The response regulator RpaB binds to the upstream element of photosystem I genes to work

for positive regulation under low-light conditions in *Synechocystis* sp. PCC 6803

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running title: regulation of PSI genes by RpaB in *Synechocystis*

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ABSTRACT

The coordinated high-light response of genes encoding subunits of photosystem I (PSI genes) is achieved by the AT-rich region located just upstream of the core promoter in *Synechocystis* sp. PCC 6803. The upstream element enhances the basal promoter activity under low-light conditions, whereas this positive regulation is lost immediately after the shift to high-light conditions. In this study, we focused on a high light regulatory 1 (HLR1) sequence included in the upstream element of every PSI gene examined. Gel mobility shift assay revealed that a response regulator RpaB binds to the HLR1 sequence in PSI promoters. Base substitution in the HLR1 sequence or decrease in copy number of the *rpaB* gene resulted in decrease in the promoter activity of PSI genes under low-light conditions. These observations suggest that RpaB acts as a transcriptional activator for PSI genes. It is likely that RpaB binds to the HLR1 sequence under low-light conditions and works for positive regulation of PSI genes and for negative regulation of high-light inducible genes depending on the location of the HLR1 sequence within target promoters.

INTRODUCTION

In photosynthesis, light energy is absorbed by the light-harvesting antennae pigments and converted into chemical energy by the reaction centers. However, when supply of light energy exceeds its consumption, elevated excitation pressure results in excess production of reactive oxygen species, leading to severe damage to many cellular processes (3, 8, 20). Thus, absorption of excess light energy must be avoided under light saturated conditions by decreasing the amount of light harvesting antenna complexes per a reaction center or amount of reaction center complexes itself.

In cyanobacteria, the decrease of photosystem (PS) content as well as phycobilisome content is typically observed under high light (HL) conditions, and the main component to be

2

down-regulated is not PSII but PSI (14, 22). The physiological significance of the selective repression of 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 keeping their PSI content at low level under HL conditions (9, 14). 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 (14). Under prolonged HL conditions, however, growth of the mutants was severely inhibited (9, 14, 30), 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 *Synechocystis* sp. PCC 6803, PSI complex is comprised of 11 subunits and genes encoding these subunits (PSI genes) are dispersed throughout the genome (11, 17). PSI genes are actively transcribed under LL conditions, whereas their transcription is coordinately and strictly down-regulated upon the shift to HL conditions preceding the decrease in protein level (13, 15, 16, 23, 31). We conducted the deletion analysis of PSI promoters and found that AT-rich upstream region from -70 to -46, relative to the transcription start site, is involved in up-regulation of the promoter activity in every PSI gene (24, 25). The addition of AT-rich upstream element to the core promoter region stimulated the promoter activity 5- to 100-fold under LL conditions, whereas this positive regulation was suppressed within 1 h after the shift to HL. This change in the activity of the upstream element was well correlated with changes in PSI transcript levels upon the shift from LL to HL conditions, showing that the upstream element is responsible for the coordinated HL response of PSI genes. However, transcriptional factors involved in the regulation have remained unknown.

In this study, we found that a response regulator RpaB binds to the AT-rich upstream region of PSI genes. Although RpaB has so far been reported as a repressor for HL-inducible

genes (19, 29), our results suggest the activation of PSI genes by RpaB under LL conditions. It is likely that RpaB is active under LL conditions and works for positive regulation of PSI genes and for negative regulation of high-light inducible genes depending on the location of its binding site within target promoters.

MATERIALS AND METHODS

Strains and culture conditions

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was grown at 31°C on solid BG-11 medium containing 5 mM TES-KOH, pH 8.2, 0.3% (x/v) sodium thiosulfate and 1.5% (x/v) agar under continuous illumination provided by fluorescent lamps at 20 μmol photons $m^{-2} s^{-1}$. To maintain the *rpaB*-disrupted strain and reporter-transformed strains, chloramphenicol (25 μg/ml) and spectinomycin (20 μg/ml) were added, respectively. Cell density was estimated at OD_{730} using a spectrophotometer (model UV-160A; Shimadzu).

Escherichia coli and DNA manipulation

XL1-Blue MRF' (Stratagene) was the host for all plasmids constructed in this study. When required, ampicillin (100 μg/ml), chloramphenicol (25 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*. (28). Sequencing of plasmids was carried out by the dideoxy-chain termination method using dye terminator cycle sequencing kit (Applied Biosystems).

Overexpression and purification of His-RpaB

The *rpaB* coding region was PCR-amplified using the primers NdeI-rpaBcod-F

(5'-AACATATGGTCGATGACGAGGCC -3') and BamHpa-rpaBcod-R (5'-

AAGGATCCGTTAACTTACGGTTCTTCCCCCGG-3'), cloned into the pT7Blue T-Vector

(Novagen), digested with *Nde*I and *Bam*HI (sites underlined) and subcloned into the same

restriction sites in pET28a (Novagen) to create pETrpaB for expression of a fusion protein

with an N-terminal His-tag. The nucleotide sequence was confirmed by DNA sequencing.

    E. coli BL21(DE3) harboring pETrpaB was grown overnight to an OD_{600} of 10 in

500 ml of 2 x yeast extract-tryptone (YT) medium containing 20 μg/ml kanamycin. Cells

were harvested by centrifugation, resuspended in 20 ml of buffer S (50 mM Tris-HCl, pH 8.0,

50 mM NaCl, 1 mM DTT) and disrupted by eight rounds of sonication for 30 sec each at 4ºC.

The inclusion body fraction containing the overexpressed protein was pelleted by

centrifugation at 12,000 g for 30 min. The pellet was washed several times with buffer S

containing 4% (w/v) Triton X-100, then with distilled water until the supernatant became

clarified. Then the pellet was solubilized in 8 M urea solution (50 mM Tris-HCl, pH 8.0, 1

mM DTT, 8 M urea) at room temperature for 30 min and centrifugated at 18,000 g for 30 min

to remove insoluble materials. The supernatant was sequentially dialyzed in three steps: first

against 50 mM Tris-HCl, pH 8.0, containing 1 mM DTT and 4 M urea for 1 h, then the same

buffer containing 2 M urea for 1 h, and finally against 20 mM phosphate buffer, pH7.4,

containing 0.5 M NaCl and 10 mM imidazole for overnight.

    After centrifugation at 18,000 g for 30 min, the supernatant was applied to a HiTrap

Chelating HP column (GE Healthcare) that was preequilibrated with 20 mM phosphate buffer,

pH 7.4, containing 0.5 M NaCl and 10 mM imidazole. After washing with 20 mM phosphate

buffer, pH 7.4, containing 0.5 M NaCl and 100 mM imidazole, His-RpaB was eluted with 20

mM phosphate buffer, pH 7.4, containing 0.5 M NaCl and 300 mM imidazole. Purified

His-RpaB was desalted by a HiTrap Desalting column (GE Healthcare). Protein composition was examined by SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250.

Gel mobility shift assay

Probes and competitor DNA fragments for gel mobility shift assays were obtained by PCR amplification using genomic DNA as a template. *psaE*sub1 and *psaE*sub2 fragments were obtained using mutated forward primers: PpsaEsub1-F 5'-TAGAACCACTCCCAGGAGCAGGGACCCCTAAAGAATTGTTTT-3' and PpsaEsub2-F 5'-TAGAACCACTCCCAGGAGCAGGGATATGTAAAGACCCCTTTT-3', 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 using a DIG Gel Shift kit as previously described (25).

Generation of *luxAB* reporter strains

All plasmids used for the reporter assay were derivatives of pPT6803-1, which is a recombinational plasmid carrying the promoterless *luxAB* genes, the neutral site sequence of *Synechocystis* sp. PCC 6803 (the downstream region of the *ndhB* gene) and the spectinomycin-resistance cassette (1, 24). Reporter construct E1 containing the (-70 to +90) region of *psaE*, E2 containing the (-45 to +90) region of *psaE* and A61 containing the (-69 to +2) region of *psaA* were generated as described in Muramatsu and Hihara (25). Base substituted constructs, E1sub1 and E1sub2, were generated using KOD-Plus Mutagenesis kit (TOYOBO) according to manufacturer's instruction. Namely, E1 construct was mutagenized by inverse PCR using a primer set PpsaE-luxSub1 (5'-CGCCCCTAAAGAATTGTTTTGGGAAAG -3') and luxAB-3

(5'-GCTTTCAATTTCCGCTTT-3') to generate E1sub1, and using a primer set

PpsaE-luxSub2 (5'- CGTATGTAAAGACCCCTTTTGGGAAAGTCGGGGGGA -3') and

luxAB-3 to generate E1sub2.

Measurement of bioluminescence from cells harboring luciferase reporter genes

For *in vivo* bioluminescence measurements of *Synechocystis* cells, cells grown on solid BG11

medium were suspended in distilled water. Aliquot (200 μl) was transferred to a reaction tube

and set immediately in the luminescence counter (Lumi-counter model 2500;

Microtech-Nichion). 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. Specific luciferase activities were calculated as relative units / OD_{730}.

Generation of the *rpaB*-disrupted strain

For generation of the *rpaB*-disrupted strain, a cosmid clone having insertion of a

chloramphenicol-resistance cassette into the coding region of *rpaB* (nucleotide 2014496

according to numbering in CyanoBase) was selected from the transposon-mutagenized

cosmid library of *Synechocystis* sp. PCC 6803 (27) and transformed to the wild-type and A61

reporter strains. Transformants were selected by addition of chloramphenicol (25 μg/ml).

Determination of Pigment Contents

Cells grown on solid BG11 medium were suspended in distilled water and *in vivo* absorption

spectra were measured at room temperature using a spectrophotometer (model 557; Hitachi)

with an end-on photomultiplier. Chlorophyll and phycocyanin contents were calculated from

the peak heights of absorption spectra using the equations of Arnon et al. (2).

RESULTS

To identify transcriptional regulators that bind to the light responsive AT-rich upstream region of PSI genes, we searched for a common regulatory sequence located in this region. As shown in Fig. 1, there exists a high light regulatory 1 (HLR1) motif, an imperfect direct repeat comprised of two octamers (G/T)TTACA(T/A)(T/A) separated by two nucleotides (18), in the upstream region of every PSI gene. The HLR1 motif was initially identified by Eriksson et al. (7) as a common sequence located upstream of HL-inducible genes such as *psbA2*, *psbA3*, *hliA* and *nblA* in *Synechocystis* sp. PCC 6803. Promoter analysis revealed that deletion of the HLR1-containing region resulted in elevated expression of *psbA2* in *Synechocystis* sp. PCC 6803 (7) and of *hliA* in *Synechococcus elongatus* PCC 7942 (18) under LL conditions. These observations indicate that the HLR1 sequence is recognized by a repressor protein working under LL conditions. Recently, RpaB (Slr0947, Ycf27, Rre26) response regulator was reported to bind to the HLR1 sequence of *hliB* in *Synechocystis* sp. PCC 6803 (19) and of *rpoD3* in *Synechococcus elongatus* PCC 7942 (29). Unlike above mentioned HL-inducible genes, PSI genes are down-regulated under HL and their AT-rich upstream element is likely to be recognized by an activator protein under LL conditions (25). Thus, it is intriguing to see if RpaB, the repressor for HL-inducible genes, can bind to PSI promoters to work for positive regulation under LL conditions.

In order to test whether RpaB binds to the upstream region of PSI genes, we overexpressed RpaB protein with N-terminal histidine tag (His-RpaB) in *E. coli*. His-RpaB exclusively accumulated in the insoluble fraction of the overnight culture without induction with isopropyl β-D-thiogalactoside. The insoluble fraction was repeatedly washed with 4% Triton X-100 and the resulted inclusion body fraction was solubilized by 8 M urea. After centrifugation, the supernatant was renatured by dialysis and His-RpaB was purified to near

homogeneity through nickel affinity chromatography. We first checked the activity of purified His-RpaB by gel mobility shift assay with various promoter fragments (Fig. 2). When the promoter region of *hliB*, one of the target genes of RpaB (19), was used as a probe, addition of 0.3 µg of His-RpaB was sufficient for the formation of a shifted complex and the disappearance of the free probe. On the other hand, no shifted complex was observed when 5'-UTR and the coding region of *psaAB* (+80 to +179) was used as a probe. The HLR1-containing fragments of *psaK1* and *psaLI* promoters formed a shifted complex with His-RpaB and specificity of this complex formation was tested by competition assays. Unlabeled probe fragments ("self") significantly competed for binding with the labeled probe, but unlabeled coding region fragments having the same length as the probe fragments ("non-self") were not sufficient to compete for binding.

Then, binding of His-RpaB protein to each PSI promoter was tested. When DNA fragment containing the upstream element from -70 to -46 was used as a probe, a shifted complex was clearly observed in every PSI gene (Fig. 3A). To obtain the completely shifted band, addition of 0.5 µg of His-RpaB was enough in the case of *psaD*, *psaFJ*, *psaK1* and *psaLI*, whereas higher amount of His-RpaB was required in the case of *psaAB*, *psaC* and *psaE*. Next, the upstream element was deleted from each probe used in Fig. 3A and gel mobility shift assay was performed again. As shown in Fig. 3B, no shifted complex was observed, showing that His-RpaB binds to the HL-responsive upstream element of PSI genes.

In order to confirm the binding of His-RpaB to the HLR1 sequence, gel mobility shift experiment was performed with probes having base substitution within the HLR1 sequence. Figure 4A shows the effect of four base substitution in each half-site of the HLR1 sequence in the *psaE* probe (*psaE*sub1 and *psaE*sub2). Either substitution resulted in the complete loss of the binding of His-RpaB. This clearly shows that binding of His-RpaB requires the HLR1 sequence. Next, the effect of the same base substitution on the promoter

activity of the *psaE* gene under LL conditions was examined using *luxAB* reporter constructs (Fig. 4B). Previously, we reported that E1 strain containing the *luxAB* genes fused to the (-70 to +90) region of the *psaE* gene showed much higher reporter activity than E2 strain containing the *luxAB* genes fused to the (-45 to +90) region (25). In E1 strain, a part of the HLR1 sequence (-73 to -71) was not contained. Instead, the addition of *Bsi*WI site (CGTACG) to 5'-terminus of the promoter fragment resulted in the formation of TACGTAAA (-73 to -66) that seems to be recognized as a half-site of the HLR1 sequence. When this half-site was changed to CCCCTAAA (E1sub1), the promoter activity largely decreased nearly to the level of E2 strain. Similarly, the base substitution in the other half-site (E1sub2) resulted in the decline in the promoter activity. These observations suggest that RpaB binds to the HLR1 sequence of PSI genes and acts as a transcriptional activator under LL conditions.

To examine *in vivo* role of RpaB, a gene disrupted mutant was created by the insertion of a chloramphenicol resistance cassette into *rpaB*. However, as reported in Ashby and Mulllineux (4), *rpaB* was an essential gene in *Synechocystis* sp. PCC 6803 and only the transformant with decreased copy number of *rpaB* was obtained. The transformant formed small yellowish colonies on BG11 agar plates, while its growth rate and pigment contents showed a substantial increase by the addition of 5 mM glucose. In liquid culture, no propagation was observed irrespective of the presence or absence of glucose. During the cultivation on agar plates, large green colonies of suppressor mutants emerged in high frequency. We collected yellowish colonies of the original transformant grown on the agar plate without glucose, measured absorption spectra and calculated cellular pigment contents. Cellular chlorophyll content (pg/cell) of the wild type and the mutant was 0.047 ± 0.003 and 0.036 ± 0.004, respectively. Similarly, cellular phycocyanin content (pg/cell) of the wild type and the mutant was 0.282 ± 0.014 and 0.205 ± 0.015, respectively. When the *luxAB* reporter genes fused to the *psaAB* promoter fragment from -69 to +2 was introduced, the reporter

activity from the mutant cells was only 26% of the wild-type level. Thus, it seemed that decrease of the copy number of *rpaB* resulted in decrease of *psaAB* promoter activity, cellular pigment contents and growth rate under LL conditions.

DISCUSSION

In this report, we showed that the response regulator RpaB binds to the HLR1 sequence located in the AT-rich upstream element of PSI genes (Figs. 3 and 4A). Base substitution in the HLR1 sequence (Fig. 4B) or decrease in copy number of the *rpaB* gene resulted in the decrease in the promoter activity of PSI genes under LL conditions. Previously, we observed that base substitution of TTTTT (-61 to -57) or TTATT (-54 to -50) but not of GGGGC (-68 to -64) resulted in drastic decrease of the *psaAB* promoter activity under LL conditions (24). This can be another example that the HLR1 sequence (-62 to -45, in the case of *psaAB*) is essential for the activation of PSI promoters. Taken together, our observations suggest that RpaB is an activator for PSI genes working under LL conditions.

RpaB has so far been reported as a negative regulator for HL-inducible genes working under LL conditions (19, 29). Here, we reported for the first time that RpaB can work for positive regulation under the same LL conditions. The regulatory role of RpaB is likely to be determined by the location of the HLR1 sequence in the target promoters. In the case of HL-inducible genes, the HLR1 sequence is located within the core promoter region (7, 18) or within 5' -UTR region (29) and it was proposed that binding of RpaB to the HLR1 sequence prevents the interaction between RNA polymerase and the core promoter sequence. On the other hand, we found that the HLR1 sequence is located upstream of the core promoter region in the case of PSI genes (Fig. 1). In various bacterial species, transcriptional activators including several response regulators were reported to bind upstream of the core promoter region. They interact with the C-terminal domain of the α subunit of RNA polymerase

11

(α-CTD), leading to increase in the rate of transcription initiation (5, 6, 10). It is possible that RpaB interacts with α-CTD at the HLR1 sequence of PSI genes to enhance the promoter activity.

Recently, Hanaoka and Tanaka (12) showed by chromatin immunoprecipitation analysis that binding of RpaB to its target promoters, *hliA* and *rpoD3* in *Synechococcus elongatus* PCC 7942, was promptly lost upon the shift to HL. Thus, it is supposed that the coordinated down-regulation of PSI genes, as well as up-regulation of HL-inducible genes, is accomplished by release of RpaB from the HLR1 sequence under HL conditions in *Synechocystis* sp. PCC 6803. Then, how is the change in light intensity transmitted to RpaB? Seki et al. (29) observed that overexpression of the truncated RpaB protein harboring only the phosphoreceiver domain resulted in the de-repression of *hliA* and *rpoD3* genes under LL conditions. This phenomenon could be due to decrease in phosphorylation level of the native RpaB protein, and authors proposed that RpaB is phosphorylated by its cognate histidine kinase under LL to work for negative regulation. The prime candidate for the cognate histidine kinase is thought to be NblS (also known as Hik33 or DspA), since the HLR1 sequence is found in the promoter region of genes regulated by NblS (18, 19). However, phosphorylation of RpaB by NblS/Hik33 under LL conditions is still controversial. Based on their extensive studies using DNA microarray technique, Los et al. (21) concluded that Hik33 is inactive under non-stress conditions and is activated in response to the environmental stress. We observed that the effect of disruption of Hik33 is far smaller than that of RpaB under LL conditions (not shown). The mechanism of the perception of the change in light intensity and the following signal transduction to modulate the RpaB activity remain to be ducidated.

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FIGURE LEGENDS

Fig. 1. (A) Nucleotide sequences of the core promoter and the light-responsive AT-rich

upstream element of PSI genes in *Synechocystis* sp. PCC 6803. The sequences are aligned

according to the major transcription start site noted as +1. Putative -35 and -10 hexamers are

boxed. The direct repeat of the HLR1 motif is indicated by arrows. (B) Alignment of the

HLR1 sequences found in the upstream region of PSI promoters. Residues identical to the

HLR1 consensus sequence (18) are shaded in black. The position relative to the transcription

start site and the orientation of HLR1 sequence are shown for each PSI gene.

Fig. 2. Gel mobility shift assay of various DNA fragments with His-RpaB. DIG-labeled

upstream DNA fragments of *hliB* (-69 to +51), *psaAB* (+80 to +179), *psaK1* (-90 to +63) and

psaLI (-90 to +54) were incubated for 30 min with His-RpaB added at indicated

concentrations. Samples were separated on a 6% polyacrylamide gel. For the competition

assay of *psaK1*, unlabeled probe fragment (-90 to +63: self) or non-specific fragment from the

coding region (+61 to +213: non-self) was added at 150-fold excess of the probe

concentration. For the competition assay of *psaLI*, unlabeled probe fragment (-90 to +54: self)

or non-specific fragment from the coding region (+121 to +264: non-self) was added at

200-fold excess of the probe concentration.

Fig. 3. Gel mobility shift assay of the promoter segments of PSI genes with His-RpaB. (A)

DIG-labeled promoter segment of each PSI gene containing the upstream element from -70 to -46 was incubated for 30 min with His-RpaB added at indicated concentrations. Samples were separated on a 6% polyacrylamide gel. (B) The upstream element was deleted from each probe used in (A) and gel mobility shift assay was performed in the same procedure.

Fig. 4.   Effect of base substitution within the HLR1 sequence of the *psaE* promoter. (A) Gel mobility shift assay of the *psaE* promoter segments with or without base substitution. DIG-labeled *psaE* promoter fragments (-97 to +23) with or without base substitution were incubated for 30 min with His-RpaB added at indicated concentrations. Samples were separated on a 6% polyacrylamide gel. Nucleotide sequences of promoter fragments used for the experiment are shown at the top of the panel. The direct repeat of HLR1 sequence in the *psaE* promoter is indicated by arrows and sites of base substitution in *psaE*sub1 and *psaE*sub2 fragments are shaded in gray. (B) Bioluminescence level from low-light grown *Synechocystis* cells harboring *luxAB* reporter genes fused to the *psaE* promoter with or without base substitution. Error bars represent the standard deviation among three independent measurements. Nucleotide sequence of 5'-terminal region of each reporter fusion is shown at the top of the panel. *Bsi*WI sites included in E1 (-70 to +90) and E2 (-45 to +90) are shown in italic. Sites of base substitution in E1sub1 and E1sub2 are shaded in gray.
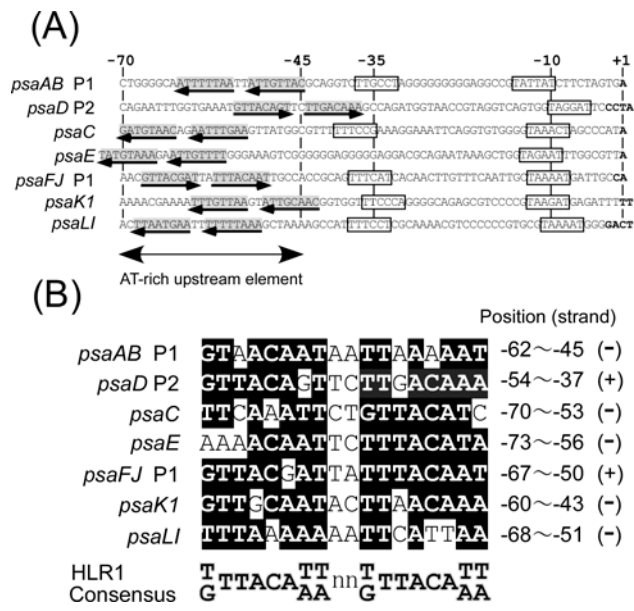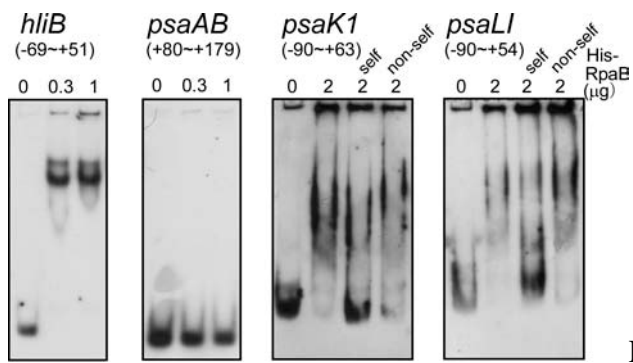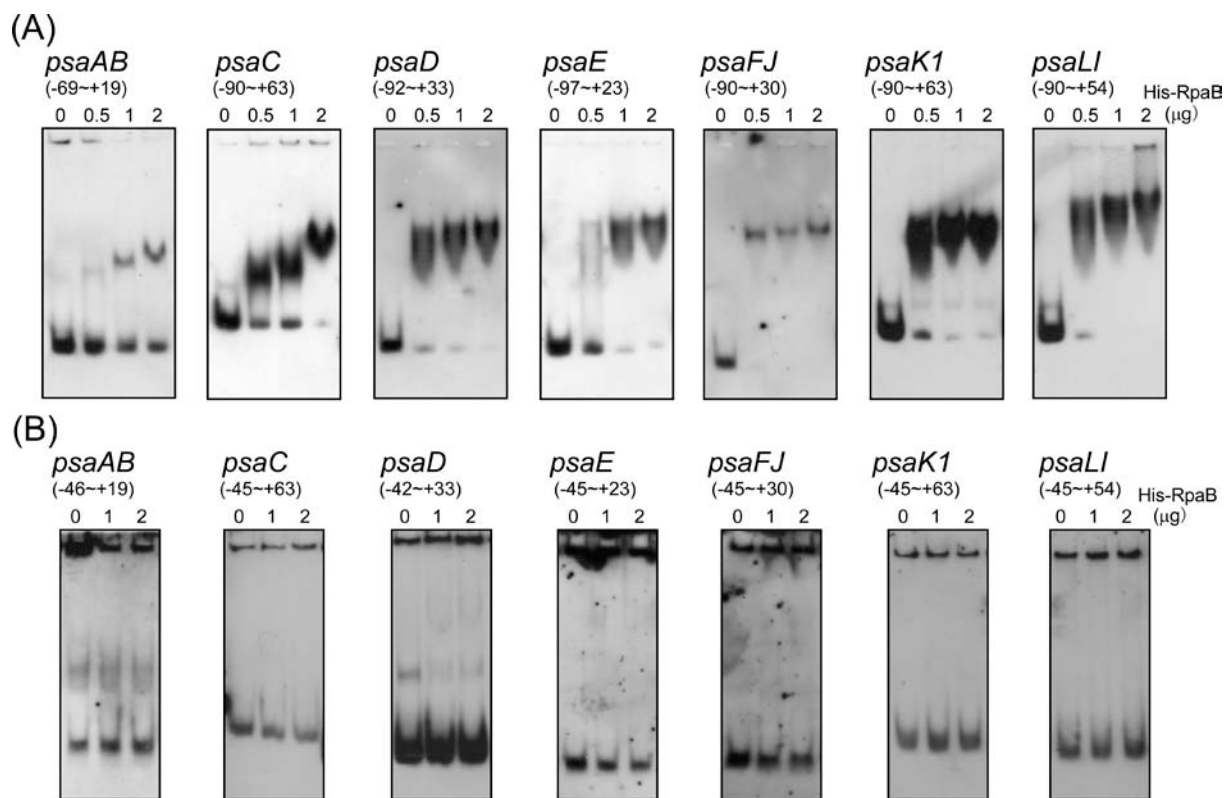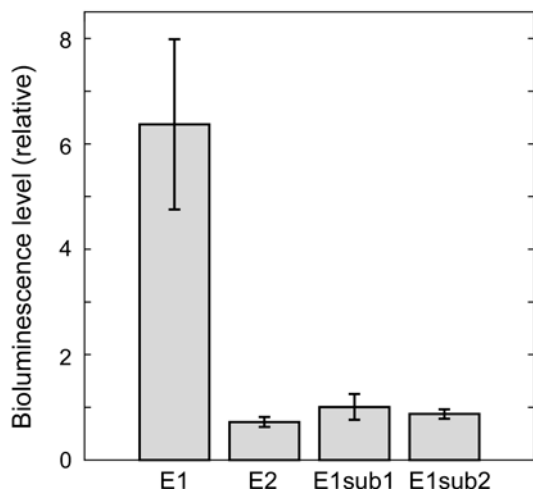
Fig. 1

Fig. 2

(A)

psaAB
(-69~+19)

psaC
(-90~+63)

psaD
(-92~+33)

psaE
(-97~+23)

psaFJ
(-90~+30)

psaK1
(-90~+63)

psaLI
(-90~+54)   His-RpaB

0 0.5 1 2   0 0.5 1 2   0 0.5 1 2   0 0.5 1 2   0 0.5 1 2   0 0.5 1 2   0 0.5 1 2 (µg)

(B)

psaAB
(-46~+19)

psaC
(-45~+63)

psaD
(-42~+33)

psaE
(-45~+23)

psaFJ
(-45~+30)

psaK1
(-45~+63)

psaLI
(-45~+54)   His-RpaB

0 1 2   0 1 2   0 1 2   0 1 2   0 1 2   0 1 2   0 1 2 (µg)

Fig. 3

21

Fig. 4