

Positive Regulation of *psbA* Gene Expression by cis-Encoded Antisense RNAs in *Synechocystis* sp. PCC 6803^{1[OA]}

Isamu Sakurai, Damir Stazic, Marion Eisenhut, Eerika Vuorio, Claudia Steglich, Wolfgang R. Hess, and Eva-Mari Aro*

Department of Biochemistry and Food Chemistry, Molecular Plant Biology, University of Turku, FI-20014 Turku, Finland (I.S., E.V., E.-M.A.); Faculty of Biology, University of Freiburg, D-79104 Freiburg, Germany (D.S., C.S., W.R.H.); and Heinrich-Heine-Universität Duesseldorf, Plant Biochemistry, D-40225 Duesseldorf, Germany (M.E.)

The D1 protein of photosystem II in the thylakoid membrane of photosynthetic organisms is encoded by *psbA* genes, which in cyanobacteria occur in the form of a small gene family. Light-dependent up-regulation of *psbA* gene expression is crucial to ensure the proper replacement of the D1 protein. To gain a high level of gene expression, *psbA* transcription can be enhanced by several orders of magnitude. Recent transcriptome analyses demonstrated a high number of cis-encoded antisense RNAs (asRNAs) in bacteria, but very little is known about their possible functions. Here, we show the presence of two cis-encoded asRNAs (PsbA2R and PsbA3R) of *psbA2* and *psbA3* from *Synechocystis* sp. PCC 6803. These asRNAs are located in the 5' untranslated region of *psbA2* and *psbA3* genes. Their expression becomes up-regulated by light and down-regulated by darkness, similar to their target mRNAs. In the PsbA2R-suppressing strain [PsbA2R(-)], the amount of *psbA2* mRNA was only about 50% compared with the control strain. Likewise, we identified a 15% lowered activity of photosystem II and a reduced amount of the D1 protein in PsbA2R(-) compared with the control strain. The function of PsbA2R in the stabilization of *psbA2* mRNA was shown from in vitro RNase E assay when the AU box and the ribosome-binding site in the 5' untranslated region of *psbA2* mRNA were both covered by PsbA2R. These results add another layer of complexity to the mechanisms that contribute to *psbA* gene expression and show PsbA2R as a positively acting factor to achieve a maximum level of D1 synthesis.

psbA genes encode the D1 reaction center protein of water-splitting PSII. The PSII structure from *Thermosynechococcus vulcanus*, a thermophilic cyanobacterium, has been resolved at 1.9 Å resolution (Umena et al., 2011). All the cofactors of PSII electron transfer are bound to the D1 protein and to a homologous D2 protein (Nanba and Satoh, 1987), forming together the reaction center core of PSII. The unique and highly oxidizing chemistry of PSII and the concomitant formation of reactive radicals induce lethal damage on the D1 protein, which needs to be replaced rapidly and properly by de novo synthesized D1 to avoid irreversible

damage to the photosynthetic apparatus. Consequently, the D1 protein shows faster turnover than any other thylakoid protein, particularly under conditions where cells are exposed to light in excess of that safely used for photochemistry (Aro et al., 1993). In cyanobacteria, the major control of *psbA* gene expression has been identified to occur at the level of transcription (Golden et al., 1986; Mohamed and Jansson, 1989; Mulo et al., 2012).

Cyanobacteria in general have up to five *psbA* gene copies (Cyanobase; <http://genome.kazusa.or.jp/cyanobase>). These different copies respond differentially to changes in environmental conditions (Clarke et al., 1993; Mohamed et al., 1993; Máté et al., 1998; Kós et al., 2008; Summerfield et al., 2008; Sicora et al., 2009), emphasizing the importance of transcriptional regulation. Indeed, at high light intensities, at least one of the *psbA* genes belongs to the most highly expressed genes of the cyanobacterial cell. Besides the general transcriptional regulation whose components still remain unknown, there are several other molecular mechanisms that impact *psbA* gene expression. One such mechanism involves RNase E/G-type endoribonuclease-mediated cleavage at the "AU box" and the ribosome-binding site (RBS; Horie et al., 2007).

Recent transcriptome analyses have demonstrated a high number of noncoding transcripts in bacteria. In addition to the transacting regulatory sense RNAs, in many bacteria several hundreds of distinct cis-antisense

¹ This work was supported by the Academy of Finland (project nos. 118637 and 133299 to E.-M.A. and M.E.), the Deutsche Forschungsgemeinschaft Focus program "Sensory and Regulatory RNAs in Prokaryotes" (grant no. SPP1258), the Bundesministerium für Bildung und Forschung (grant no. 0315274D to W.R.H.), and the Ministerium für Wissenschaft, Forschung und Kunst Baden-Württemberg Program Innovation Fund and Research Seed Capital to C.S.

* Corresponding author; e-mail evaaro@utu.fi.

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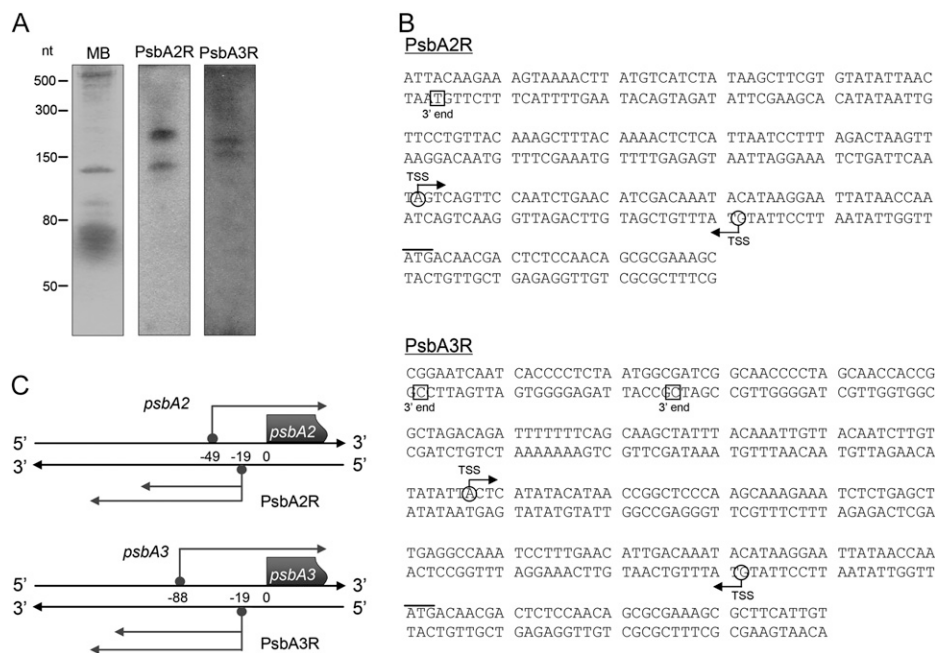


Figure 1. Identification of the asRNAs PsbA2R and PsbA3R. A, Typical detection pattern of PsbA2R and PsbA3R by northern analysis. Five micrograms of total RNA was loaded on each lane. The blot was stained by methylene blue before being hybridized with RNA probes (MB). B, Determination of the 5' and 3' ends of the asRNAs by RACE analysis. mRNA and asRNAs are shown in the top and bottom DNA strands, respectively. Transcription start sites (TSS) and the 3' ends of mRNAs or asRNAs are indicated by circles together with an arrow and by squares, respectively. The start codon of translation is indicated by a bar in the top DNA strand. C, Schematic presentation of PsbA2R and PsbA3R. Numbers within the two DNA strands indicate the positions counted from the ATG translation start codon of the mRNAs.

transcripts have been reported (for review, see Georg and Hess, 2011). This matches a recent observation for plant chloroplasts, where antisense transcripts (asRNAs) were found for 35% of all genes (Zhelyazkova et al., 2012). Moreover, natural antisense transcripts are also abundant in the plant nuclear genome (Zhou et al., 2009; Zhang et al., 2012b).

In cyanobacteria, asRNAs amounting to 26% of all genes were reported for the unicellular *Synechocystis* sp. PCC 6803 (Georg et al., 2009; Mitschke et al., 2011a) and to 39% of all genes in the nitrogen-fixing *Anabaena* sp. PCC 7120 (Mitschke et al., 2011b). However, the functional relevance of specific antisense transcripts in plants, chloroplasts, as well as bacteria has scarcely been addressed. In *Anabaena* sp. PCC 7120, *furA*, the gene for the ferric uptake regulator, is covered by a long asRNA that originates by read through from the neighboring gene *alr1690* (Hernández et al., 2006). In a $\Delta alr1690$ strain, increased levels of the Fur protein and an iron deficiency phenotype were observed, suggesting the biological relevance of this asRNA (Hernández et al., 2010). The 177-nucleotide asRNA IsrR in *Synechocystis* sp. PCC 6803 controls the expression of *isiA*, which encodes the iron stress-induced protein A, in a codegradation mechanism (Dühring et al., 2006). In this case, the transcript accumulations of IsrR and *isiA* mRNA are strictly inverse to each other. However, IsrR is transcribed from a constitutive promoter, whereas the *isiA* promoter is induced upon iron, redox, or light stress. When both transcripts are expressed simultaneously, they form an RNA duplex that is immediately degraded. Therefore, IsiA proteins are not observed before the number of *isiA* mRNA molecules has titrated out the number of IsrR molecules. As a result, IsrR filters short-time environmental stress signals out and causes a delay of *isiA*

expression in the early stress phase but also a faster depletion during the recovery from stress, in a regulatory mode known as the threshold linear response (Legewie et al., 2008). Yet another cis-asRNA was recently characterized from the *flv4-2* operon in *Synechocystis* sp. PCC 6803 and shown to be transiently expressed under inorganic carbon limitation (Eisenhut et al., 2012). Proteins encoded by the *flv4-2* operon

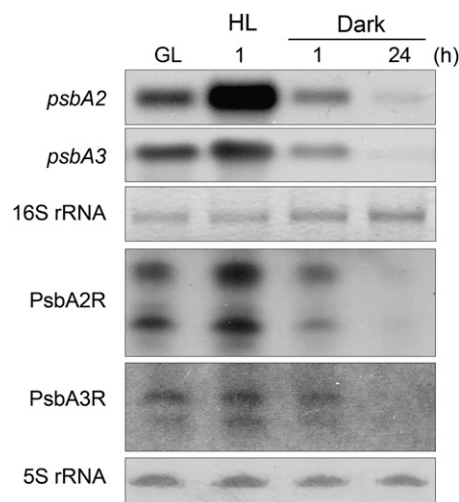


Figure 2. Response of the expression of *psbA2* and *psbA3* genes and their respective asRNAs to changes in light intensity. Total RNA was isolated from the cells grown under standard GL, 1 h after the shift to HL ($1,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), and 1 or 24 h after the shift to darkness. One microgram of total RNA was used for the detection of *psbA2*, and 5 μg of total RNA was applied to each lane in the case of PsbA2R, *psbA3*, and PsbA3R.

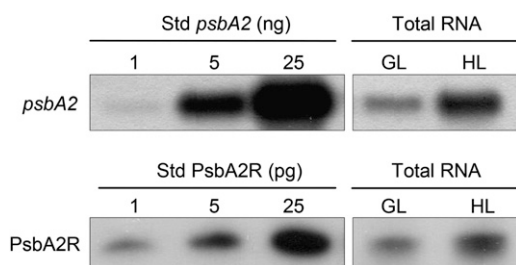


Figure 3. Molecular ratio of mRNA and asRNA of *psbA2* in *Synechocystis* sp. PCC 6803 cells. RNA molecules synthesized by the in vitro transcription system were used as a standard series (Std; 1, 5, and 25 ng). Total RNA was isolated from cells grown under GL and from cells treated under HL for 30 min. One and 5 μg of total RNA were used for the detection of *psbA2* mRNA and PsbA2R, respectively.

have been demonstrated as crucial in photoprotection of PSII under inorganic carbon limitation and high-light conditions (Zhang et al., 2009, 2012a), and the respective asRNA functions as a buffer mechanism to avoid premature expression of the *flv4-2* operon when the stress conditions are only transient, thus avoiding the initiation of long-term acclimation processes upon short-term environment fluctuations.

Thus far, distinct functions have been identified for only a few and strongly expressed bacterial asRNAs, but many occur at relatively low abundance (for review, see Georg and Hess, 2011), raising the question of whether these low-abundance transcripts just constitute some kind of “transcriptional noise” or provide some selective advantage. In this study, we identified and characterized two short asRNAs, PsbA2R and PsbA3R, which originate from the 5' untranslated region (UTR) of genes *psbA2* and *psbA3* in *Synechocystis* sp. PCC 6803. Despite low steady-state abundance, we show that PsbA2R acts as a positive regulator of the *psbA2* expression and a positive effector of cell growth on solid medium.

RESULTS

Two asRNAs Originating in the 5' UTR of *psbA2* and *psbA3*

Analysis of the *Synechocystis* sp. PCC 6803 transcriptome by differential RNA sequencing revealed two transcription start sites immediately upstream of the *psbA2* and *psbA3* genes on the reverse complementary strand (Mitschke et al., 2011a). Both asRNAs (designated as PsbA2R and PsbA3R, respectively) were confirmed by northern analysis using strand-specific RNA probes (Fig. 1A) and by 5' and 3' RACE analyses to determine the full length of PsbA2R and PsbA3R (Fig. 1B). Two distinct signals with fragment lengths of approximately 130 and 220 nucleotides as well as approximately 160 and 180 nucleotides were detected for PsbA2R and PsbA3R, respectively (Fig. 1B). For both asRNAs, one transcription start site was found at

position -19 counted from the ATG start codon of *psbA* mRNAs, corresponding to genome positions 7,210 and 1,820,016 on the reverse and forward strands, respectively (Fig. 1, B and C). This result is consistent with previous transcription start site mapping by differential RNA sequencing (Mitschke et al., 2011a). On the contrary, 3' RACE analysis identified one reasonable 3' end for PsbA2R at the position -147 and two 3' ends for PsbA3R at positions -175 and -199 (Fig. 1B). However, no proper 3' end was identified for the approximately 220-nucleotide signal of PsbA2R. Thus, there are two variants of PsbA2R (129 and about 220 nucleotides) and PsbA3R (157 and 181 nucleotides; Fig. 1C).

Light-Dependent Expression of PsbA2R and PsbA3R

Since the transcription of the *psbA* genes is strictly regulated by light, we investigated whether PsbA2R and PsbA3R show any transcriptional response to changes in light intensity. As shown in Figure 2, the accumulation of PsbA2R was strongly enhanced upon a shift of cells from growth light (GL; 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) to high light (HL; 1,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), whereas the accumulation of PsbA3R transcripts was less drastic. This mimicked the expression patterns of the *psbA2* and *psbA3* genes, respectively. Likewise, the asRNAs were down-regulated in darkness similar to their target mRNAs.

Molecular Ratio between mRNA and asRNA of *psbA2* under Steady-State Conditions

The molecular ratio between the target mRNA and the respective asRNA provides important information about the putative regulatory function of the asRNA on its target gene. To this end, we focused on the *psbA2* gene, which is the most active one of the three *psbA* genes in *Synechocystis* sp. PCC 6803 at HL, and on the

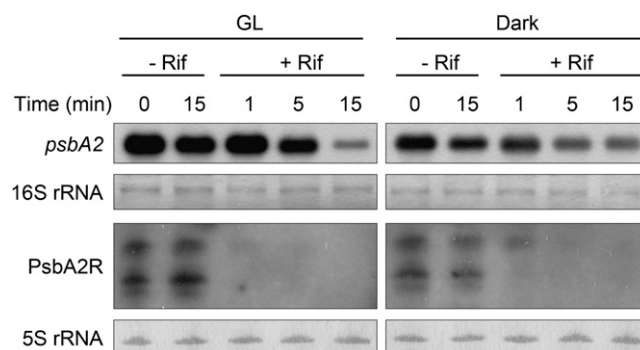


Figure 4. Turnover of *psbA2* mRNA and PsbA2R. Cells treated by HL for 30 min were transferred to GL or darkness in the absence or presence of Rif. Cells were harvested for RNA analysis at the designated time points. One and 5 μg of total RNA were used for the detection of *psbA2* mRNA and PsbA2R, respectively.

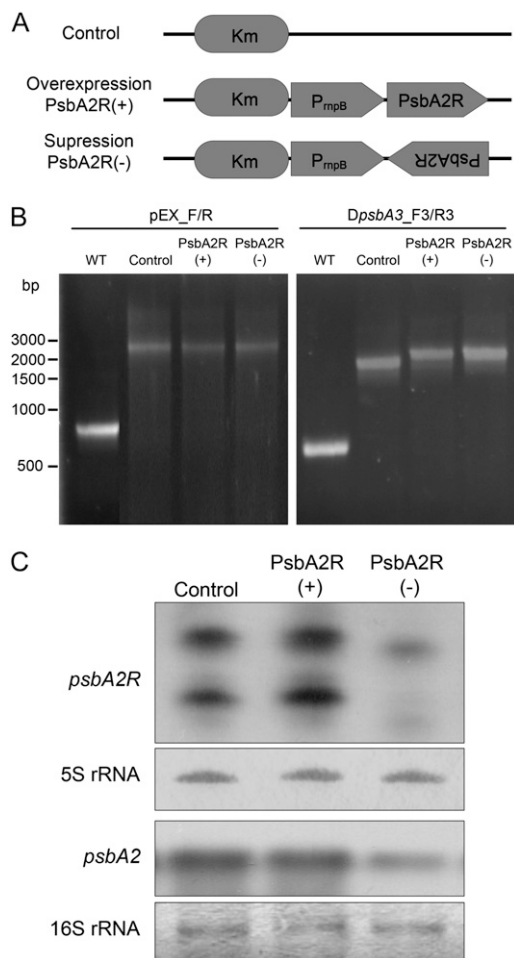


Figure 5. Overexpression and suppression of PsbA2R. A, The sense and antisense fragments of PsbA2R were fused to the *mpb* promoter, yielding overexpressor [PsbA2R(+)] and suppressor [PsbA2R(-)], respectively. The control $\Delta psbA3$ strain contains only the kanamycin (Km) resistance cassette. B, Segregation check of transformants. Disruption of the *psbA3* gene and replacement of the *slr2031* region were checked by PCR. WT, Wild type. C, Expression of PsbA2R and *psbA2* mRNA in the overexpressor [PsbA2R(+)] and suppressor [PsbA2R(-)] mutant strains, respectively. One and 5 μ g of total RNA isolated from the cells grown under GL were used for the detection of *psbA2* mRNA and PsbA2R, respectively.

respective asRNA PsbA2R. The molecular ratio between mRNA and asRNA of *psbA2* was estimated by comparing their amounts in the total RNA extract from *Synechocystis* sp. PCC 6803 cells with in vitro-synthesized RNA molecules as a reference. Such analyses revealed that 1 μ g of total RNA isolated from the HL-treated cells contained about 3 ng of *psbA2* mRNA and 1 pg of PsbA2R (Fig. 3). To estimate the molecular ratio between mRNA and asRNA of *psbA2*, the lengths of the *psbA2* and PsbA2R transcripts, 1,083 and 129 nucleotides, respectively, were taken into account. This resulted in a steady-state molecular ratio between mRNA and asRNA of *psbA2* at approximately 300:1.

Rapid Turnover of asRNA PsbA2R Compared with *psbA2* mRNA

The low steady-state amount of PsbA2R prompted us to investigate the turnover rates of the asRNA and the target mRNA. To determine the half-lives of the *psbA2* mRNA and the asRNA PsbA2R, the cell suspension was supplemented with rifampicin (Rif) to prevent de novo RNA synthesis. Furthermore, for practical reasons, the amounts of both *psbA2* mRNA and PsbA2R were first left to increase by HL illumination for 30 min, and only thereafter were the measurements of *psbA2* mRNA and PsbA2R amounts initiated (Fig. 4, 0 min). In the absence of Rif, the steady-state amount of *psbA2* mRNA slightly decreased both in GL and in darkness, whereas the content of PsbA2R decreased only in darkness. On the contrary, in the presence of Rif, a fast RNA degradation occurred and distinct differences in the disappearance of *psbA2* mRNA and PsbA2R were evident. Although the degradation of *psbA2* mRNA was faster in GL compared with darkness, there was a clear signal of *psbA2*

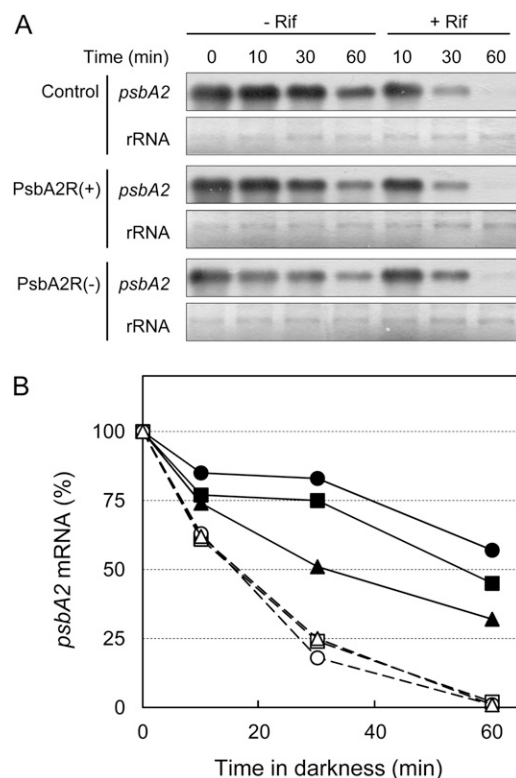


Figure 6. Effect of PsbA2R on the stability of *psbA2* mRNA. A, Cells treated by HL for 1 h were transferred to darkness in the presence or absence of Rif. Cells were harvested for RNA analysis at the designated time points. One microgram of total RNA was used for the detection of *psbA2* mRNA. B, Quantification of signal intensity. Signal intensity at 0 min (1 h of incubation of cells at HL) was set as 100%. Control, PsbA2R(+), and PsbA2R(-) are shown in squares, circles, and triangles, respectively, in the presence (closed symbols with solid lines) and absence (open symbols with dashed lines) of Rif.

mRNA still present after 15 min of Rif treatment in GL. In sharp contrast to the behavior of *psbA2* mRNA, PsbA2R had nearly disappeared already within 1 min under GL conditions. The degradation of both the *psbA2* mRNA and PsbA2R occurred more slowly in darkness, yet, again, there was a clear difference, with the asRNA disappearing faster. These results indicated that the turnover of PsbA2R is more than 1 order of magnitude faster, particularly in light, than that of the *psbA2* mRNA (Fig. 4).

Suppression of PsbA2R Decreases the Steady-State Amount of *psbA2* mRNA

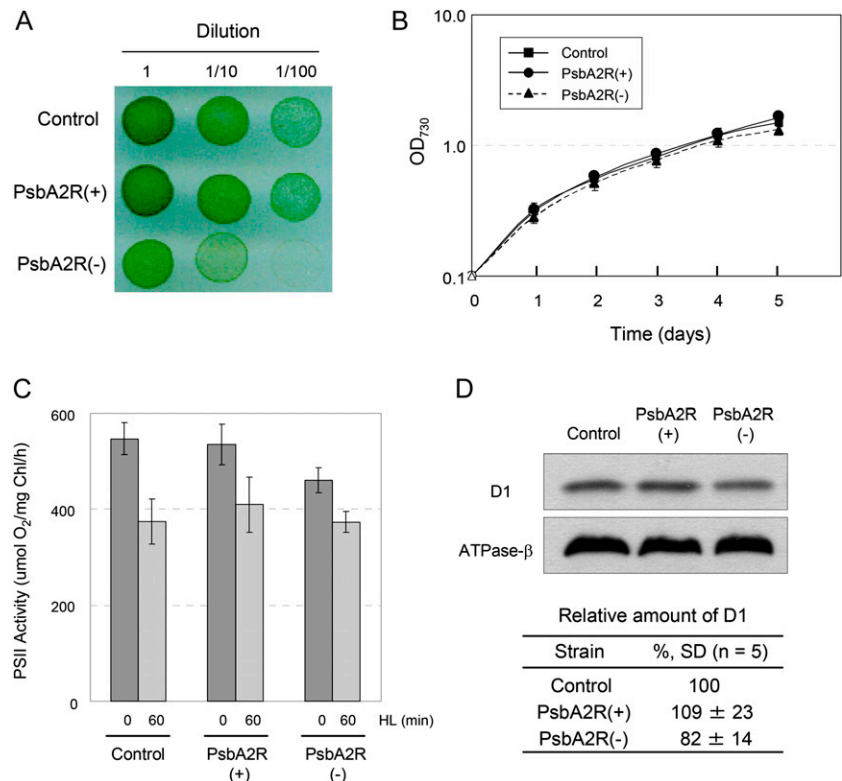
To further study the relationship between *psbA* gene expression and that of the respective asRNA, we prepared overexpressor and suppressor mutants of PsbA2R, referred to as PsbA2R(+) and PsbA2R(-), respectively (Fig. 5A). PsbA2R was selected for these analyses because a significant part, nearly 90% of the total *psbA* mRNA, is transcribed from the *psbA2* gene, the putative target of PsbA2R (Mohamed et al., 1993). As mentioned above, the *psbA3* gene also has a similar type of asRNA in the 5' UTR as *psbA2*. Therefore, in order to eliminate the possibility that PsbA3R would compensate for the function of PsbA2R, the $\Delta psbA3$ strain that lacks PsbA3R was used as a parental strain to construct the PsbA2R(+) and PsbA2R(-) strains (Fig. 5B). Figure 5C shows the expression level of PsbA2R in each strain. PsbA2R(+) contained a slightly

elevated amount of PsbA2R compared with the control strain. However, in the PsbA2R(-) strain, nearly complete suppression of PsbA2R occurred. To investigate the effect of PsbA2R on the expression of *psbA2*, the amount of *psbA2* mRNA was also analyzed in the cells. As shown in Figure 5C, the PsbA2R(+) strain contained almost the same amount of *psbA2* mRNA as the control strain. Intriguingly, however, the level of *psbA2* mRNA in the PsbA2R(-) strain was significantly lower, being only about 50% of that in the control strain. These results provided strong evidence that PsbA2R, despite its low steady-state amount, is an important factor in controlling the cellular amount of *psbA2* mRNA.

Effect of PsbA2R on the Stability of *psbA2* mRNA

The balance between de novo synthesis and degradation determines the amount of mRNA. As the level of *psbA2* mRNA was lower in the PsbA2R(-) strain as compared with the control strain, two possible functions of PsbA2R are conceivable. The asRNA PsbA2R either enhances the de novo synthesis of *psbA2* mRNA or suppresses the degradation of *psbA2* mRNA. To check the latter possibility, the stability of *psbA2* mRNA was analyzed in the strains with modulated PsbA2R contents. Addition of Rif prevents the de novo synthesis of RNA, thus allowing the determination of transcript degradation rates. However, degradation of PsbA2R antisense transcripts is extremely fast in light as compared

Figure 7. The effect of PsbA2R on growth and photosynthesis of *Synechocystis* sp. PCC 6803 cells. A, Growth of mutants on an agar plate. The cells were incubated for 4 d under GL conditions. B, Growth of mutants in liquid culture. OD₇₃₀, Optical density at 730 nm. C, PSII-dependent oxygen-evolving activity. Oxygen evolution was measured in the presence of 2 mM 2,6-dimethyl-*p*-benzoquinone and 1 mM potassium ferricyanide at 30°C from the suspension culture grown under GL conditions and from cells illuminated under HL for 1 h prior. D, Amount of D1 protein in the mutants. Thylakoid membranes containing 0.5 μ g of chlorophyll were solubilized, and the supernatant was applied to the gel. The same blot was used for the detection of the D1 protein and the β -subunit of ATPase. The table shows the relative amount of the D1 protein in each transformant. Data represent means \pm SD from more than three independent experiments.



with the *psbA2* mRNA (Fig. 4) and therefore would mask the putative effect of PsaA2R on the stability of *psbA2* mRNA when the cells are incubated in light and transcription is blocked by Rif (dashed lines in Fig. 6B). Nevertheless, Figure 4 also showed that de novo transcription of *psbA2* is somehow suppressed in darkness, where PsaA2R is likewise more stable. Consequently, the stability of *psbA2* mRNA in PsaA2R(+) and PsaA2R(-) was analyzed in darkness in the absence of Rif. As shown in Figure 6, the degradation of *psbA2* mRNA occurred more slowly in the PsaA2R(+) strain compared with the control strain. In contrast, the degradation rate of *psbA2* mRNA was clearly faster in the PsaA2R(-) strain than in the control strain. These results provide strong evidence that PsaA2R functions in maintaining the stability of *psbA2* mRNA.

Growth and Photosynthesis of PsaA2R(+) and PsaA2R(-) Strains

Next, we analyzed the physiological phenotypes of PsaA2R(+) and PsaA2R(-). On agar plates, the growth of PsaA2R(+) was similar to the control strain. On the contrary, PsaA2R(-) showed clearly slower growth compared with the control strain and PsaA2R(+) (Fig. 7A). However, the growth phenotype was lost

when the PsaA2R(-) strain was grown in liquid medium (Fig. 7B).

PSII activity of the PsaA2R(+) and PsaA2R(-) strains was analyzed using a Clark-type oxygen electrode (Fig. 7C). Under normal GL conditions, the control strain and PsaA2R(+) showed similar oxygen evolution activity of PSII, 550 and 535 $\mu\text{mol oxygen mg}^{-1}$ chlorophyll h^{-1} , respectively. PsaA2R(-) showed lower oxygen-evolving activity, approximately 85% of that measured in the control strain. In line with this result, PsaA2R(-) also contained a reduced amount of the D1 protein as compared with the control or the PsaA2R(+) strain (Fig. 7D).

In Vitro Indication for the Protection of *psbA2* mRNA by PsaA2R against RNase E Attack

RNase E has an important role in the degradation of *psbA2* mRNA, as shown previously (Horie et al., 2007). Since our experiments pointed to the fact that PsaA2R possibly functions in stabilizing the *psbA2* mRNA (Fig. 6), we used an RNase E in vitro assay to gain further insights into the protective role of PsaA2R. Artificially synthesized *psbA2* mRNA (110 nucleotides, positions -49 to +61, as described by Horie et al. [2007]) was incubated with purified RNase E both in the absence

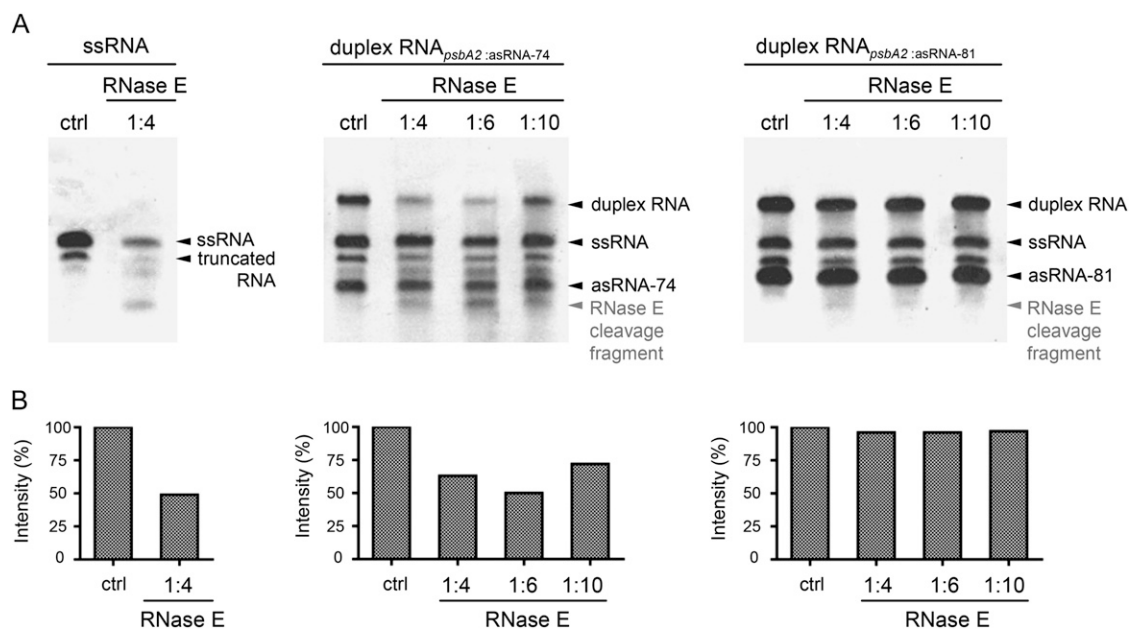


Figure 8. Protection of *psbA2* mRNA from RNase E attack by PsaA2R. A, Single-stranded *psbA2*-110 RNA (left panel) and duplex *psbA2*-110 RNA (middle and right panels) were treated without (ctrl) or with recombinant *Synechocystis* sp. PCC 6803 RNase E in a designated dilution. Duplex formation with *psbA2* asRNA-74 (middle panel) and *psbA2* asRNA-81 (right panel) served to mask one RNase E site (AU box) and two RNase E sites (AU box and RBS) in *psbA2* mRNA (Horie et al., 2007), respectively. Following incubation with RNase E for 15 min, RNA cleavage patterns were visualized on ethidium bromide-stained 7 M urea-6% polyacrylamide gels. B, *psbA2*-110 RNA was quantified with Quantity One software (Bio-Rad). Signal intensities of each single-stranded RNA fraction and duplex fraction were combined to estimate total abundance of *psbA2*-110 RNA. Comparison of RNase E-treated samples and nontreated (ctrl) samples showed no decline in signal intensity for asRNA fractions. Thereby, the decline in signal intensity in duplex fractions exclusively accounts for the cleavage of *psbA2*-110 RNA.

and presence of PsbA2R asRNA (74 nucleotides, positions -19 to -92). RNase E cleaved the single-stranded form of *psbA2* mRNA into specific RNA fragments (Fig. 8A). Different from our expectations, however, *psbA2* mRNA was degraded by RNase E also in the presence of PsbA2R (Fig. 8, middle panels). PsbA2R covers the AU box but not the RBS in the 5' UTR of *psbA2* mRNA (Horie et al., 2007). Both sites are recognized by RNase E, yet only the AU box can be masked by duplex formation. Nevertheless, Horie et al. (2007) demonstrated that *psbA2* mRNA is successfully protected against RNase E also at the RBS, which would not be covered by PsbA2R. It is thus conceivable that in vivo binding of ribosomes protects *psbA2* mRNA from RNase E attack at the latter site. To elucidate this question, we synthesized a "long and artificial" form of PsbA2R (81 nucleotides, positions -12 to -92), which covers both RNase E cleavage sites, and analyzed the protective effect of such an artificial PsbA2R. As shown in Figure 8 (right panels), the amount of the RNase E degradation products from *psbA2* mRNA is substantially diminished in the presence of the long and artificial form of PsbA2R as compared with the presence of natural PsbA2R species. These data suggested that masking of both the AU box and the RBS is necessary to protect *psbA2* mRNA from degradation by RNase E.

DISCUSSION

Expression of the D1 protein of PSII is strictly regulated, and in general, no free D1 protein accumulates in the thylakoid membrane. A sufficient number of *psbA* mRNAs is expected to be always available, being tightly controlled by the intensity of light. In light, the *psbA* mRNAs reside in translation initiation complexes in the cytoplasm (Tyystjärvi et al., 2001), being ready for cotranslational insertion of the new D1 copy directly into the partially disassembled PSII complex whose D1 protein has been degraded. In cyanobacteria, regulation of *psbA* gene expression was postulated to occur mainly at the level of transcription (Golden et al., 1986; Mohamed and Jansson, 1989; Mulo et al., 2012). Yet, regulatory mechanisms at the molecular level are largely unresolved. Here, we provide novel data on *psbA* gene regulation in *Synechocystis* sp. PCC 6803 based on positive interaction between the *psbA* mRNAs and their respective asRNAs.

The asRNAs of the *psbA2* and *psbA3* genes were first discovered by deep sequencing of the *Synechocystis* sp. PCC 6803 transcriptome (Mitschke et al., 2011a). These asRNAs, named PsbA2R and PsbA3R, are located in the 5' UTR of *psbA2* and *psbA3* genes, respectively, and thus belong to cis-type asRNAs (Waters and Storz, 2009). The expression of PsbA2R and PsbA3R is shown here to be regulated by light, the asRNAs accumulating with increasing light intensity, similar to the expression of their target *psbA2* and *psbA3* genes (Fig. 2). Corresponding responses of

both the mRNA and asRNA upon changes in environmental conditions are so far unique for the *psbA2* and *psbA3* genes. Indeed, many asRNAs studied thus far have revealed an opposite expression pattern compared with their target mRNAs (Kawano et al., 2002; Silvaggi et al., 2005; Dühning et al., 2006). A clearly retarded growth of the PsbA2R(-) strain was evident on agar plates, which provides evidence for an important function of PsbA2R under some environmental conditions. Suppression of PsbA2R also induced a decrease in the abundance of *psbA2* mRNA (Fig. 5C), which, based both on in vivo and in vitro experiments, was interpreted to result from a decrease in *psbA2* mRNA stability (Fig. 6). From the cellular physiology point of view, such a decrease in the expression of the *psbA2* gene was not detrimental for the growth of *Synechocystis* sp. PCC 6803 cells under standard growth conditions (Fig. 7B). Nevertheless, the down-regulation of *psbA2* transcripts in the PsbA2R(-) strain resulted in lowering of the maximal PSII activity and in reduction in amount of the D1 protein (Fig. 7, C and D). Therefore, it is highly likely that the expression of PsbA2R has an important function in *Synechocystis* sp. PCC 6803 cells in maintaining the cellular capacity to rapidly respond to

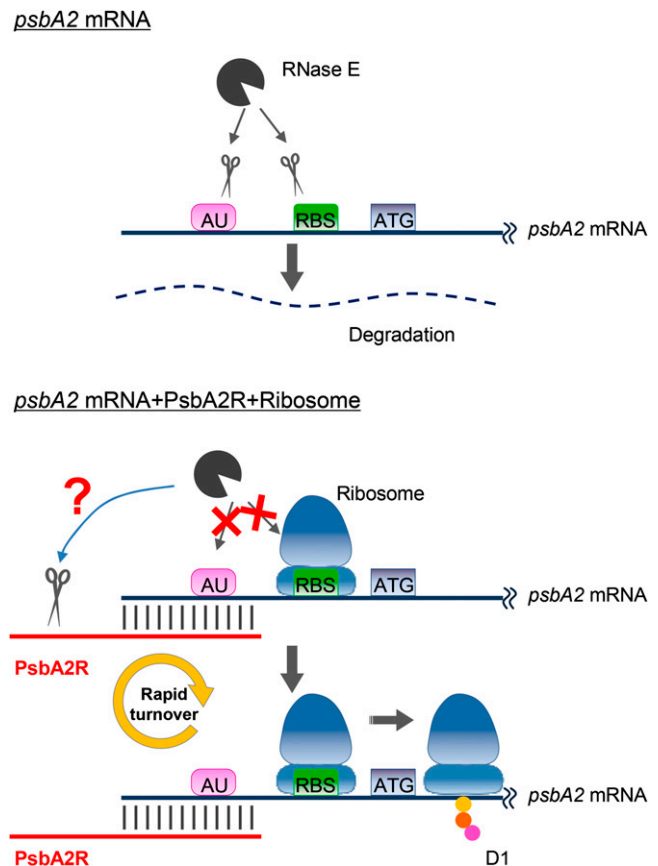


Figure 9. Hypothetical model on the regulation of *psbA2* gene expression by PsbA2R. *psbA2* mRNA is stabilized both by the annealing of PsbA2R and the binding of ribosomes. For more details, see text.

Table 1. Primers used in this work

Restriction sites are underlined. fw, Forward; rev, reverse.

| Name | Sequence (5'→3') | Experiment |
|------------------------------|---|---|
| <i>psbA2_F</i> -T7 | TAATACGACTCACTATAGGGAGCGCGCTGTTGG AGAGTCGTTGTC | <i>psbA2</i> probe |
| <i>psbA2_R</i> | AGTCAGTTCCAATCTGAACATCGAC | <i>psbA2</i> probe |
| PsbA2R_F-T7 | TAATACGACTCACTATAGGGAAAACCTCATTAAATCCTT | PsbA2R probe |
| PsbA2R_R | TGTATTTGTCGATGTTGACA | PsbA2R probe |
| <i>psbA3_F</i> -T7 | TAATACGACTCACTATAGGGAGTATTTGTCAATG TTCAAAGGAT | <i>psbA3</i> probe |
| <i>psbA3_R</i> | ATACATAACCGGCTCCCAAGC | <i>psbA3</i> probe |
| PsbA3R_F-T7 | TAATACGACTCACTATAGGGAAAACCTCATATACA TAACCGGCTCC | PsbA3R probe |
| PsbA3R_R | GTATTTGTCAATGTTCAAAGGATTTG | PsbA3R probe |
| 5' RACE_RNA adaptor | GAUAUGCGCGAAUUCUGUAGAACGAACAC UAGAAGAAA | 5' RACE |
| 3' RACE_RNA adaptor | UUCACUGUUCUUAGCGGCCGCAUGCUC | 3' RACE |
| 5' RACE_RNA Adaptor_comp_F | GAATTCCTGTAGAACGAACACTAG | 5' RACE, PCR |
| 3' RACE_RNA adaptor_comp_R | CATGCGGCCGCTAAGTTCAGTGAA | 3' RACE, PCR |
| PsbA2R_5' RACE_R1 | ACTTATGTCATCTATAAGCTTCCG | 5' RACE, first PCR |
| PsbA2R_5' RACE_R2 | CGTGTATATAACTTCCTGTTAC | 5' RACE, second PCR |
| PsbA2R_3' RACE_F | AGATTGGAAGTACTAAAC | 3' RACE, PCR |
| PsbA3R_5' RACE_R1 | TTCAGCAAGCTATTTACAAATTTG | 5' RACE, first PCR |
| PsbA3R_5' RACE_R2 | TTACTCATATACATAACCGGC | 5' RACE, second PCR |
| PsbA3R_3' RACE_F | AAAGGATTTGGCCTCAAGC | 3' RACE, PCR |
| <i>psbA2_Std_F</i> -T7 | TAATACGACTCACTATAGGGAGTCAGTCCAATCTGAACATCGA | <i>psbA2</i> standard |
| <i>psbA2_Std_R</i> | CCAAGGAATTAACCGTTGACAG | <i>psbA2</i> standard |
| PsbA2R_Std_F-T7 | TAATACGACTCACTATAGGGAGTATTTGTCGATG TTCAGATTGGA | PsbA2R standard |
| PsbA2R_Std_R | ACAAGAAAGTAAAACTTATGTCATCA | PsbA2R standard |
| Δ <i>psbA3_F1</i> | ACATGCATGCTAGTGGTGTCTGTTGGCGTTG | Mutagenesis (<i>psbA3</i> , upstream) |
| Δ <i>psbA3_R1</i> | CCCAAGCTTATCTGTCTAGCCGGTGGTTG | Mutagenesis (<i>psbA3</i> , upstream) |
| Δ <i>psbA3_F2</i> | CCCAAGCTTTATCGGTTTGCACCTCTACC | Mutagenesis (<i>psbA3</i> , downstream) |
| Δ <i>psbA3_R2</i> | AACTGCAGTAACCGTTGACAGCAGGAGC | Mutagenesis (<i>psbA3</i> , downstream) |
| Δ <i>psbA3_F3</i> | TGCAGGATTAGTGGCTTTCC | Mutagenesis (<i>psbA3</i> , segregation check) |
| Δ <i>psbA3_R3</i> | CCATCAGAGAAGGACTTTGACCA | Mutagenesis (<i>psbA3</i> , segregation check) |
| PsbA2R (+)_F1 | GGCACACTAGAAAAAGTATTTGTGCGATGTTGAGATTGG | Mutagenesis (two-step PCR) |
| PsbA2R (+)_R1 | ACGCGTCGACACAAGAAAGTAAAACCTTATGTCATC | Mutagenesis (two-step PCR) |
| PsbA2R (-)_F1 | GGCACACTAGAAAAACAAAGCTTTACAAAACCTCTCAT | Mutagenesis (two-step PCR) |
| PsbA2R (-)_R1 | ACGCGTCGACGGAATAAAAAACGCCCGCGGCAAC CGAGCGTTGATTTGTGCGATGTTGAGATTGG | Mutagenesis (two-step PCR) |
| <i>rnpB_F1</i> | AACTGCAGTTCAATGCGGCTCCAATACCTCC | Mutagenesis (two-step PCR) |
| <i>rnpB(+)_R1</i> | AACATCGACAAATACTTTTTCTAGTGTGCCATTGATTAG | Mutagenesis (two-step PCR) |
| <i>rnpB(-)_R1</i> | TTTGTAAGCTTTGTTTTTCTAGTGTGCCATTGATTA | Mutagenesis (two-step PCR) |
| pEX_F1 | GGGCCCAGTACTATTGGGGTCAG | Mutagenesis (<i>slr2031</i> , upstream) |
| pEX_R1 | GGTACCGGTTGTTATAACAGGCGATC | Mutagenesis (<i>slr2031</i> , upstream) |
| pEX_F2 | TCTAGAGATCAAACCGCTGTGGCG | Mutagenesis (<i>slr2031</i> , downstream) |
| pEX_R2 | ATGCATCAATGTCATATCGTCTCGGGC | Mutagenesis (<i>slr2031</i> , downstream) |
| pEX_F3 | GATGCCGATGGCAGTGC | Mutagenesis (<i>slr2031</i> , segregation check) |
| pEX_R3 | ACAATACTCCAGGGCACC | Mutagenesis (<i>slr2031</i> , segregation check) |
| T7- <i>psbA2</i> -110 fw | TAATACGACTCACTATAGGGAGTCAGTCCAATCTGAACATC | In vitro RNase E assay |
| <i>psbA2</i> -110 rev | CCCCTGACAAAACCTGTTCC | In vitro RNase E assay |
| T7- <i>psbA2</i> asRNA-74 fw | TAATACGACTCACTATAGGGGATTTGTGCGATGTTGAGATTG | In vitro RNase E assay |
| <i>psbA2</i> asRNA-74 rev | ACAAAGCTTTACAAAACCTCTCAT | In vitro RNase E assay |
| T7- <i>psbA2</i> asRNA-81 fw | TAATACGACTCACTATAGGGTCTTATGTTGTCGATGTTG | In vitro RNase E assay |
| <i>psbA2</i> asRNA-81 rev | ACAAAGCTTTACAAAACCTCTCAT | In vitro RNase E assay |

transient changes in environmental conditions that require higher performance of PSII than occurs under standard growth conditions.

It is also worth emphasizing that the steady-state amount of PsbA2R is much less than that of the *psbA2* mRNA (1:300; Fig. 3). This is apparently compensated

for by rapid turnover of the PsbA2R asRNA, which is at least 1 order of magnitude faster compared with the *psbA2* mRNA. Our recent genome-wide analysis of the cyanobacterium *Prochlorococcus* sp. MED4 demonstrated the presence of many short-lived asRNAs (Steglich et al., 2010). Thus, a short lifetime appears as

a common feature of many asRNAs, which further means that the molecular mechanism of regulation of the target mRNA depends on freshly synthesized antisense transcripts.

Elucidation of the mechanism for positive posttranscriptional regulation of the *psbA2* gene by the cis-type PsaA2R transcript needs a closer sequence inspection of both the target gene *psbA2* and the respective asRNA PsaA2R, their cleavage by RNases, as well as their interaction with ribosomes. In Figure 9, we have summarized the present understanding of the function of PsaA2R in the regulation of *psbA2* gene expression.

Single-stranded *psbA2* mRNA is cleaved by RNase E at the AU box and RBS, both located in the 5' UTR of the mRNA. Deduced from sequence information, PsaA2R has a capacity to protect the AU box by duplex formation with the *psbA2* mRNA. Our in vitro RNase E assay, however, showed no protection of the *psbA2* mRNA by the longest naturally occurring form of PsaA2R in cells. Only the long and artificial form of PsaA2R, covering both the AU box and RBS, had a protective effect against RNase E cleavage of the *psbA2* mRNA. In the cellular environment in vivo, one would expect that the binding of the ribosome prevents cleavage at the RBS. It is thus conceivable that, in vivo, the *psbA2* mRNA is stabilized by both PsaA2R and ribosome binding at the AU box and RBS, respectively. The double protection mechanism is likely important in stabilizing the initiation step of D1 protein translation. The requirement for PsaA2R drops sharply upon translational initiation, which is a very fast event, and would be consistent with the extremely short lifetime and low abundance of PsaA2R compared with the *psbA2* mRNA, which then resides on polysomes during translation. Another feature of PsaA2R with possible mechanistic implications is that it is longer than the 49-nucleotide-long 5' UTRs of the *psbA2* mRNA and thus lacks an annealing partner. This raises the possibility that the single-stranded region of PsaA2R is subject to degradation in the cell by RNases more efficiently than the *psbA2* mRNA during formation of the duplex structure and the subsequent translation event.

Besides clarification of the function of PsaA2R in regulation of the abundance of *psbA2* transcripts, we also recognized the presence of PsaA3R. From the sequence similarity of these asRNAs, especially the 15 to 20 nucleotides at the 5' end where the AU box was found, it is highly likely that the mechanism to regulate the expression of their target mRNAs is similar between PsaA2R and PsaA3R. On the other hand, PsaA2R and PsaA3R sequences are very different in other regions, providing evidence that both asRNAs specifically regulate the expression of their cis-encoded target gene.

In summary, asRNAs of *psbA* genes, PsaA2R and PsaA3R, were identified in the 5' UTR of the *psbA2* and *psbA3* genes, respectively. These asRNA molecules are found in low abundance in the cell, yet they are shown here to act as important positive regulators to achieve a stable level of D1 protein synthesis that

maintains the functionality of water-splitting PSII. Such an effect of the PsaA2R is reached by stabilizing the *psbA2* mRNA. Collectively, our results strongly suggest that PsaA2R functions as a positive regulator in the expression of the *psbA2* gene, which maintains photosynthesis in *Synechocystis* sp. PCC 6803 cells upon changes in environmental conditions, particularly at high light intensities. The results obtained in this study add a new layer of complexity to the mechanisms that contribute to the regulation of *psbA* gene expression. They also provide evidence that low-abundance asRNAs cannot be regarded as transcriptional noise but probably have important regulatory functions in cell physiology and indeed call for thorough investigation.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Synechocystis sp. PCC 6803 was grown photoautotrophically at 30°C in BG-11 medium (Allen, 1968) supplemented with 20 mM HEPES-NaOH (pH 7.5). The BG-11 medium for mutant strains (see below) was supplemented with 25 $\mu\text{g mL}^{-1}$ kanamycin and 15 $\mu\text{g mL}^{-1}$ spectinomycin. Cell growth was monitored by determination of optical density at 730 nm. As normal GL conditions, 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ white light was provided from a fluorescent lamp, and 1,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ white light provided from a slide projector was used as HL. For the dark treatment, the flask was covered with aluminum foil. Precultured cells were collected and resuspended in fresh BG-11 at the cell concentration of optical density at 730 nm = 0.6. These cultures were incubated under the normal GL conditions for 3 h before the experiments. Rif was used at 200 $\mu\text{g mL}^{-1}$ to prevent de novo transcription.

RNA Extraction and Northern-Blot Analysis

Total RNA was isolated from 40 mL of culture using TriSure reagent (Bioline) according to the company's instructions and treated with DNase (Ambion Turbo DNase kit) to remove the genomic DNA. *psbA2* and *psbA3* mRNAs were analyzed as described by Tyystjärvi et al. (2001), and PsaA2R and PsaA3R were analyzed as described by Axmann et al. (2005). All primers used to amplify the DNA templates for RNA probes and standard RNA are listed in Table I. RNA probe and standard RNA molecules were prepared with the MAXIscript kit (Ambion) according to the company's instructions. [α - ^{32}P] UTP (Perkin-Elmer) was used for the labeling of probes.

5' and 3' RACE

The 5' end and 3' end RACE was performed according to Argaman et al. (2001). Sequences of RNA adaptors and DNA primers are listed in Table I.

Mutagenesis

Sequences of DNA primers used for mutagenesis are listed in Table I. The ΔpsbA3 mutant was prepared as a parental strain to generate PsaA2R(+) and PsaA2R(-) as well as the control strain. Two DNA fragments, *psbA3*-up and *psbA3*-down, were amplified by PCR with the primer sets $\Delta\text{psbA3_F1}/\Delta\text{psbA3_R1}$ and $\Delta\text{psbA3_F2}/\Delta\text{psbA3_R2}$, respectively. These fragments were treated with *SphI/HindIII* and *HindIII/PstI*, respectively. The spectinomycin resistance cassette was cut out from pHP45 by *HindIII*. pGEM-T vector was treated with *SphI/PstI*, and the fragment containing the ampicillin resistance cassette was extracted. These four fragments were ligated using the T4 DNA ligase (New England Biolabs), and the obtained plasmid was used to generate the ΔpsbA3 mutant by homologous recombination (Golden et al., 1987). The segregation of genomic DNA was confirmed by PCR using a $\Delta\text{psbA3_F3}/\Delta\text{psbA3_R3}$ primer set.

To generate PsaA2R(+) and PsaA2R(-) expression strains, sense or antisense versions of PsaA2R were amplified with the primer sets PsaA2R(+)_F1/PsaA2R(+)_R1 and PsaA2R(-)_F1/PsaA2R(-)_R1, respectively. PsaA2R(-)

_R1 contains the sequence of the *oop* terminator of the bacteriophage λ . On the other hand, the *rnpB* promoter region was amplified with the primer sets *rnpB_F1/rnpB(+)_R1* and *rnpB_F1/rnpB(-)_R1* for *PsbA2R(+)* and *PsbA2R(-)*, respectively. These fragments were fused in a two-step PCR with the primer sets *rnpB_F1/PsbA2R(+)_R1* and *rnpB_F1/PsbA2R(-)_R1*. After digestion with *PstI/SalI*, these fragments were cloned into the *PstI/SalI* double-digested pEX6803 together with the kanamycin resistance cassette obtained by *PstI* digestion of pUC4K. The pEX6803 plasmid was prepared as below for homologous recombination at the *slr2031* region. Segments upstream and downstream of *slr2031* were amplified with the primer sets pEX_F1/pEX_R1 and pEX_F2/pEX_R2, respectively. These fragments were subcloned into pGEM-T vector and cut out by double digestion with *ApaI/KpnI* and *XbaI/NsiI*, respectively, for the following ligation. pDrive and pGEM-T were treated with *KpnI/XbaI* and *ApaI/NsiI* to get the multiple cloning site or ampicillin resistance cassette-containing fragments. Ligation of these four fragments gave pEX6803. The pEX6803 vector containing only the kanamycin resistance cassette was used for generation of the control strain. After homologous recombination, the segregation was checked by PCR with the pEX_F3/pEX_R3 primer set.

Thylakoid Membrane Isolation and Western-Blot Analysis

Thylakoid membranes were isolated from 30 mL of cell culture according to Kashino et al. (2002). Chlorophyll concentration was measured by the method of Arnon et al. (1974).

Western analysis was done by standard procedure using the thylakoid membranes containing 0.5 μg of chlorophyll. The D1 protein and β -subunit of ATPase were detected by the chemiluminescence method. The signal intensities of the D1 protein obtained from western analysis were quantified (NIH ImageJ software) and normalized by the amount of β -subunit of ATPase.

Measurement of PSII Activity

PSII activity was measured with a Clark-type oxygen electrode using 5 μg of chlorophyll-containing cells in the presence of 2 mM 2,6-dimethyl-*p*-benzoquinone and 1 mM potassium ferricyanide at 30°C.

Overexpression and Purification of *Synechocystis* sp. PCC 6803 Recombinant RNase E from *Escherichia coli*

pQESlr1129 (courtesy of M. Asayama; Horie et al., 2007) was transformed in *E. coli* M15 (pREP4) for the expression of recombinant *Synechocystis* sp. PCC 6803 RNase E containing a C-terminal hexa-His fusion. Strains were grown in Lysogeny broth supplemented with ampicillin (100 $\mu\text{g mL}^{-1}$) and kanamycin (25 $\mu\text{g mL}^{-1}$). Overnight cultures were diluted in Lysogeny broth and grown at 37°C to an optical density at 600 nm of 0.5 to 0.8. Following induction of heterologous expression with 1 mM isopropyl β -D-1-thiogalactopyranoside, incubation continued at 22°C for 24 h.

Recombinant *Synechocystis* sp. PCC 6803 RNase E was affinity purified under native conditions according to Stazic et al. (2011).

In Vitro RNase E Assay

In vitro cleavage assays were carried out according to Stazic et al. (2011) with the following modifications. The reaction mixture, containing 13 pmol of single-stranded RNA and 26 pmol of duplex RNA, was buffered with 25 mM Tris-HCl (pH 7.6). Transcripts for RNase E cleavage assays were synthesized with the MEGAShortsript system (Ambion) according to the manufacturer's instructions. Removal of residual template DNA was conducted by incubating reaction mixtures with 2 units of TURBO DNase (Ambion) at 37°C for 15 min. Subsequently, RNA was purified by phenol-chloroform extraction and quantified with a NanoDrop ND-1000 spectrofluorometer (PEQLAB Biotechnology). The following in vitro RNAs were synthesized: *psbA2-110*, *psbA2* asRNA-74, and *psbA2* asRNA-81. Oligonucleotides used for template generation are listed in Table I.

ACKNOWLEDGMENTS

We thank Munehiko Asayama (Ibaraki University) for providing plasmid pQESlr1129.

Received June 19, 2012; accepted July 29, 2012; published August 2, 2012.

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