Running title: promoter analysis of *psaAB* in *Synechocystis*

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Characterization of high-light responsive promoters of the *psaAB* genes in *Synechocystis* sp. PCC 6803

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Abbreviations: DTT, dithiothreitol; HL, high light (250 μmol photons m^{-2} s^{-1}); LL, low light (20 μmol photons m^{-2} s^{-1}); UP-element, upstream promoter element.

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Abstract

In cyanobacteria, transcription of genes encoding subunits of PSI is tightly repressed under high-light conditions. To elucidate the molecular mechanism, we examined the promoter architecture of the *psaAB* genes encoding reaction center subunits of PSI in a cyanobacterium *Synechocystis* sp. PCC 6803. Primer extension analysis showed the existence of two promoters, P1 and P2, both of which are responsible for the light-intensity dependent transcription of the *psaAB* genes. Deletion analysis of the upstream region of *psaAB* fused to bacterial luciferase reporter genes (*luxAB*) indicated that the light response of these promoters is achieved in totally different manner. The *cis*-element required for the light response of P1, designated as PE1, was located just upstream of the –35 element of P1 and was comprised of AT-rich sequence showing significant homology to UP-element often found in strong bacterial promoters. PE1 activated P1 under low-light conditions and the down-regulation of P1 was achieved by rapid inactivation of PE1 upon the shift to high-light conditions. On the other hand, the *cis*-element required for the light response of P2, designated as HNE2, was located upstream of P1 region, far from the basal promoter of P2. The down-regulation of P2 seemed to be attained through the negative regulation by HNE2 activated only under high-light conditions. DNA gel mobility shift assays showed that at least five regions in *psaAB* promoters were responsible for the binding of putative regulatory protein factors.

Key words: cyanobacteria, high light, photosystem I, promoter, *psaAB*, *Synechocystis* sp. PCC 6803

Introduction

Photosynthetic organisms must acclimate to their light environment by changing themselves. In response to changing light intensity, they modulate capacities of light harvesting, photosynthetic electron transport, CO_2 fixation (Anderson 1986, Melis 1991, Anderson 1995, Walters 2005) and scavenging system of reactive oxygen species (Grace and Logan 1996, Niyogi 1999). Under low-light (LL) conditions, amount of light harvesting antenna complexes is maintained at high level, because maximal capture of light energy is required to fulfill energy demand of cells. Under high-light (HL) conditions, however, energy supply tends to exceed its consumption and photosynthetic electron transport components become relatively reduced. This may result in excess production of reactive oxygen species leading to severe damage to many cellular processes (Asada 1994). Thus, absorption of excess light energy must be avoided under HL conditions by decreasing the amount of light harvesting antenna complex per reaction center or amount of reaction center complexes *per se*.

In cyanobacteria, the decrease of photosystem content is typically observed under HL conditions, and the main component to be down-regulated is not PSII but PSI (Murakami and Fujita 1991, Hihara et al. 1998). The physiological significance of the decrease in PSI content during HL acclimation has been demonstrated by the characterization of the two mutants of *Synechocystis* sp. PCC 6803, disruptants of *pmgA* (sll1968) and sll1961, both of which have defect in decreasing their PSI content during HL acclimation (Hihara et al. 1998, Fujimori et al. 2005). The phenotype of these mutants could not be distinguished from that of the wild-type strain under LL conditions, and normal decrease in amount of PSI was observed just after the shift to HL conditions. However, after 12 h of HL exposure, the selective repression of PSI content was lost in these mutants. They grew better than the wild-type cells during a short–term exposure (e.g. 24 h) to HL because higher amount of PSI

accelerated the rate of photosynthetic electron transport (Hihara et al. 1998). Under long-term exposure to HL, however, growth of the mutants was severely inhibited (Hihara et al. 1998, Sonoike et al. 2001, Fujimori et al. 2005), presumably due to the generation of reactive oxygen species at the acceptor side of PSI. These observations strongly suggest that the repression of PSI content is indispensable for growth under continuous HL conditions.

In contrast to the accumulation of the data showing the physiological importance of down-regulation of PSI content during HL acclimation, the molecular mechanisms controlling the process have been poorly understood. However, some recent reports have implied the involvement of transcriptional control in decrease of PSI content during HL acclimation. In *Synechocystis* sp. PCC 6803, coordinated down-regulation of genes encoding subunits of PSI (PSI genes) was observed upon the shift to HL conditions preceding the decrease of PSI content (Hihara et al. 2001, Huang et al. 2002, Muramatsu and Hihara 2003, Tu et al. 2004). Herranen et al. (2005) showed by pulse-labeling experiment that light-induced modulation in the translation rate of a reaction center subunit of PSI, PsaA protein, closely parallels the transcription rate of the *psaAB* genes. Furthermore, it was shown that the defect in repression of the transcript levels of the *psaAB* genes might cause the aberrant accumulation of PSI in *pmgA* disruptant under prolonged HL conditions (Muramatsu and Hihara 2003). These results indicate that the decrease in transcript levels of PSI genes, in particular that of the *psaAB* genes, should be one of the prerequisite factors for down-regulation of PSI content under HL conditions. Since the stability of the *psaAB* transcripts does not decrease so much upon the shift to HL (Muramatsu and Hihara 2003, Herranen et al. 2005), the decrease in the *psaAB* transcripts under HL seems largely dependent on the regulation of promoter activities. Therefore, in this study, as the first step for elucidation of light-intensity dependent mechanism of transcriptional regulation of PSI genes, we analyzed the promoter architecture of the *psaAB* genes using *luxAB* reporter genes. We show that the *psaAB* genes have two light responsive promoters, P1 and P2.

Although activity of both promoters is coordinately down-regulated under HL, the mechanisms to achieve the light response are different between them.

Results

Mapping of the transcriptional starting points of the psaAB genes

The transcriptional starting points of the *psaAB* genes were determined by primer extension analysis. When the [γ-^{32}P]-labeled psaA-13 primer (Table 1) that primed 35 bp downstream of the *psaA* start codon was used, three 5' ends of transcript were detected and each of them was designated as ep (end point) 1, ep2, and ep3 (Fig. 1A). The location of ep1 was corresponding to 144 nucleotides upstream of the *psaA* start codon. There existed putative -35 and -10 elements upstream of ep1 (Fig. 1B). These elements, TTGCCT and TATTAT, were separated by a 16 bp spacer and deviated from the consensus -35 (5'-TTGACA-3') and -10 (5'-TATAAT-3') elements of *E. coli* σ^{70}-type promoters (Harley and Reynolds 1987) in 3 out of 12 positions. The location of ep2 was corresponding to 45 nucleotides upstream of the *psaA* start codon. The putative -35 and -10 elements located upstream of ep2, TTCCCT and TACACT, were separated by an 18 bp spacer and deviated from the consensus -35 and -10 elements in 5 out of 12 positions (Fig. 1B). The spacing between the putative -35 and -10 regions of both ep1 and of ep2 was consistent with the 17±1 bp spacing reported for canonical *E. coli* σ^{70} promoters (Harley and Reynolds 1987). In general, conserved –10 element (5'-TANNNT-3') is found in cyanobacterial promoters, whereas –35 element is weakly conserved or totally absent (Curtis and Martin 1994, Vogel et al. 2003). It is noted that not only –10 but also –35-like element is found at the appropriate position relative to ep1 and ep2 of the *psaAB* genes.

We observed that both of the two independent upstream regions including ep1 or ep2

could yield bioluminescence when they were fused to promoterless bacterial luciferase genes (*luxAB*) as described later. This indicates that the *psaAB* genes have at least two promoters and ep1 and ep2 are transcriptional starting points originated from these promoters. Thus, the upper and the lower promoters were designated as P1 and P2, respectively. As for ep3 corresponding to 16 nucleotides upstream of the *psaA* start codon, it was probably originated from the processed product, since the cells harboring *luxAB* reporter fused to the fragment covering just upstream of ep3 exhibited only background level of bioluminescence (data not shown). In addition, putative promoter elements were not found in the upstream region of ep3.

Under HL conditions, the *psaAB* genes were transcribed from the same residues as that under LL conditions (Fig. 1A). However, the amount of the products from ep1 and ep2 greatly decreased after 12 h of exposure to HL. This indicates that both promoters of the *psaAB* genes can respond to changes in light intensity.

In vivo reporter assay for identification of cis-elements required for the light-intensity dependent transcription of the psaAB genes

Previously, we showed that the amount of *psaAB* transcripts drastically decrease upon the shift from LL to HL conditions (Muramatsu and Hihara 2003). Considering that the amount of the products from ep1 and ep2 was low in HL-incubated cells compared to that in LL-acclimated cells (Fig. 1A), both P1 and P2 should contribute to this regulation. To identify *cis*-promoter elements required for the light response, a series of 5'- and/or 3'- deleted fragments of the upstream region of the *psaAB* genes was cloned into pPT6803-1 carrying promoterless *luxAB* genes. These constructs were transformed into *Synechocystis* cells and inserted into the neutral site of the chromosome by homologous recombination.

First, we assessed the correlation among the transcript level of the endogenous *psaAB*, that

of *luxAB*, and the level of bioluminescence under LL and HL conditions in A0 strain harboring full length of the intergenic region between *psaAB* and the divergently transcribed sll1730. As shown in Fig. 2A, the amount of the endogenous *psaAB* transcripts drastically decreased upon the shift to HL conditions, and gradually increased by further incubation under HL. This response could be traced through analyzing the change in the *luxAB* transcript levels (Fig. 2B, A0). However, we found that change in bioluminescence levels was inconsistent with that of *psaAB* and *luxAB* transcript levels. Namely, the extent of the decrease in the bioluminescence level in A0 strain 1 h after the shift to HL conditions (Fig. 2C, A0) was far smaller than that in the *psaAB* and *luxAB* transcript levels (Fig. 2A, B, A0). This might be ascribed to higher stability of LuxAB protein compared to that of the *luxAB* transcripts as shown by Liu et al. (1995). Another discrepancy is observed after 3 h of HL exposure. Namely, the bioluminescence level continued to decrease (Fig. 2C, A0) although the transcript level began to increase (Fig. 2A, B, A0). This tendency was also observed when levels of the *luxAB* transcript and bioluminescence in A1 strain harboring 5'-deleted promoter fragment (-176 to +144) were examined. In this strain, continuous decrease in bioluminescence levels was observed after the shift to HL conditions (Fig. 2C, A1), in spite of almost constant level of the *luxAB* transcripts (Fig. 2B, A1). Recently, Koga et al. (2005) reported that the activity of LuxAB expressed under the control of a *lac*UV5 promoter in *E. coli* declined upon entry into the stationary phase irrespective of the constant amount of LuxA and LuxB proteins. They demonstrated that this phenomenon, ADLA (Abrupt Decline of Luciferase Activity), was due to the decline in the supply of intracellular $FMNH_2$ required for luciferase activity. The aberrant decrease of the bioluminescence level shown in Fig. 2C might be also due to the loss of reducing power during HL incubation.

Thus, in this study, we assessed the changes of promoter activities upon the shift from LL to HL conditions by monitoring the level of the *luxAB* transcripts in reporter strains. However, as far as cells were incubated under LL conditions, the level of bioluminescence and that of *luxAB*

transcripts in each strain showed good correlation (Fig. 2D). Hence, for LL acclimated cells, we employed the measurement of bioluminescence to compare the promoter activities among reporter strains.

The architecture of P1

As mentioned above, the transcriptional starting point of P1 was corresponding to 144 nucleotides upstream of the *psaA* start codon (noted as +1), and P1 had conserved –35 and –10 elements (Fig. 1B). Strain A62, which possessed the (–46 to +2) fragment including –35 and –10 elements, showed very low but substantial bioluminescence (1.5×10^6 relative units/OD_{730}) compared with that from the control cells having promoter-less *luxAB* genes (1.0×10^5 relative units/OD_{730}) (Fig. 3B). Thus, in this study, we assigned the (-46 to +2) region to the minimal sequence for P1. The promoter activities of A62 (-46 to +2) and A63 (-46 to +19) were constitutively low irrespective of light intensity (Fig. 3C). In contrast, strain A61 (-69 to +2), which carries the additional sequence, (–69 to –47), to the minimal sequence, exhibited typical light response for PSI genes. Thus, the (-69 to –47) region is a positive regulating element working under LL conditions and alone is sufficient to confer light response to P1. This positive regulating element, designated as PE1, seems to be inactivated just after the shift to HL conditions, and reactivated after 3 h of HL exposure. To identify the nucleotide sequences critical for the light response, base-substitutions were performed within the PE1 region of A61 (Fig. 4A). When the sequence TTTTT (from -61 to -57) or TTATT (from -54 to -50) was replaced with the sequence, CGCGC (strain A61mt2 and A61mt3, respectively), the bioluminescence levels under LL conditions were drastically decreased (Fig. 4B). This indicates that these AT-rich pentamers, TTTTT and TTATT, are the core sequences for light response of P1. It should be noted that the nucleotide sequence of PE1 showed significant homology to UP-element often found in strong bacterial promoters (Ross et al. 1993, Estrem et al. 1998). This

feature will be discussed later.

Successive elongation at 3’ end from +19 to +51, while maintaining the 5’ end at -91 (Fig. 3, compare strain A6 and A7), decreased P1 activity both under LL and HL conditions, although further elongation at 3’ end to +83 (A8) did not affect P1 activity so much. This indicates that the (+20 to +51) region harbors a negative regulating element (NE1), which is not involved in light response. Further elongation at 3’ end from +83 to +115 (compare strain A8 and A9) increased promoter activity both under LL and HL conditions, which was probably due to the addition of –10 element of P2 to the reporter fragment.

In summary, the analysis of P1 architecture suggests that P1 was controlled by at least two *cis*-elements. The basal promoter (-46 to +2) was up-regulated by PE1 located just upstream of –35 element under LL conditions and probably under prolonged HL conditions. On the other hand, the negative element (NE1) located downstream of the basal promoter decreased P1 activity irrespective of light intensity.

The architecture of P2

The transcriptional starting point of P2 was corresponding to 45 nucleotides upstream of the *psaA* start codon (noted as +100), and P2 had conserved –35 and –10 elements (Fig. 1B). A region required for the basal activity of P2 was found to be located within (+58 to +101) region (Fig. 3B, A42). Elongation at 3’ end from +101 to +123, while maintaining the 5’ end at +58 (compare strain A42 and A43), decreased P1 activity irrespective of light intensity. This indicates that the (+102 to +123) region works as a negative regulating element for P2. This element was designated as NE2B. Further elongation of 3’ end from +123 to +144 increased the *luxAB* expression both under LL and HL conditions (compare strain A43 and A44), indicating that there exists a positive regulating element in the (+124 to +144) region. This region was designated as PE2B.

Next, 5’ end was elongated from +58 to +20, while maintaining 3’ end at +144. The (+20 to +57) region seems to work as a positive regulating element of P2, because the expression level of the *luxAB* genes in A4 was higher than that in strain A44 both under LL and HL conditions. Thus, the (+20 to +57) region was designated as PE2A. Considering that A4 strain which possessed PE2A, NE2B, and PE2B did not show light response, these elements are unlikely to be involved in the light response. Further elongation of the 5’ end from +20 to –46 decreased *luxAB* expression both under LL and HL conditions (compare strain A4 and A3), in spite of inclusion of P1 to the fragment. This indicates that there exits a negative regulating element for P2 in the (-46 to +19) region, designated as NE2A. NE2A was also not involved in light-induced decrease of P2 activity. A2 and A1 strains showed constitutive *luxAB* expression although both strains possessed PE1, the *cis*-element required for the light response of P1. It is likely that the light response of P1 was masked by the constitutively high activity of P2 in these strains. However, total promoter activities of *psaAB* after 1 h of HL exposure gradually decreased by the successive elongation of 5’ end from –176 to –271, with fixed 3’ end at +144 (compare strain A1, A13, A12, and A0). This indicates that P2 can respond to changes in light intensity when the (-271 to-177) region is added. Thus, we designated the region as HNE2 because it is the HL condition-specific negative regulating element for P2. It is noticeable that HNE2 is located very far from the core of P2. HNE2 might contact with P2 by means of the change in the secondary structure of DNA. Whether the upstream of *psaAB* can bend in response to change in light intensity to allow HNE2 contact with P2 should be tested in future.

In conclusion, P2 was also controlled by multiple *cis*-elements, three NEs and two PEs. Except for HNE2 which functioned to repress P2 activity under HL conditions, other two NEs and two PEs affected the activity of basal promoter (+58 to +101) in light-intensity independent manner.

Protein factors bound to the promoter region of the psaAB genes

To detect the binding of putative regulatory proteins to the promoter region of *psaAB*, DNA gel mobility shift assays were performed with the crude extract from *Synechocystis* cells grown under LL conditions. As summarized in Fig. 5A, at least five regions, (-271 to –242), (-161 to –126), (-125 to –92), (around +20), and (+80 to +123) were found to be responsible for the binding of protein factors, and were designated as BR (Binding Region) 1, BR2, BR3, BR4, and BR5, respectively. When the crude extract from cells grown under HL conditions was used, the observed DNA-protein complexes were totally the same as those obtained by the crude extract from LL-grown cells (data not shown). However, we could not exclude the possibility that the result was due to the loss of the intracellular redox environment of HL-grown cells during preparation of the crude extract. To know whether redox environment affects the binding activity of putative regulatory proteins, DNA gel mobility shift assays were carried out under reduced condition with dithiothreitol (DTT) using protein extract prepared in the presence of DTT or under oxidized condition without DTT using protein prepared without DTT. In Fig. 5B-E, the results of DNA gel mobility shift assays with F/29, 27/23, 14/9 and 32/20, which are DNA fragments covering five BRs are shown. When F/29 or 27/23 was used as a probe, a large shifted band was detected only under the oxidized condition (Fig. 5B, C, lane 3). As the extent of the band shift observed with F/29 and 27/23 was similar, we checked whether these shifts were ascribed to the same protein. By addition of 300-fold excess of unlabeled 27/23 fragments to the reaction mixture, formation of the large shifted band of F/29 was completely prevented (Fig. 5B, lane 4). This indicates that the same protein factor is bound to BR1 and BR2. The nucleotide sequences of BR1 and BR2 were shown in Fig. 5F. As common features between them, AT-rich sequences, T(C/G)AAAAT(C/G)C and TT(C/T)(T/G)TAACA, were found.

Interestingly, when 5/8 or 27/23 probe was used, only a small shifted band was detected under the reduced condition (Fig. 5C, lane 2) instead of the large shifted band observed under the

oxidized condition (Fig. 5C, lane 3). The protein factor responsible for the small shifted band must be bound to BR3, considering that the shifted band was not observed with 27/25 probe.

When 14/9 or 6/11 was used as a probe, a single shifted band was detected irrespective of the addition of DTT (Fig. 5D). On the other hand, the shifted band was detected with neither 6/8 nor 7/18 probe. This indicates that a protein factor could be bound to BR4.

When 7/13, 32/20 or 32/13 was used as a probe, a clear shifted band was observed irrespective of the addition of DTT (Fig. 5E). Since no shifted band was observed with 7/18 or 19/13 probe, the protein factor must be bound to BR5. Noticeably, the amount of the crude extract required for the band shift at BR5 was very low compared to that required for the band shift at the other BRs, indicating that the protein factor bound to BR5 may abundantly exist in *Synechocystis* cells, or may have higher affinity compared to other protein factors bound to other BRs.

Discussion

The two light responsive promoters in the psaAB genes

In this study, to elucidate the mechanism of light intensity dependent regulation of the *psaAB* gene expression in *Synechocystis* sp. PCC 6803, we analyzed *psaAB* promoter architecture using transcriptional fusions of a series of *psaAB* upstream regions to the promoterless *luxAB* reporter genes (Fig. 3). We found that two promoters (P1 and P2) were responsible for transcription of the *psaAB* genes. As for the *psaB* gene, it does not appear to have its own promoter as suggested by Herranen et al. (2005), since the intergenic region between *psaA* and *psaB* did not yield luciferase reporter activity (data not shown).

Although activities of both P1 and P2 decreased upon the shift to HL conditions (Fig. 1), the mechanisms to achieve light response were completely different between them as shown in Fig.

6. The key element for the light response of P1 is PE1 involved in positive regulation under LL conditions. P1 activity under LL is highly up-regulated by PE1. Upon a shift to HL conditions, PE1 is rapidly inactivated, which resulted in the drastic decrease of P1 activity. As for P2, the key element for the light response is HNE1 involved in negative regulation under HL conditions. Under LL where HNE1 is inactivated, P2 shows high activity. When shifted to HL, HNE1 becomes active and the decrease of P2 activity is observed. As a result, activity of both P1 and P2 are greatly suppressed under HL conditions. However, considering that the *psaAB* transcripts accumulated again about 3 h after the shift to HL conditions, these regulations seem to be lost gradually during incubation under HL conditions.

Until now, there are some reports on the promoter architectures of HL- responsive genes in cyanobacteria, such as *psbAI* (Nair et al. 2001), *psbAII*, *psbAIII* (Li and Golden 1993, Li et al. 1995), and *psbDII* genes (Anandan and Golden 1997) of *Synechococcu*s sp. PCC 7942 and *gap2* (Figge et al. 2000), *secA* (Mazouni et al. 1998), and *fedI* genes (Mazouni et al. 2003) of *Synechocystis*. sp. PCC 6803. These genes possess only a single promoter except for *psbAII* having two promoters. Promoters of *psbAII* are different from those of *psaAB* in that only one promoter shows light response. The physiological significance of existence of two light-responsive promoters in the *psaAB* genes is still unclear. It is reported that the transcript levels of the *psaAB* genes are also down-regulated under stress conditions such as irradiation of UV-B (Huang et al. 2002) or high salt conditions (Marin et al. 2004). P1 and P2 might be differently regulated under such conditions.

The mechanism of light response of P1

The light response of P1 was achieved by PE1 located in the (-69 to –47) region just upstream of –35 element (Fig. 3). By base substitution analysis of PE1 region, we found that the AT-rich sequence of PE1 was indispensable for the light response of P1 (Fig. 4). In *Synechococcus*

sp. PCC 7942, the *psbAI* promoter, whose activity is known to decrease upon the shift from LL to HL conditions, also possessed AT-rich sequence, 5'-AGCTAAAAATTA-3', located in the (-54 to –43) region just upstream of –35 element (Nair et al. 2001). This segment was implicated in both promoter activation *per se* and light-responsive regulation of *psbAI* just like PE1 for P1 of *psaAB* in *Synechocystis* sp. PCC 6803. In eubacterial promoters, AT-rich sequences of about 20 bp located just upstream of –35 hexamer (-59 to –38), called UP-element, play a role in increasing promoter activity by up to 300-fold (Ross et al. 1993, Estrem et al. 1998). UP-element can interact with α–subunit of RNA polymerase and has been referred to as the third promoter recognition element besides the –35 and –10 hexamers (Ross et al. 1993, Rao et al. 1994, Czarniecki et al. 1997, Ross et al. 1998, Tagami and Aiba 1999, Ozoline et al. 2000). The UP element is suggested to function in two parts, the distal subsite extending from -59 to –46, and the proximal subsite extending from –46 to –38. The consensus sequence of the distal subsite is 5'-NNAWWWWWTTTTTN-3' and that of the proximal subsite is 5'-AAAAAARNR-3', each of which is sufficient for binding α-subunit and can increase promoter activity by itself (Estrem et al. 1998, Gourse et al. 2000). In fact, numerous *E.coli* promoters, driving expression of a wide array of gene products, contain one of these subsites of UP-element (Estrem et al. 1998). The –62 to -49 region of PE1, 5'-ATTTTTAATTATTG-3', matches the sequence of the distal site (12 out of 14), and also its location is suited as the distal site, suggesting that PE1 comprises the UP-element and might work as a platform for the interaction with α-subunit of RNA polymerase. The AT-rich sequence of *psbAI* in *Synechococcus* PCC 7942 may also belong to the UP-element, since the sequence of the (-51 to –43) region matches to the consensus of the proximal subsite in 7 out of 9. These findings implicated that P1 of *psaAB* in *Synechocystis* and the promoter of *psbAI* in *Synechococcus* possess the common mechanism to exhibit light response although how the up-regulation by these putative UP-elements is inactivated upon the shift to HL remains to be solved. One explanation is that some *trans*-factors might affect

PE1 activities. It should be noted that a protein factor bound to UP-element was reported in *E. coli*. Methylated Ada protein binds to an UP-like element in *ada* and *aidB* promoters to stimulate transcription by modifying the nature of the RNA polymerase-promoter interaction (Landini and Volkert 1995). Also, it has been reported that the UP-element of *deoP2* promoter of *E. coli* overlaps with the DNA binding site of CytR, a repressor of *deoP2* (Shin et al. 2001).

The *mechanism of light response of P2*

The light response of P2 is found to be regulated by the region designated as HNE2 located far from the core sequence of P2 (Fig. 3). HNE2 repressed P2 activity under HL conditions. Although how HNE2 modulates P2 activity is totally unknown, there is a possibility that DNA bending is involved in this regulatory mechanism considering the distance between HNE2 and the core sequence of P2. There are some reports of participation of DNA bending in transcriptional regulation both in eukaryote and prokaryote. For example, in *gal* operon of *E. coli*, DNA bending is known to be involved in the negative control of promoter activity (Irani et al. 1983). In the case of cyanobacteria, the occurrence of DNA bending is reported in *psbA2* in *Microcystis aeruginosa* K-81. This DNA bending, called CIT, is suggested to affect the basal activity of *psbA2* transcription (Asayama et al. 2002).

Protein factors bound to the psaAB promoter region

DNA gel mobility shift assay revealed that at least four different protein factors were bound to five regions of *psaAB* promoters (Fig. 5). We showed that the same protein factor could be bound to BR1 and BR2 under oxidized conditions. Under reduced conditions, however, the binding activity to BR1 and BR2 was not detected. Taking into consideration that BR1 was located within HNE2, the protein factor bound to BR1 might be involved in the regulation of light response of P2

through perception of the change in cellular redox state. As for BR2, we could not assign any regulatory roles in this region by the deletion analysis. However, there remains a possibility that BR2 works as a *cis*-element involved in light response because BR2 bound the same protein factor as BR1. To ascertain this possibility, mutational analyses in BR2 should be required. As well as BR2, we could not assign any regulatory roles in BR3 region. However, considering that the binding of a protein to BR3 was only observed in the oxidized condition where no binding activity was detected at BR1 and BR2, the proteins bound to BR3 and BR1/2 might work competitively in response to the cellular redox state. In contrast to the above mentioned binding factors, the binding activities observed with BR4 and BR5 were not affected by the presence or absence of DTT. This feature is consistent with the fact that NE2A, PE2A, NE1, and NE2B affected promoter activity regardless of light intensity (Fig. 3, 5A).

Based on the information obtained in this study, we are going to isolate putative regulators that bound to *psaAB* promoters. Characterization of null mutants of these regulators will provide further information on the direct role of these proteins in the regulation of light-responsive gene expression. In addition, analysis of the promoter architectures of other PSI genes is now in progress. This analysis must lead to understanding of the mechanism of coordinated light response of PSI genes scattered throughout the whole genome.

Materials and Methods

Strains and culture conditions

A glucose-tolerant wild-type strain and reporter-transformed strains of *Synechocystis* sp. PCC 6803 were grown at 31°C in BG-11 liquid medium (Stanier et al. 1971) with 20 mM

HEPES-NaOH, pH 7.0. Unless stated otherwise, cultures were grown under continuous illumination provided by fluorescent lamps at 20 μmol photons m^{-2} s^{-1}. To maintain reporter strains, spectinomycin (20 μg/ml) was added to cultures. Cells were grown in volumes of 50 ml with test tubes (3 cm in diameter) and bubbled with air. Cell density was estimated at OD_{730} using a spectrophotometer (model UV-160A; Shimadzu, Kyoto, Japan). HL shift experiments were performed by transferring cells at the exponential growth phase (OD_{730}=0.1 - 0.2) from LL (20 μmol photons m^{-2} s^{-1}) to HL conditions (250 μmol photons m^{-2} s^{-1}).

Escherichia coli and DNA manipulation

XL1-Blue MRF' (Stratagene, La Jolla, CA, U.S.A.) was the host for all plasmids constructed in this study. When required, ampicillin (100 μg/ml) or spectinomycin (20 μg/ml) was added to Terrific Broth medium for selection of plasmids in *E. coli*. Procedures for the growth of *E. coli* strains and for the manipulation of DNA were as described in Sambrook et al. (1989). Sequencing of plasmids was carried out by the dideoxy-chain termination method using dye terminator cycle sequencing ready reaction kit (ABI PRISM; Applied Biosystems, Foster City, CA, U.S.A.).

Primer extension analysis

Primer extension analysis of the *psaAB* genes was carried out using 10 μg of total RNA as the template. The primer, psaA-10 or psaA-13 (Table 1), was labeled with [γ^{-32}P]-ATP (ICN Biomedicals, Irvine, CA, U.S.A.) using T4 polynucleotide kinase (USB, Cleveland, OH, U.S.A.). Reverse transcription was performed as follows. Total RNA was incubated at 70°C for 10 min in a 15 μl reaction mixture containing 5×First Strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$) and 2 pmol of labeled primer. Then, 2 μl of 0.1 M DTT and 2 μl of 10 mM

dNTPs were added and reaction mixture was further incubated at 42°C for 5 min. After addition of 1 μl of Superscript II (Invitrogen, Carlsbad, CA, U.S.A.), reverse transcription reaction was performed at 42°C for 60 min. The extension products were ethanol precipitated, resuspended in loading buffer [98% formamide (v/v), 0.5 mM EDTA, 0.3% xylene cyanol (w/v), 0.3% bromophenol blue (w/v)], and denatured at 95°C for 3 min. Samples were electrophoresed alongside DNA ladders on a gel containing 6% polyacrylamide and 8 M urea. DNA ladders were created using T7 sequencing Kit (USB) with the same end-labeled primer as that used for reverse transcription.

RNA isolation and RNA blot analysis

RNA isolation and Northern blot analysis were performed as described previously (Muramatsu and Hihara 2003). For dot blot analysis, five μg of total RNA per each dot was spotted onto the nylon membrane (Hybond N+; Amersham Biosciences, Uppsala, Sweden). To generate gene specific probes, the *psaA* and *luxA* genes were amplified by PCR from genomic DNA of *Synechocystis* and from pPT6803-1 vector (see below), respectively. The following primers were used: *psaA* forward (5'-TTCCCCTTCCCCACGAGT-3'), *psaA* reverse (5'-ACCGGCCCCTACGATGAG-3'), *luxA* forward (5'-ACTTATCAGCCACCTGAG-3'), and *luxA* reverse (5'-TATCTTTGGCTCTATTTG-3'). 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. *In vitro* transcription reaction was carried out with a digoxigenin (DIG) RNA labeling kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Construction of luxAB reporter strains

All plasmids used for transformation of *Synechocystis* cells were derivatives of pPT6803-1,

which is a recombinational plasmid having the promoterless *luxAB* genes, the neutral site of *Synechocystis* sp. PCC 6803 (the downstream region of the *ndhB* gene) and the spectinomycin-resistance cassette (Aoki et al. 2002, Muramatsu and Hihara 2003).

Each promoter fragment was generated by PCR with primers containing the *Bsi*WI site at their 5'-termini, and cloned into the unique *Bsi*WI site of pPT6803-1 to produce transcriptional fusions with the promoterless *luxAB* gene. All primers used in this study are shown in Table 1. The nucleotide sequence and direction of the promoter region in the reporter constructs were verified by sequencing. Wild-type *Synechocystis* was transformed with the pPT6803-1 derivatives, and transformants were selected and propagated in liquid BG-11 with spectinomycin. All of reporter strains used in this study are shown in Table 2.

Measurement of luminescence from cells harboring luciferase reporter genes

For *in vivo* bioluminescence measurements, liquid culture of reporter strains grown under LL conditions was diluted with distilled water to OD_{730} =0.2. Aliquot (200 μl) was transferred to a reaction tube and set immediately in the luminescence counter (Lumi-counter model 2500; Microtech-Nichion, Chiba, Japan). 100 μl of 0.15% *n*-decanal (v/v) was injected into a reaction tube with a syringe and bioluminescence from the cells was measured during 120 s after the injection of *n*-decanal.

Preparation of protein extract

LL or HL exposed *Synechocystis* cells at the exponential growth phase (OD_{730}=0.4) were harvested by centrifugation at 30,000×g for 5 min. The pellet was resuspended in 1 ml of TE buffer with or without 0.5 mM DTT. The cell suspension was mixed with approximately 500 μl volume of zircon beads (diameter 0.1 mm; BioSpec Products Bartlesville, OK, U.S.A.) and disrupted by three

times of vigorous vortexing for 2 min followed by cooling on ice for 1 min. After removal of the zircon beads, 200 μl of 5 M NaCl was added to the crude extract and centrifuged at 17,400×g for 20 min. Proteins were precipitated by adding 0.4 g of solid $(NH_4)_2SO_4$ to 1 ml of extract. After resuspension in 1 ml of extraction buffer, the extract was dialyzed twice for 12 h against 1 liter of the extraction buffer. All manipulations were carried out at 4°C. Protein concentration was determined using Protein Assay Kits II (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to manufacturer's instructions.

DNA gel mobility shift assays

DNA fragments used as probes or competitors in gel mobility shift assays were generated by PCR. To prepare probes for assays, the 3' end of DNA fragments were labeled with Digoxigenin (DIG)-ddUTP by the terminal transferase using DIG Gel shift Kit (Roche, Mannheim, Germany) according to manufacturer's instructions. Assays were performed using DIG Gel shift Kit (Roche). Cell extract from *Synechocystis* was incubated with 30 fmol DIG-labeled DNA fragment in a 20 μl reaction mixture containing 1 μg poly d[I-C], 0.1 μg poly $_L$-lysine, 20 mM HEPES-KOH, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 0.2% Tween 20 (v/v), 30 mM KCl and 1 mM DTT. DTT was not added to the reaction mixture when the binding reaction was performed under oxidized conditions. After overnight incubation at 4℃, 5 μl of gel loading buffer consisting of 60% of 1×TBE (v/v) and 40% glycerol (v/v) was added to the reaction mixture. Samples were then applied onto a 6% polyacrylamide gel and subjected to electrophoresis at 95 V for 2.5 h at 4°C. DNA and protein were transferred to a nylon membrane (Hybond N+; Amersham Biosciences) by capillary transfer method and fixed by baking at 80°C for 2 h. Detection of DIG-labeled probe was performed according to the standard protocol for DIG Luminescent Detection kit (Roche).

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Table 1. Primers used in this study

Name[a]	location[b]	Sequence	Use[c]
PpsaA-F	-271→	AACGTACGTGTCAAAATCCGCTTCTT	C, M
PpsaA-R	←+144	AACGTACGGCAGGGTTCTCCTCGCTC	C
psaA-3	-91→	AACGTACGAGGAGCTAGAACTGGTCA	C
psaA-5	-176→	AACGTACGATCAGTTGTAAGTTGATG	C, M
psaA-6	-46→	AACGTACGACGCAGGTCTTGCCTAGG	C, M
psaA-7	+20→	AACGTACGATCTGTGCAAGGTTTAAC	C, M
psaA-8	←+19	AACGTACGAGGCGTTTTCAGCAAACA	C, M
psaA-9	←+51	AACGTACGTTCATAATAACGATGTTA	C, M
psaA-10	←+83	AACGTACGGAGGAAGCGTAAAAAAGG	C, P
psaA-11	←+115	AACGTACGATTAAGGGTTTCCTATGC	C, M
psaA-13	←+179	AACGTACGGCCTTAGCCTCTCTTTCG	P, M
psaA-14	-69→	AACGTACGTGGGGCAATTTTTAATTA	C, M
psaA-15	←+2	AACGTACGATCACTAGAAGATAATAC	C
psaA-17	+58→	AACGTACGACTAATTCCCTTTTTTAC	C
psaA-18	←+101	AACGTACGATGCAGAATAGTGTAATA	C, M
psaA-19	+102→	AACGTACGAGGAAACCCTTAATAGTT	M
psaA-20	←+123	AACGTACGAATGAACTATTAAGGGTT	C, M
psaA-23	←-92	AGATTTTGCAAAGACTTA	M
psaA-24	-211→	AACGTACGTAATTTCTATCTAATTTT	C, M
psaA-25	←-126	AACGTACGTTGAACTTTGTTACAAA	M
psaA-27	-161→	AACGTACGATGAAAATGCTGTAAATT	M
psaA-28	-241→	AACGTACGAATTAAAGCTGTTATAGC	C, M
psaA-29	-177→	AACGTACGAGCAATGGGCACCGTCAA	M
psaA-32	+80→	CCTCTATTACACTATTCT	M
psaA-61mt1	-69→	AACGTACGTCCCCGAATTTTTAATTATTGTT	S
psaA-61mt2	-69→	AACGTACGTGGGGCAACGCGCAATTATTGTT	S
psaA-61mt3	-69→	AACGTACGTGGGGCAATTTTTAACGCGCGTT ACGCAGG	S

[a]All primers except for psaA-23 and psaA-32 include *Bsi*WI site (CGTACG) for cloning and additional two adenine nucleotides at their 5' end.

[b]The starting point and direction of each primer are indicated. Numbers refer to the nucleotide positions relative to the transcriptional starting point of P1.

[c]These primers were used for C: Cloning, M: DNA gel mobility shift assay, S: Site directed mutagenesis, P: Primer extension analysis.

Table 2. Strains used in this study

Strain	Description of promoter fragment	Insert size	Primer combination
Control	*luxAB* reporter vector (pPT6803-1*)* without promoter fragment		
A0	(-271 to +144) region of *psaAB*	415 bp	PpsaA-F / PpsaA-R
A12	(-241 to +144) region of *psaAB*	385 bp	psaA-28 / PpsaA-R
A13	(-211 to +144) region of *psaAB*	355 bp	psaA-24 / PpsaA-R
A1	(-176 to +144) region of *psaAB*	320 bp	psaA-5 / PpsaA-R
A2	(-91 to +144) region of *psaAB*	235 bp	psaA-3 / PpsaA-R
A3	(-46 to +144) region of *psaAB*	190 bp	psaA-6 / PpsaA-R
A4	(+20 to +144) region of *psaAB*	125 bp	psaA-7 / PpsaA-R
A42	(+58 to +101) region of *psaAB*	44 bp	psaA-17/ psaA-18
A43	(+58 to +123) region of *psaAB*	66 bp	psaA-17/ psaA-20
A44	(+58 to +144) region of *psaAB*	87 bp	psaA-17/ PpsaA-R
A61	(-69 to +2) region of *psaAB*	71 bp	psaA-14 / psaA-15
A62	(-46 to +2) region of *psaAB*	48 bp	psaA-6 / psaA-15
A63	(-46 to +19) region of *psaAB*	65 bp	psaA-6 / psaA-8
A6	(-91 to +19) region of *psaAB*	110 bp	psaA-3 / psaA-8
A7	(-91 to +51) region of *psaAB*	142 bp	psaA-3/ psaA-9
A8	(-91 to +83) region of *psaAB*	174 bp	psaA-3/ psaA-10
A9	(-91 to +115) region of *psaAB*	206 bp	psaA-3/ psaA-11
A61mt1	pA61 with GGGGC (-68～)→CCCCG	71 bp	psaA-61mt1/ psaA-15
A61mt2	pA61 with TTTTT (-61～)→CGCGC	71 bp	psaA-61mt2/ psaA-15
A61mt3	pA61 with TTATT (-54～)→CGCGC	71 bp	psaA-61mt3/ psaA-15

Each insert DNA was amplified by PCR using primers indicated and cut with *Bsi*WI prior to cloning in *Bsi*WI site of pPT6803-1.

Figure legends

Figure 1. Mapping of the 5’ ends of the *psaAB* transcripts. (A) Total RNA (10 μg) was extracted from cells acclimated to LL and those exposed to HL for 12 h, and used for primer extension analysis. Detected 5’ ends of transcripts are indicated by arrowheads and shown with dideoxy sequencing ladders. (B) Nucleotide sequence upstream of ep1 and ep2 of the *psaAB* genes. Positions of nucleotides are shown relative to ep1 noted as +1.

Figure 2. Validation of bacterial luciferase reporter assay under different light conditions. (A) Northern blot analysis of the *psaAB* genes upon the shift to HL conditions in wild-type (WT) cells. Two μg of total RNA was loaded in each lane. DIG-labeled *psaA*-specific probe was used for detection. (B) Northern blot analysis of the *luxAB* reporter genes upon the shift to HL conditions in A0 and A1 reporter strains harboring full-length (-271 to +144) and 5’-deleted (-176 to +144) fragment of the *psaAB* promoter, respectively. Two μg of total RNA was loaded in each lane. DIG-labeled *luxA*-specific probe was used for detection. (C) Changes in bioluminescence levels from A0 and A1 strains upon the shift to HL conditions. (D) Relationship between transcript levels of *luxAB* and bioluminescence levels in reporter strains acclimated to LL (closed cycle) and those exposed to HL for 6 h (open cycle). *luxAB* transcript level was examined by Northern blot analysis and quantified with Scion Image software.

Figure 3. Deletion analysis of the *psaAB* promoter region. (A) A schematic representation of a series of 5’- and/or 3’-deleted *psaAB* promoter fragments in reporter strains (A0 - A9). The numbers above the promoter fragments refer to the nucleotide positions relative to the transcriptional starting point

of P1 (tsp1), noted as +1. The arrangement of positive (PE) and negative (NE, HNE) regulatory elements in two promoters of the *psaAB* genes, P1 and P2, is shown below. (B) Bioluminescence levels from reporter strains grown under LL conditions. Error bars represent the standard deviation values obtained from at least four independent measurements. (C) Changes in levels of the *luxAB* transcripts of the reporter strains shown by dot blot analysis. Total RNA was isolated from reporter strains incubated under HL conditions for indicated period. Five μg of total RNA per each dot was spotted onto the nylon membrane and hybridized with DIG-labelled *luxAB* probe.

Figure 4. Mutational analysis of the PE1 region of the *psaAB* promoter. (A) The sequence alignment of the wild-type (A61) and the mutant (A61mt1, mt2 and mt3) promoters. Mutated bases in A61mt strains are shaded. (B) Bioluminescence levels from A61 and A61mt strains under LL conditions. Error bars represent the standard deviation values obtained from three independent measurements.

Figure 5. DNA gel mobility shift assay of the promoter region of the *psaAB* genes with crude extract from *Synechocystis* cells. (A) A schematic representation of the probes used for the DNA gel mobility shift assay and protein binding regions (BRs). End points of probes and BRs are shown as the nucleotide positions relative to tsp1 (+1). Names of each probe indicate the primer pair used for the generation of the fragment. Putative regulatory factors bound to probes are indicated as A, B, C and D. The results obtained with (B) F/29, (C) 27/23, (D) 14/9 and (E) 32/20 probes are shown. Lane 1 contained no crude protein. Lanes 2 and 3 contained 30 μg (B, C, D) or 20 μg (E) of crude protein from *Synechocystis* cells. Preparation of crude protein and binding reaction were performed with (lane 2) or without (lane 3) DTT. Lane 4 in (B) shows the competition assay with 300-fold excess amount of unlabeled 27/23 fragment. The combination of BR and the putative regulatory factor

likely to cause the band shift is shown in the right side of each panel. Asterisks indicate the non-specific band attributed to the addition of crude protein. (F) Nucleotide sequences of BR1 and BR2. Common sequences, T(C/G)AAAAT(C/G)C and TT(C/T)(T/G)TAACA, were shaded.

Figure 6. A schematic representation of the mechanism of light-intensity dependent regulation of the promoter activities of the *psaAB* genes. (A) Under LL conditions, the activity of P1 is enhanced by PE1. (B) Upon the shift to HL conditions, up-regulation of P1 activity is lost presumably through the inactivation of PE1. The activity of P2 is down-regulated by HNE2.

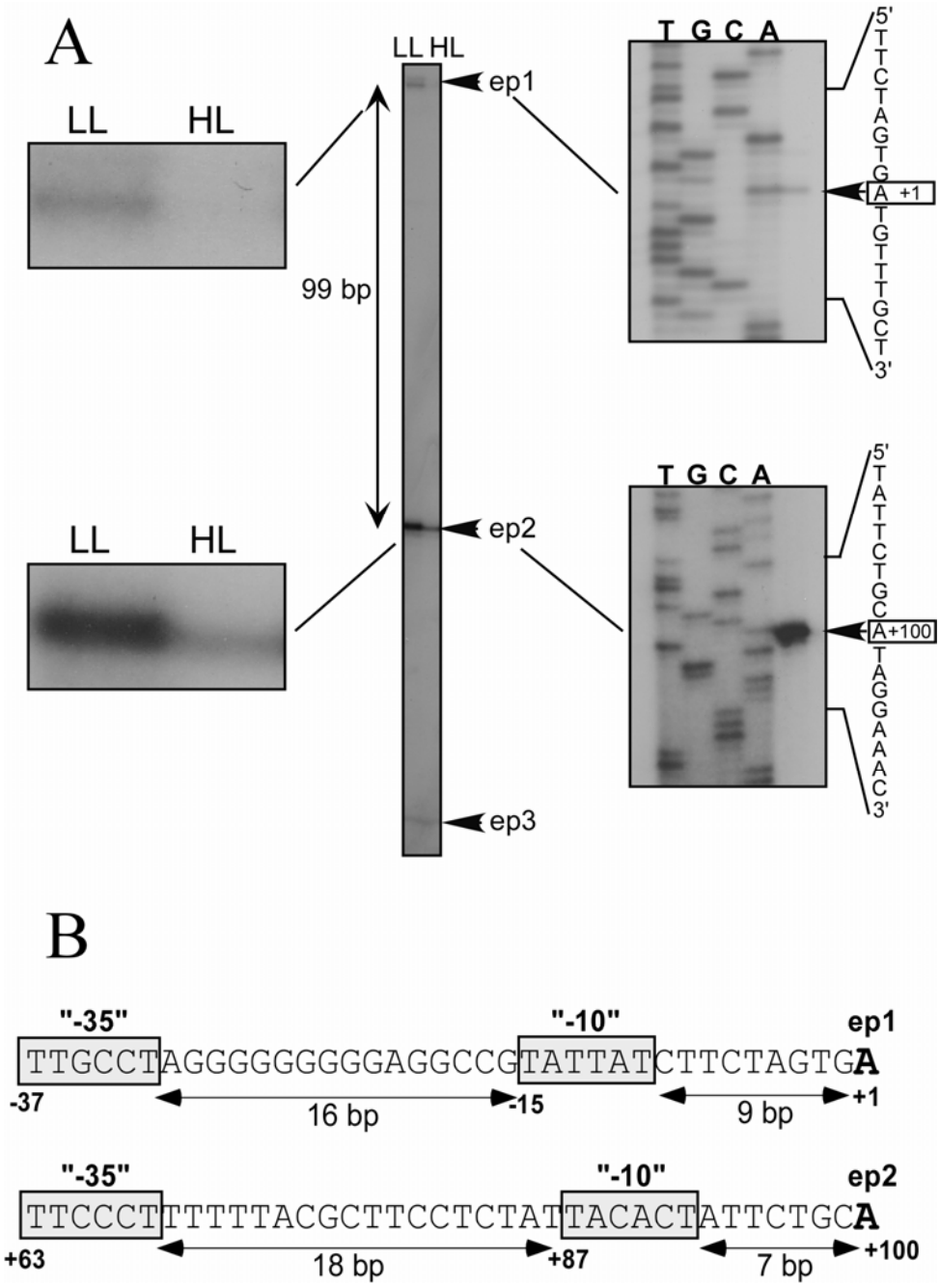
A
LL HL
ep1
T G C A
5' TTCTAGTG A +1 TGTTTGCT 3'
LL HL
99 bp
ep2
T G C A
5' TATTCTGC A+100 TAGGAAAC 3'
LL HL
ep3
B
"-35"
"-10"
ep1
TTGCCTAGGGGGGGGGAGGCCGTATTATCTTCTAGTGA
-37
16 bp
-15
9 bp
+1
"-35"
"-10"
ep2
TTCCCTTTTTTACGCTTCCTCTATTACACTATTCTGCA
+63
18 bp
+87
7 bp
+100

Fig. 1

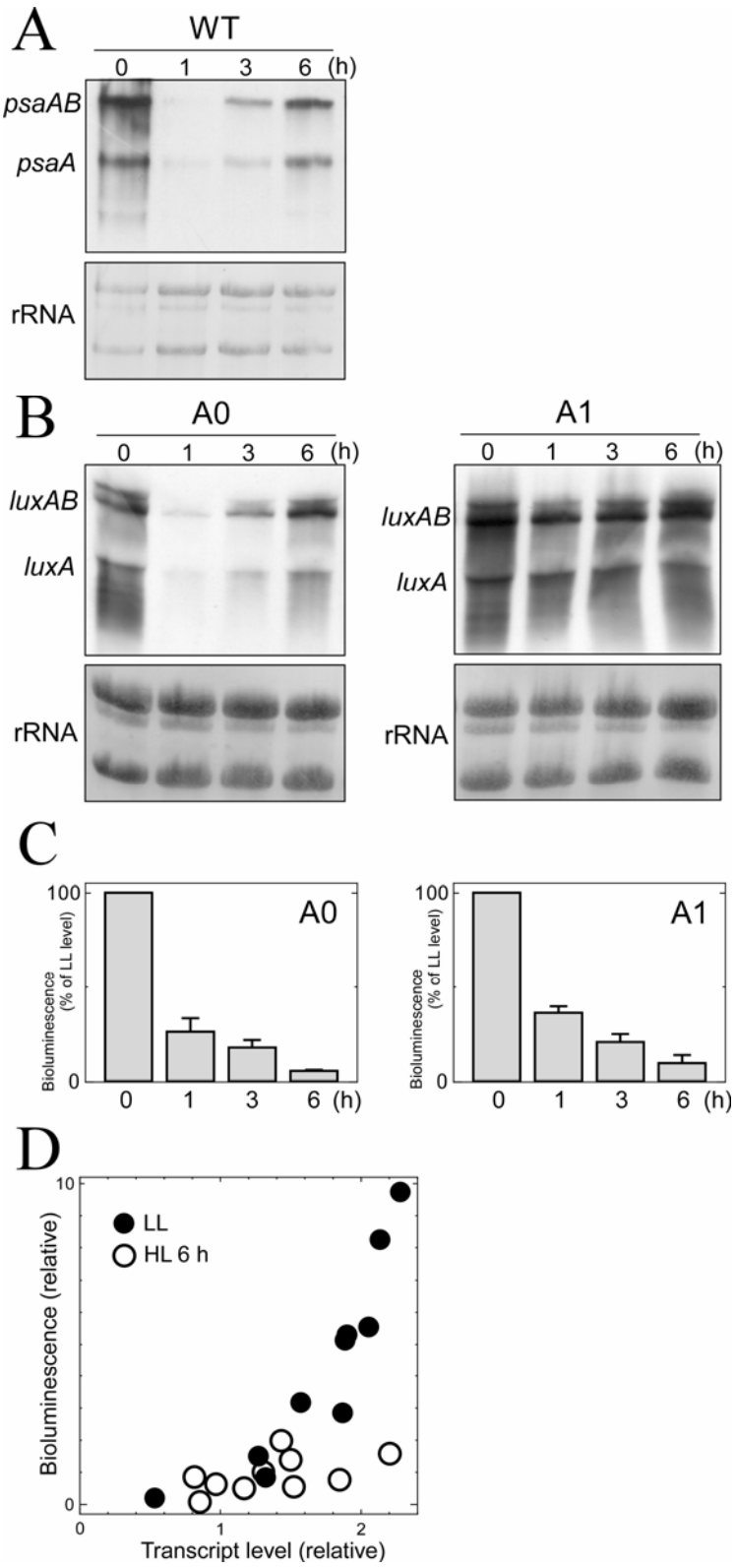
A
WT
0 1 3 6 (h)
psaAB
psaA
rRNA
B
A0
A1
0 1 3 6 (h)
luxAB
luxA
rRNA
C
A0
A1
Bioluminescence (% of LL level)
100
0
0 1 3 6 (h)
D
LL
HL 6 h
Bioluminescence (relative)
Transcript level (relative)
10
0
1
2

Fig. 2

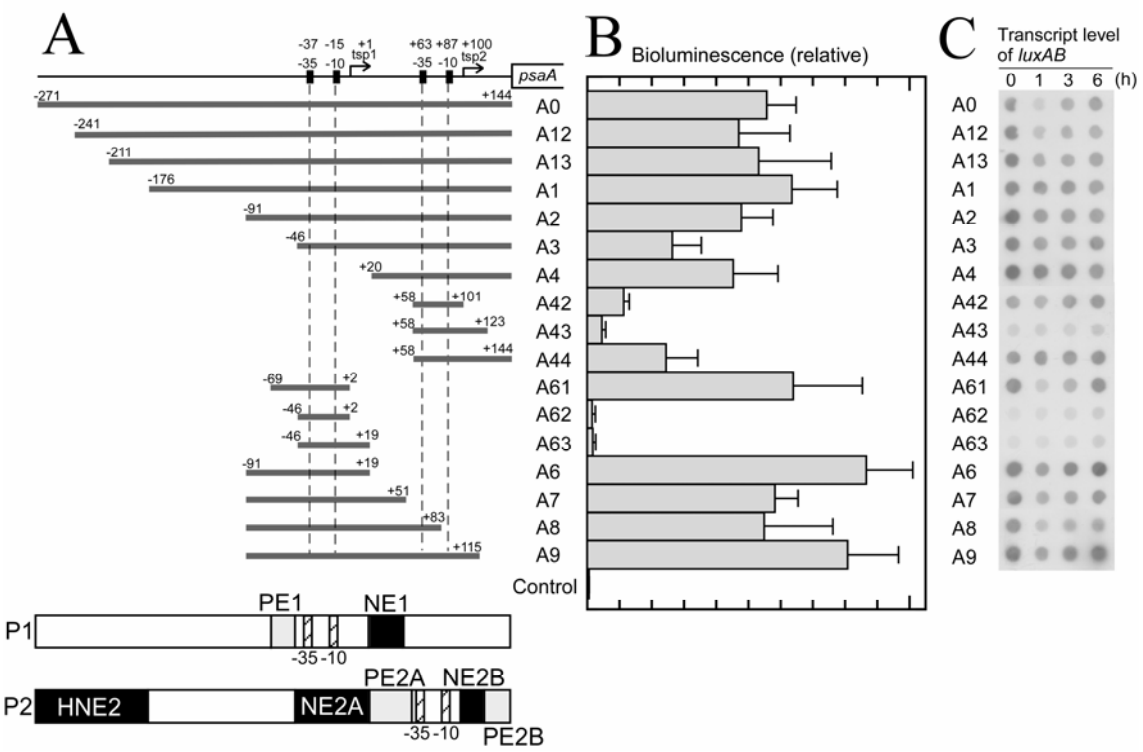
A
-37 -15 +1
-35 -10 tsp1
+63 +87 +100
-35 -10 tsp2
psaA
-271
+144
-241
-211
-176
-91
-46
+20
+58 +101
+58 +123
+58 +144
-69 +2
-46 +2
-46 +19
-91 +19
+51
+83
+115
A0
A12
A13
A1
A2
A3
A4
A42
A43
A44
A61
A62
A63
A6
A7
A8
A9
Control
PE1
NE1
P1
-35 -10
PE2A
NE2B
P2
HNE2
NE2A
-35 -10
PE2B
B
Bioluminescence (relative)
C
Transcript level of luxAB
0 1 3 6 (h)
A0
A12
A13
A1
A2
A3
A4
A42
A43
A44
A61
A62
A63
A6
A7
A8
A9

Fig. 3

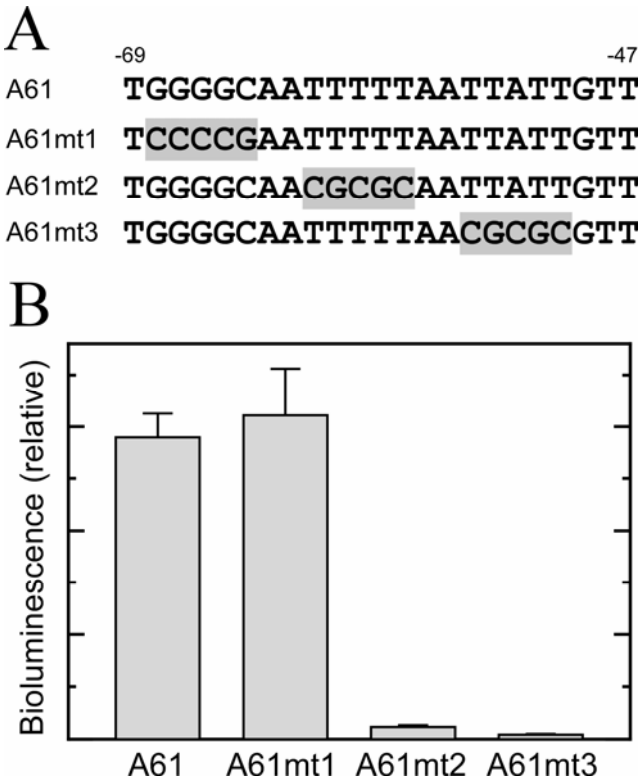
A
-69
-47
A61 TGGGGCAATTTTTAATTATTGTT
A61mt1 TCCCCGAATTTTTAATTATTGTT
A61mt2 TGGGGCAACGCGCAATTATTGTT
A61mt3 TGGGGCAATTTTTAACGCGCGTT
B
Bioluminescence (relative)
A61
A61mt1
A61mt2
A61mt3

Fig. 4

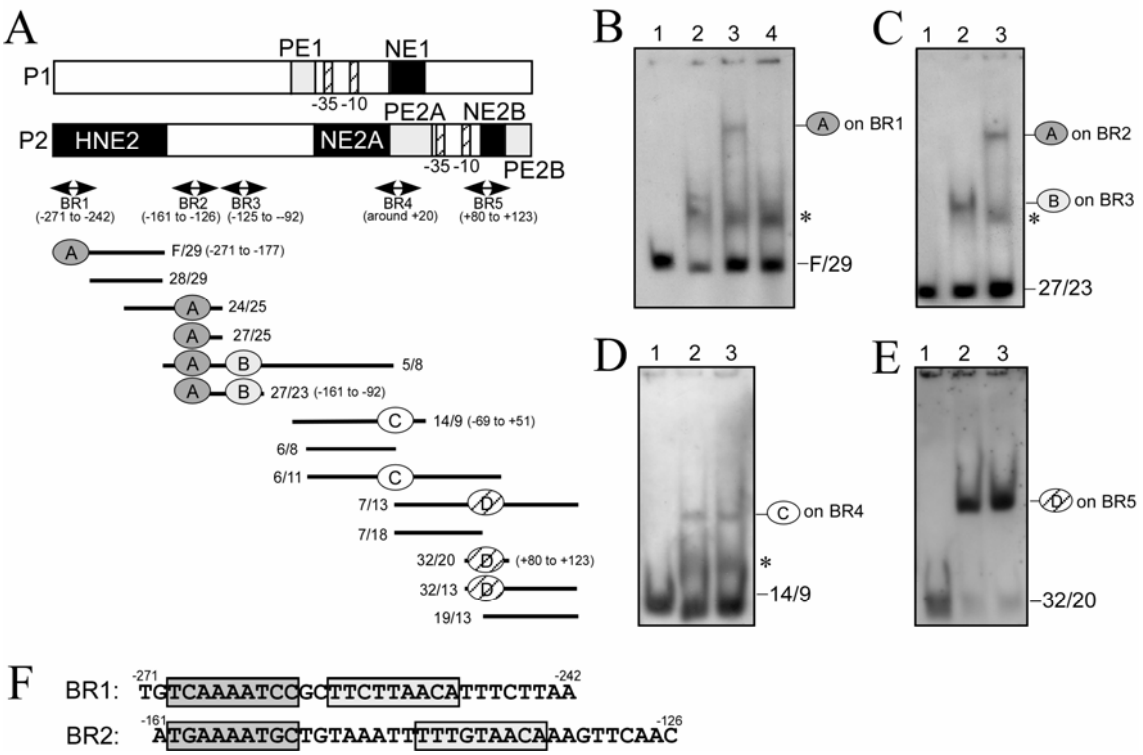
A
PE1
NE1
P1
-35 -10
PE2A
NE2B
P2
HNE2
NE2A
-35 -10
PE2B
BR1 (-271 to -242)
BR2 (-161 to -126)
BR3 (-125 to --92)
BR4 (around +20)
BR5 (+80 to +123)
F/29 (-271 to -177)
28/29
24/25
27/25
5/8
27/23 (-161 to -92)
14/9 (-69 to +51)
6/8
6/11
7/13
7/18
32/20 (+80 to +123)
32/13
19/13
B
1 2 3 4
A on BR1
F/29
C
1 2 3
A on BR2
B on BR3
27/23
D
1 2 3
C on BR4
14/9
E
1 2 3
D on BR5
32/20
F
BR1: -271 TGTCAAAATCCGCTTCTTAACATTTCTTAA -242
BR2: -161 ATGAAAATGCTGTAAATTTTTGTAACAAAGTTCAAC -126

Fig. 5

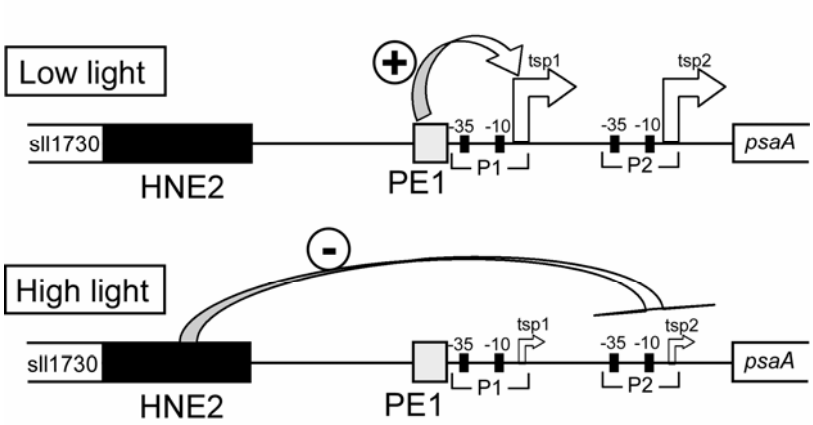
Low light
sll1730
HNE2
PE1
tsp1
tsp2
-35
-10
P1
-35
-10
P2
psaA
High light
sll1730
HNE2
PE1
tsp1
tsp2
-35
-10
P1
-35
-10
P2
psaA

Fig. 6