB strains (**Fig. 3**).

ChAP analysis was performed using the 12 × His-RpaB strain and the WT strain as a negative control. After in vivo cross-linking of DNA–protein complexes by formaldehyde and affinity purification of 12 × His-RpaB by nickel chromatography, the amount of co-purified DNA was examined by quantitative PCR (qPCR) analysis and expressed as the percentage recovery in relation to the amount of input DNA (see the Material and Methods). As shown in **Fig. 4A**, in ChAP samples prepared from the 12 × His-RpaB strain grown under LL conditions, *psaA*, *psrR1* and *psaL* promoters exhibited a higher percentage recovery (0.015–0.06%) than *cyabrB2* and *glnB* promoters not having the HLR1 sequence (0.004–0.007%). In ChAP samples prepared from the WT strain grown under LL conditions, each promoter

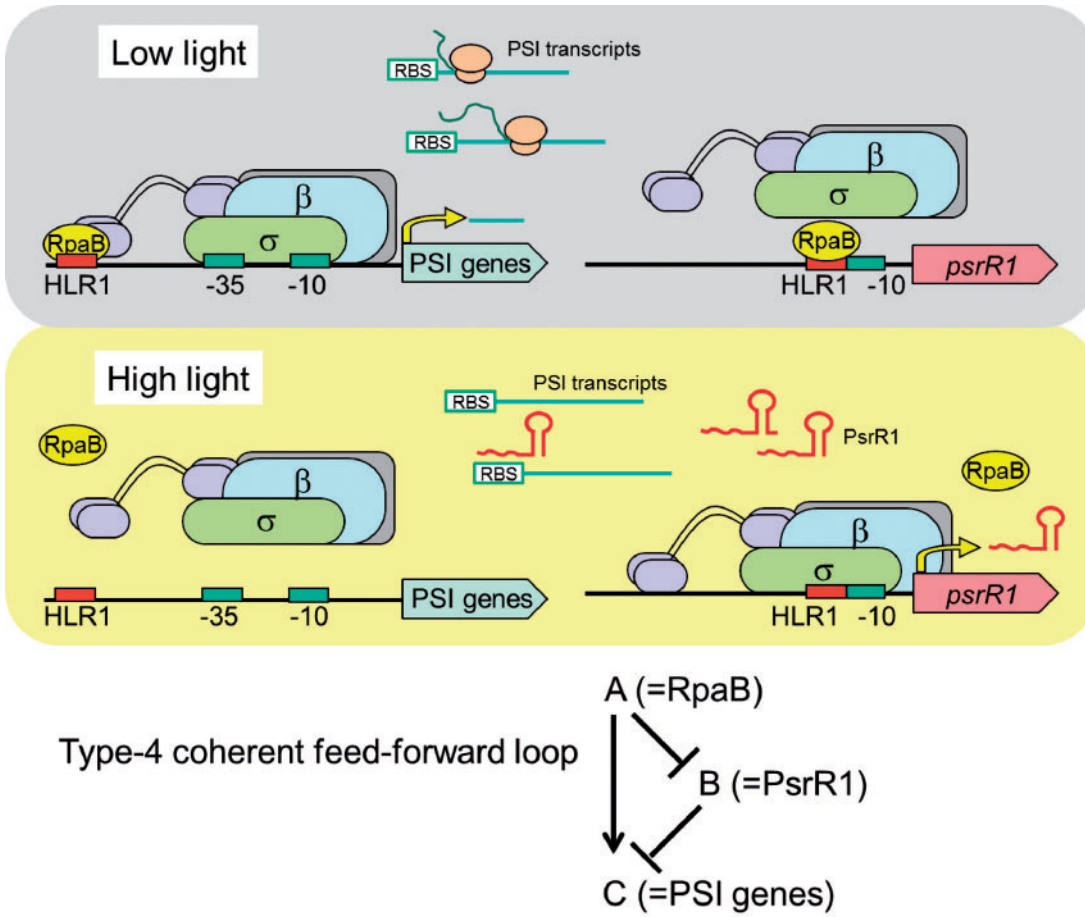

Fig. 5 Schematic representation of the dual repression of PSI genes by RpaB and PsrR1 upon the shift to HL and of the type-4 coherent feed-forward loop. RpaB activates the expression of PSI genes under LL, whereas the regulation is lost under HL, leading to the decline of transcription activity of PSI genes. Transcription of *psrR1* is repressed by RpaB under LL but de-repressed upon the shift to HL. Transcribed PsrR1 interacts with the ribosome-binding site of PSI transcripts to inhibit translation. This dual repression at the transcriptional and post-transcriptional levels achieved by RpaB and PsrR1 can be categorized into the 'type-4 coherent feed-forward loop' which is proposed to decrease leaky expression of target genes under repressing conditions.

showed a percentage recovery of<0.004% irrespective of the presence or absence of the HLR1 sequence. These results indicate that RpaB specifically binds to the promoter region of *psrR1* as well as to those of known target genes, *psaA* and *psaL*, under LL conditions.

Next, we examined the dynamic change in the RpaB binding activity to the target promoters upon the shift from LL to HL conditions. For promoters of *psaA* (**Fig. 4B**), *psrR1* (**Fig. 4C**) and *psaL* (**Fig. 4D**), 5 min of exposure to HL resulted in a decrease of binding activity of RpaB to <35% of the LL level, and the low binding activity was maintained for at least 15 min. After 30 min, recovery of binding activity to the LL level was observed. On the other hand, the change in binding activity of RpaB after HL shift was insignificant in the case of the *cyabrB2* (**Fig. 4E**) and *glnB* promoters (**Fig. 4F**). These results indicate that RpaB binds to the promoter regions of *psrR1* and other target genes under LL conditions and that HL exposure causes the prompt and transient decline of the binding activity.

Discussion

The recent progress in the functional characterization of sRNAs demonstrated that sRNA-based post-transcriptional regulation, as well as transcriptional regulation, is important for bacteria to acclimate to changing environmental conditions, including cyanobacteria (Georg et al. 2014, Klähn et al. 2015). In various bacterial species, it has been reported that sRNAs participate in global regulatory networks together with protein regulators by forming regulatory circuits (Beisel and Storz 2010). In the present study, we propose that RpaB, a key regulator for HL acclimation, and the sRNA PsrR1 constitute a feed-forward loop to regulate expression of PSI genes upon exposure to HL conditions in the cyanobacterium S.6803.

The HLR1 sequence, the recognition sequence for RpaB, is highly conserved in the upstream region of the *psrR1* gene among cyanobacterial species (**Fig. 1**). Gel mobility shift assays revealed that RpaB binds to the HLR1 sequence overlapping with the −10 box of the *psrR1* promoter in S.6803 (**Fig. 2**). It should be noted that RpaB can bind to the 'sub2'

fragment with three substituted bases within the proximal half site of HLR1, but not to the 'sub1' fragment with four base substitutions within the distal half site. Previously, we reported that RpaB cannot bind to the HLR1 sequence of the *psaE* promoter when base pair substitutions were introduced in one of the HLR1 half sites (Seino et al. 2009). Since the recombinant RpaB protein used for gel mobility shift assay was different mainly in the location of the His-tag between the previous and present studies, we re-examined the effect of base substitutions in HLR1 within the *psaE* promoter using the RpaB protein purified in this study. As shown in **Supplementary Fig. S3**, RpaB bound to the 'sub2' fragment and a slight band shift was observed in the case of the 'sub1' fragment of the *psaE* promoter. The recombinant protein used in this study showed a higher DNA binding activity than that in the previous study, and this can be explained by differences between these proteins such as the location of the His-tag (N-terminus or C-terminus), purification method (from the insoluble fraction or the soluble fraction) and location of the start codon leading to a difference in protein length (234 or 243 amino acids). The fact that RpaB can bind to the 'sub2' fragment with the mutated proximal half site is consistent with the report by Hanaoka et al. (2012). They observed that RpaB binds to the HLR1-like sequence containing only one half site in the case of the circadian clock-regulated *kaiBC* promoter in *S. elongatus* PCC 7942. Although these results suggest that one half site is sufficient for RpaB binding, our previous reporter assay examining the effect of base substitution within HLR1 revealed that RpaB requires both half sites to activate the *psaE* promoter under LL (Seino et al. 2009). Further studies are needed to determine the minimal sequence requirements for binding and promoter activation by RpaB.

RNA gel blot analysis (**Fig. 3**) together with ChAP analysis (**Fig. 4**) suggested that *RpaB* binds to the *psrR1* promoter to act as a repressor under LL conditions, and its release from the promoter results in induction of the sRNA PsrR1 within 5 min after the shift to HL conditions. This is consistent with the results of chromatin immunoprecipitation (ChIP) analysis in *S. elongatus* PCC 7942 examining the binding activity of RpaB to the upstream region of HL-inducible genes, *hliA* and *rpoD3*, upon a shift to HL conditions (Hanaoka and Tanaka 2008). RpaB dissociated already after 1 min of HL exposure from target promoters in *S. elongatus* PCC 7942. Our ChAP analysis also suggested that co-ordinated down-regulation of PSI genes under HL is regulated by the light-dependent change in binding activity of RpaB: binding of the activator RpaB under LL and its release upon the shift to HL. It has been proposed that the effect of RpaB binding to target promoters is determined by the location of the HLR1 sequence. In the case of PSI genes, binding of RpaB to the HLR1 sequence located upstream of the core promoter region may facilitate binding of RNA polymerase and increase the rate of transcription initiation. On the other hand, HL-inducible genes possess the HLR1 sequence in the 5′-UTR like *rpoD3* in *S. elongatus* PCC 7942 (Seki et al. 2007) or in the core promoter regions like *hliB* (Kappel and van Waasbergen 2007) and *psrR1* in S.6803. Binding of RpaB to the HLR1 sequence of *psrR1* may prevent the interaction between RNA polymerase and the −10 element, leading to the repression of promoter activity under LL.

Based on the results obtained in this study, we suggest that RpaB and PsrR1 constitute a dual repression system of PSI gene expression under HL at both the transcriptional and post-transcriptional level (**Fig. 5**). RpaB activates the expression of PSI genes under LL, whereas the regulation is lost under HL, leading to the decline of transcription activity of PSI genes. In addition to this transcriptional effect, there is post-transcriptional regulation through PsrR1 under HL. Transcription of *psrR1* is repressed by RpaB under LL, but release of RpaB from the promoter upon the shift to HL results in de-repression of *psrR1*. Transcribed PsrR1 interacts with the ribosome-binding site of PSI transcripts to inhibit translation (Georg et al. 2014). Although transcription activity of PSI genes is down-regulated within 5 min after the shift to HL, it takes >30 min for complete degradation of the pre-existing PSI transcripts (**Fig. 3**). PsrR1 is likely to be highly induced within 5 min and prevents translation from these transcripts under HL. Furthermore, it was shown that binding of PsrR1 to the *psaL* mRNA induces cleavage of the transcript by RNase E (Georg et al. 2014), thereby enhancing RNA degradation upon HL stress. By this dual repression at the transcriptional and post-transcriptional level, rapid and strict down-regulation of PSI genes under HL is attained.

Recent discoveries concerning how sRNAs participate in global regulatory networks in bacteria revealed the existence of several types of regulatory circuits, such as single-input modules, dense overlapping regulons, positive and negative feedback loops and feed-forward loops (Beisel and Storz 2010). Dual repression of PSI genes achieved by RpaB and PsrR1 can be categorized into the 'type-4 coherent feed-forward loop' (Mangan and Alon, 2003), where regulator 'A' activates target 'C' and represses regulator 'B', and regulator 'B' represses target 'C' (**Fig. 5**). Pairs of transcription factor 'A' and sRNA 'B' making up the type-4 feed-forward loop have been reported in *E. coli*, e.g. involving the transcription factor CRP and the sRNA Spot42 (Beisel and Storz 2011) or the response regulator OmpR and the sRNA MicC (Chen et al. 2004). They were proposed to decrease leaky expression of target 'C' under steady-state repressing conditions to allow cells to adapt rapidly to the new environment. Based on the concept of the feed-forward loop, we can speculate about the benefits of dual repression of PSI genes by RpaB and PsrR1 upon the shift to HL. By inhibiting translation and fast degradation of the remaining mRNAs, cells can divert metabolic resources and energy toward HL acclimation responses and therefore can speed up the HL response. After 30 min of incubation under HL, we observed the recovery of binding activity of RpaB (**Fig. 4B–D**). Resumption of regulation by RpaB may be required for some target genes at this stage of HL acclimation, but expression of PSI genes should still be kept at a low level. Then, highly accumulating PsrR1 molecules, together with RNase E, function in the inactivation and degradation of PSI transcripts. It is notable that binding activity of RpaB remained at a low level after 30 min in *S. elongatus* PCC 7942 (Hanaoka and Tanaka 2008) which has no PsrR1 homologs

Table 1 Oligonucleotides used in this study

Name	Sequence (5′ to 3′)	Purpose[a]
pET21-NdeI-rpaB-F	AAGGAGATATACATATGGA AACCAATAAAGAAAG	C
pET21-XhoI-rpaB-R	GGTGGTGGTGCTCGAGCGGT TCTTCCCCCG	C
rpaB-TTG-F	CATATGTTGGAAACCAATAA AGAAAG	C
rpaB-TTG21-R	TGGTTTCCAACATATGTATA TCTCCTT	C
HpaI-2031-F	GTTAACCTCACATTGGATTC	C
Km-R	GGCGAAGAACTCCAGCATGA	C
Km-PrpaB-F	CTGGAGTTCTTCGCCTCTGCC CCATCCC	C
slr2031-rpaB-R	CAATGTGAGGTTAACTTACGG TTCTTCCCCCG	C
slr2031−12 × His-rpaB-R	CAATGTGAGGTTAACTTAA TGATGATGATGATGATGAT GATGATGATGATGCGG TTCTTCCCCCGG	C
2031-F	GATGTTGAAACATGATGA	S
2031-R	TAGTTACGATCTTGACTA	S
Pslr0946-F	TGTTTTTCCCACTGAAAC	S
rpaB-R	GAACAGGTAACCGGTGC	S
PpsrR1-F	GTGGGACACGCACCACTA	G
PpsrR1-R	GTTTCCATAGCCTTATGT	G
PpsrR1-sub1-R	GTTTCCATAGCCTTATGT TTTTTATAGTAAACATAA TGGGGGTTTTTGTCAAC GAGACTT	G
PpsrR1-sub2-R	GTTTCCATAGCCTTATGTTTTT TATAGTAGGGATAATGTT AATTTTTGTC	G
PpsaE-F	TAGAACCACTCCCAGGAG	G
PpsaE-R	CCAGGCAACAACCCAGCC	G
PpsaE-sub1-F	TAGAACCACTCCCAGGAGCAG GGACCCCTAAAGAATTGTTTT	G
PpsaE-sub2-F	TAGAACCACTCCCAGGAGCAG GGATATGTAAAGACCCCTTTT	G
psrR1-F	GGCTATGGAAACCCGACAGAATTC	R
T7-psrR1-R	TAATACGACTCACTATAGGGCG ACCGAGGGCATATCTAGGAGAAC	R
hliB-F	ATGACTAGCCGCGGATTTC	R
T7-hliB-R	TAATACGACTCACTATAGGG CGATTAGAGAGAGAGCAACC AACCCA	R
psaA-F	TTCCCCTTCCCCACGAGT	R
T7-psaA-R	TAATACGACTCACTATAGGGCGA ACCGGCCCCTACGA	R
psaL-F	TGTCACGTCCCTGACGAA	R
T7-psaL-R	TAATACGACTCACTATAGGGC GACCCCTTGGAAGGTAACCAGT	R
RT-psaA-F	ACGGTGCCCATTGCTATC	Q
RT-psaA-R	CTGAAAACGCCTATCTGTGC	Q
RT-psrR1-F	CCCTCAACTTTGTCCGCATTG	Q
RT-psrR1-R	TTCTGTCGGGTTTCCATAGCC	Q
RT-psaL-F	GGAAAAGCCGCTGTTGAGTG	Q
RT-psaL-R	AGATGCCCCACAAAAGGATCG	Q
RT-cyabrB2-F	TCCTTGCCCATCAACTATCC	Q
RT-cyabrB2-R	AGCAATGCTTCGCCAGTTAG	Q
RT-glnB-F	CAAGGGTTCACCAAATCCAG	Q
RT-glnB-R	CTCGTGCAATGATCTGGTTG	Q

[a] These oligonucleotides were used for C, cloning; S, segregation check; G, gel mobility shift assay; R, RNA gel blot analysis; Q, quantitative real-time PCR analysis.

(**Supplementary Fig. S1**). Furthermore, there will be a sign-sensitive delay of the expression of the PsrR1 targets when cells encounter LL after HL illumination, because residual PsrR1 molecules will transiently repress newly transcribed target mRNAs. Thus, the feed-forward loop might enable the cells to filter out fluctuations in light intensity and other noisy signals. The high conservation of the HLR1 sequence in the upstream region of the *psrR1* gene throughout the cyanobacterial phylum (**Fig. 1A**) suggests that the feed-forward loop consisting of RpaB and PsrR1 may play a key role in light acclimation of cyanobacteria.

Materials and Methods

Bacterial strains and growth conditions

Escherichia coli XL1-blue cells were used for routine cloning, and BL21 pLys (DE3) cells (Novagen) for protein expression. Cultures of *E. coli* were grown in TB at 37°C. Glucose-tolerant WT and mutant strains of S.6803 were grown at 32°C in BG-11 medium containing 20 mM HEPES-NaOH, pH 7.0, under continuous illumination at 20 μmol photons m^{-2} s^{-1} with bubbling of air. Cell density was estimated by measuring optical density at 730 nm (OD_{730}) using a spectrophotometer (model UV-160A, Shimadzu). When necessary, antibiotics were added at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 20 μg ml^{-1}; and chloramphenicol, 25 μg ml^{-1}.

Generation of the *E. coli* strain expressing C-terminal 6 × His-tagged RpaB

pET 21a vector was linearized by digestion with *Nde*I and *Xho*I. The coding region of the *rpaB* gene (from nucleotide 2 012 754 to 2 013 485 according to the numbering in CyanoBase) was amplified by PCR using the primer pair, pET21-NdeI-rpaB-F and pET21-XhoI-rpaB-R (**Table 1**), whose 5′ sequence is complementary to the 3′ ends of the linearized pET21a vector. The linearized vector and PCR product were fused using the In-Fusion HD Cloning Kit (Clontech) to generate the pET21a-RpaB construct. A TTG start codon was additionally introduced just upstream of the ATG start codon of the *rpaB* coding sequence using the PrimeSTAR Mutagenesis Basal Kit (TAKARA BIO INC.) and the primer pair, rpaB-TTG-F and rpaB-TTG21-R (**Table 1**), according to the reported successful production of recombinant RpaB protein (Kato et al. 2011). BL21 pLys (DE3) competent cells (Novagen) were transformed with the pET21a-RpaB construct using the heat shock method to obtain the His-RpaB strain.

Purification of His-RpaB from *E. coli* cells

His-tagged RpaB protein was purified from the His-RpaB strain. The- preculture was seeded into 1 liter of 2 × YT medium. Isopropyl-β-D-thiogalactopyranoside (IPTG) at a concentration of 100 μM was added to the mid-log culture to induce the expression of His-RpaB. After cultivating for 3 h at 37°C, cells

were harvested and resuspended with 20 ml of the purification buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$) with 20 mM imidazole. All protein purification procedures were performed at 4°C. Resuspended cells were disrupted by sonication on ice and then centrifuged at 16,000 × *g* for 20 min. The resulting supernatant was loaded onto a HiTrap chelating HP column (GE Healthcare) pre-equilibrated with the purification buffer, washed with the same buffer with 40 mM imidazole and eluted with the same buffer with 300 mM imidazole. Eluates containing purified His-RpaB were desalted by a HiTrap desalting column (GE Healthcare) equilibrated with purification buffer. After the addition of 20% (v/v) glycerol, the eluate was frozen in liquid N_2 and stored at −80°C before use.

Protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad) with bovine serum albumin as the standard. The purity of the proteins was assessed by fractionating an aliquot on an SDS–PAGE gel and staining with Coomassie Brilliant Blue (CBB).

Gel mobility shift assay

The *psrR1*promoter fragment (−84 to +16, relative to the transcription start site, 100 bp) and the *psaE* promoter fragment (−97 to +23, relative to the transcription start site, 120 bp) were PCR amplified from genomic DNA using primer pairs PpsrR1-F and PpsrR1-R, and PpsaE-F and PpsaE-R, respectively (**Table 1**). P*psrR1*sub1 and P*psrR1*sub2 fragments were obtained using mutated reverse primers: PpsrR1sub1-R and PpsrR1sub2-R (**Table 1**), respectively. P*psaE*sub1 and P*psaE*sub2 fragments were obtained using mutated forward primers: PpsaEsub1-F and PpsaEsub2-F (**Table 1**), respectively. The 3′ end of the DNA fragment for each probe was labeled with digoxigenin (DIG)-ddUTP by the terminal transferase method according to the manufacturer's instructions (DIG gel shift kit; Roche). Assays were performed by using a DIG gel shift kit as previously described (Seino et al. 2009).

Generation of S.6803 strains expressing 12 × His-tagged RpaB

For expression of C-terminal 12 × His-tagged RpaB from the neutral site of the genome, the pTKP2031-12 × His-RpaB construct was generated. Vector pTKP2031 contains a kanamycin resistance cassette and the *psbA2* (*slr1311*) promoter flanked by a part of the coding regions of *slr2030* and *slr2031* as a platform for homologous recombination (Yamauchi et al. 2011). The *psbA2* promoter was removed from pTKP2031 by linearization of the vector by inverse PCR using the primer pair HpaI-2031-F and Km-R (**Table 1**). The promoter and coding region of the *rpaB* gene (from nucleotide 2 012 627 to 2 013 485 according to the numbering in CyanoBase) was amplified by PCR using the primer pair Km-PrpaB-F and slr2031-rpaB-R (**Table 1**), whose 5′ sequence is complementary to the 3′ ends of the linearized pTKP2031 vector. The linearized vector and PCR product were fused using the In-Fusion HD Cloning Kit (Clontech) to generate the pTKP2031-RpaB construct. The 12 × His-tag sequence was added just upstream of the stop codon of *rpaB* using the PrimeSTAR Mutagenesis Basal Kit (TAKARA BIO INC.) and the primer pair HpaI-2031-F and slr2031-12 × His-rpaB-R (**Table 1**) to generate the pTKP2031-12 × His-RpaB construct.

For disruption of the endogenous *rpaB* gene, the previously reported cosmid clone having an insertion of a chloramphenicol resistance cassette into the coding region of *rpaB* (Seino et al. 2009) was used. pTKP2031-12 × His-RpaB and the *rpaB*::Cm^r constructs were introduced into WT cells by natural transformation. Transformants were selected by the addition of kanamycin (20 μg ml^{-1}) and chloramphenicol (25 μg ml^{-1}).

Immunoblot analysis

Cell cultures (50 ml at OD_{730} = 0.5) were collected by centrifugation and resuspended with 200 μl of lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl). The cell suspension was mixed with 280 mg of glass beads (diameter 0.1 mm; AS ONE) in a 2 ml tube, disrupted with a Mini-Bead Beater (BioSpec) by three pulses of 10 s at 4°C, and the cell lysate was collected by centrifugation. Protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad) with bovine serum albumin as the standard. Cell lysate was separated by 12% SDS–PAGE, blotted onto a polyvinylidene diflouride (PVDF) membrane (Immobilon-P; Millipore) and probed with the polyclonal antibody raised against His-RpaB recombinant protein or that against the His-tag (Bethyl). The bound antibodies were detected with goat anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase (Bio-Rad) using the chemiluminescence detection reagent, EzWestLumi plus (Atto), as previously described (Kadowaki et al. 2015).

RNA gel blot analysis

Isolation of RNA by the hot-phenol method and RNA gel blot analyses using the DIG RNA labeling and detection kit (Roche) were performed as described previously (Muramatsu and Hihara 2003). To generate RNA probes by in vitro transcription, template DNA fragments for *psrR1*, *hliB*, *psaA* and *psaL* were amplified from the genomic DNA using the primers listed in **Table 1**. To use PCR products directly as templates for in vitro transcription, the T7 polymerase recognition site (TAATACGACTCACTATAGGGCGA) was added to the reverse primers at their 5′ termini.

Preparation of whole-cell extracts for ChAP analysis

Preparation of whole-cell extracts for ChAP analysis was performed as described previously (Ishikawa et al. 2007, Hanaoka and Tanaka 2008) with some modifications. Cultures of S.6803 at OD_{730} = 0.2 incubated under LL or HL were treated with formaldehyde as a cross-linker to a final concentration of 1% at room temperature under room light conditions for 15 min with occasional shaking. To stop the fixation, glycine was added to a final concentration of 125 mM and cultures were incubated at room temperature for 5 min. Cells were then harvested by centrifugation at 8,000 r.p.m. for 3 min at 4°C, and washed twice with cold Tris-buffered saline (TBS; 20 mM Tris–HCl, pH 7.5, 500 mM NaCl). The resulting pellets were resuspended in 500 μl of UT buffer [50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 4 M urea, 1% Triton X-100, 5 mM imidazole, supplemented with Complete EDTA-free protease inhibitor cocktail (Roche)] at 4°C. After addition of 500 mg of zircon beads (diameter 0.1 mm; BioSpec), cells were broken by vigorous vortexing at 4°C for 45 min in a micro tube mixer (MT-360; TOMY). The cell lysate was sonicated for fragmentation of genomic DNA to an average size of 0.6–0.7 kb with a setting of 20% output, 90% duty cycle, 5 s sonication 15 times at 1 min intervals (Sonifier 450; Branson). After centrifugation twice at 14,000 × *g* for 15 min at 4°C to remove cell debris, the whole-cell extract was collected for affinity purification. Protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad) with bovine serum albumin as the standard. These extracts were stored at −80°C until use.

Affinity purification of His-RpaB and DNA purification

A 1 mg aliquot of protein of the whole-cell extract was mixed with the UT buffer up to 500 μl and subsequently added to 100 μl of Ni^{2+}-Sepharose resin (COSMOGEL His-Accept; Nacalai tesque) pre-equilibrated with UT buffer. After rotation overnight at 4°C, the sample was centrifuged at 1,500 × *g* for 5 min and the pellet was resuspended in 1.5 ml of UT buffer. This washing step was repeated five times. Proteins were then eluted from the resin with 100 μl of the elution buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 1% SDS, 500 mM imidazole) by incubation at 4°C for 20 min. The supernatant was collected and the elution step was repeated once. Then 8.3 μl of 5 M NaCl was added to the 200 μl of pooled eluate, and the mixture was incubated at 65°C for 5 h to revert cross-linking. To obtain input DNA required to calculate the original DNA quantity, 1/10th of the volume of whole-cell extract was subjected to reversion. After ethanol precipitation, washing with cold 70% ethanol and drying, the pellet was dissolved in 100 μl of TE and 100 μl of 2 × proteinase K buffer (20 mM Tris–HCl, pH 7.8, 10 mM EDTA, 1% SDS). Incubation at 50°C for 30 min was followed by extraction twice using phenol/chloroform/isoamylalcohol (25 : 24 : 1). Ethanol-precipitated DNA was finally dissolved in 100 μl of TE, and subjected to quantitative real-time PCR analysis.

Quantitative real-time PCR analysis

Quantitative real-time PCR analysis was performed using the Opticon 2 real-time qPCR monitoring system (MJ Research) according to its SYBR Green protocol. A 12 μl reaction mixture contains 5 pmol each of forward and reverse primers, 1 μl of purified DNA and 6 μl of Thunderbird SYBR qPCR Mix (TOYOBO). The primers used for amplification of the promoter region of

each gene are listed in **Table 1**. PCR amplification was performed according to the manufacturer's instructions. For quantification, standard curves were plotted using several dilutions of input DNA, and the percentage recovery for each purified DNA by ChAP relative to the input DNA was calculated as described previously (Hanaoka and Tanaka 2008). The percentage recovery is shown as the ratio of the quantity of purified DNA against that of input DNA contained in the same volume of whole-cell extract before affinity purification. As a negative control, the PCR without template DNA was performed.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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