Post-transcriptional regulation of the *psbA* gene family in the cyanobacterium *Synechococcus* sp. PCC 7942

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Abstract In the cyanobacterium *Synechococcus* sp. PCC 7942, the photosystem II reaction center protein D1 is encoded by three *psbA* genes. The *psbAI* gene encodes the D1:1 protein that is the prevailing form under steady state conditions, whereas the expression of the *psbAII* and *psbAIII* genes, encoding the D1:2 protein, is enhanced under many stress conditions. Here, we show that in addition to transcriptional control, the synthesis of D1 protein forms is regulated at the levels of membrane targeting of *psbA* mRNA ribosome complexes and translation elongation, whereas the formation of translation initiation complexes does not have a significant regulatory role.

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Keywords: Translation; Photosystem II; D1 protein; Light stress

1. Introduction

Photosystem II (PSII) is a large pigment-protein complex performing light-driven oxidation of water and reduction of plastoquinone in the thylakoid membranes of cyanobacteria and plants. The PSII complex is damaged in the light, the rate of damage directly depending on light intensity [1]. The concurrent repair of the PSII complex consists of degradation of the damaged D1 protein and insertion of a newly synthesized D1 protein copy into the PSII complex [2,3]. The presence of two different forms of the D1 protein further complicates the repair cycle in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (referred to here as *Synechococcus*) [4].

The two forms of the D1 protein in *Synechococcus* differ by 25 amino acid residues [4]. The D1:1 protein, encoded by the *psbAI* gene, is the predominant form under standard growth conditions (32 °C, 40 μmol photons m^{-2} s^{-1}), while many stress conditions induce an exchange of the D1:1 protein to D1:2 protein that is encoded by *psbAII* and *psbAIII* genes [5–7]. Under mild stress conditions, the exchange to D1:2 protein is only transient [8] but under more severe stress, only the D1:2 protein can be found in the thylakoid membrane [9,10].

The replacement of the damaged D1 protein in PSII is a tightly regulated process and free D1 copies do not accumulate [11]. D1 protein is translated on membrane-bound ribosomes as a 34 kDa precursor form [12]. Insertion of D1 protein into the thylakoid membrane and its assembly with other PSII proteins occurs co-translationally [13–16]. In chloroplasts, the initiation of *psbA* mRNA translation is a key step regulating D1 protein synthesis [17–19]. The rate of *psbA* mRNA translation elongation, in turn, varies according to environmental conditions both in cyanobacteria [16] and in chloroplasts [15,20,21].

The exchange of the D1 protein forms in PSII, induced by environmental cues, is regulated at the level of transcription in *Synechococcus*. During acclimated growth, the *psbAI* gene produces the majority of *psbA* mRNAs [4] whereas high light [5,22], low temperature [23], anoxia [24] and UV-light [7] upregulate transcription of the *psbAII/III* genes. The involvement of regulatory levels other than transcription was suggested by data showing that almost no D1:1 proteins accumulated in thylakoid membranes after long high-light treatments or exposure of cells to UV-light [7,9,25,26], despite the presence of high amounts of *psbA1* mRNA. Furthermore, the thylakoid membranes lack the D1:2 protein under anoxia [24] and when the cells are treated with an photosynthetic electron transfer inhibitors under thiol reducing conditions [10], even though the *psbAII/III* messages do accumulate. Here, we report the operation of translational regulation in the expression of *psbA* genes in *Synechococcus* under stress conditions.

2. Materials and methods

Synechococcus cells were grown as described previously [26]. The cells were subjected to high-light stress by illuminating for 5, 30, 60 or 180 min under 400 μmol photons m^{-2} s^{-1} using a slide projector as a light source. For some experiments, as indicated, the growth medium was supplemented with 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 5 mM 1,4-dithiothreitol (DTT) just before the onset of high-light illumination. A temperature shift to 18 °C was performed under constant illumination at 40 μmol photons m^{-2} s^{-1}.

The membrane and the cytosolic fractions of the cells as well as the membrane-bound and free ribosomes were isolated according to [16]. Total RNA and RNA from different cell fractions were isolated as in [16]. RNAs were separated on 1.2% agarose-glyoxal gels and subsequently transferred to Hybond-N membrane (Amersham) according to standard procedures [27]. For total RNA determination, 5 μg of RNA was loaded per lane. In studies of different cell fractions, all RNA rescued from one fraction (starting with cells containing 50 μg chlorophyll) was loaded per lane. Northern blot hybridization was performed using *psbAI* and *psbAII/III* specific 5′ end probes as described in [10].

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DTT, 1,4-dithiotreitol; PSII, Photosystem II

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Thylakoid isolation and immunodetection of the D1 protein by Western blotting was done as described in [10]. Antibodies raised against the first 22 amino acid residues of the N-termini of the D1:1 and D1:2 proteins were used as the D1 protein form-specific antibodies [11].

3. Results

Translation of *psbA* mRNAs begins on cytosolic ribosomes, followed by targeting of the ribosome-nascent D1 polypeptide chain complexes to the membranes, where the D1 polypeptide is co-translationally inserted into the membrane and assembled into the PSII complex [12,14,28]. To study the possible translational regulation, we first analyzed the distribution of *psbAI* and *psbAII/III* mRNAs in the cytosolic and membrane fractions of *Synechococcus* cells under different environmental conditions.

Under standard growth conditions, the abundant *psbAI* mRNAs were equally distributed to the membrane and cytosolic fractions of the cell and only a low amount of *psbAII/III* mRNAs was detected in both fractions (Fig. 1A). The high amount of membrane-bound *psbAI* mRNA and low amount of membrane-bound *psbAII/III* mRNA were compatible with the high amount of D1:1 protein and low amount of D1:2 protein in the thylakoid membrane under the standard growth conditions (Fig. 1).

Transfer of *Synechococcus* cells from growth light to high light induced a rapid and permanent induction of *psbAII/III* mRNAs but only transient downregulation of *psbA1* mRNA (Fig. 1B). A 7-fold increase in the amount of membrane-bound *psbAII/III* mRNA was measured within 5 min of high-light illumination and the level increased gradually during the entire high-light treatment reaching finally, after 3 h of illumination, at least 20 times higher level than under the standard growth conditions (Fig. 1A). In the cytosolic fraction, the amount of *psbAII/III* mRNA increased throughout the high-light treatment. The *psbAI* mRNAs, in turn, gradually disappeared from the membrane fraction upon high-light illumination: the amount of membrane-bound *psbAI* mRNA was approximately 90% and 10% of that measured under the acclimated growth conditions after 5 and 30 min of high-light treatments, respectively. The amount of membrane-bound *psbAI* mRNA remained low also in 1 h sample (Fig. 1A), and in accordance with the low amount of *psbAI* mRNA in the membrane fraction, only traces of the D1:1 protein were detected in thylakoid membranes after 1 h of high-light illumination (Fig. 1C). After prolonged high-light treatment (3 h) the amount of the D1:1 protein remained very low (Fig. 1C), although the *psbAI* mRNAs were as abundant as under the standard growth conditions, and 70% of *psbAI* mRNAs were detected from the membrane fraction (Fig. 1A).

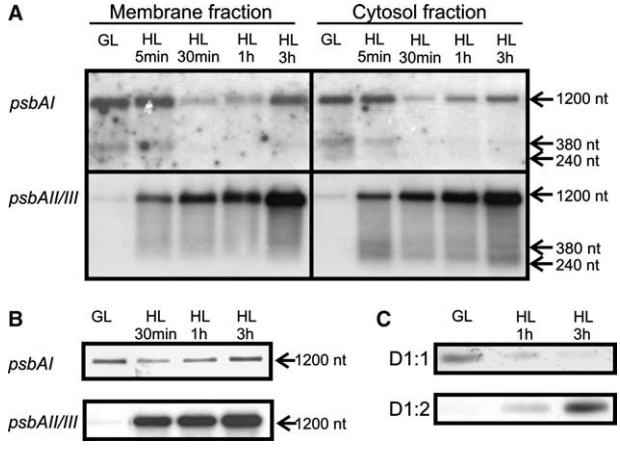

Fig. 1. High-light induced changes in the distribution of the psbAI and psbAII/III mRNAs between the membrane and cytosolic fractions of the cells, and in the amounts of the D1:1 and D1:2 proteins in the thylakoid membrane. (A) Distribution of the *psbAI* and *psbAII/III* mRNAs on cytosolic and membrane fractions at acclimated growth light conditions (GL, 40 μmol photons m^{-2} s^{-1}) and after 5 min, 30 min, 1 h and 3 h high light (HL) illumination at 400 μmol photons m^{-2} s^{-1}. Northern blots were probed with *psbA1* and *psbAII/III* gene-specific probes. (B) The total amount of *psbAI* and *psbAII/III* mRNAs at acclimated growth light and after high light illumination of 30 min, 1 h and 3 h. (C) Western blot of the D1:1 and D1:2 proteins in membranes isolated from cells acclimated to standard growth-light conditions and from cells treated for 1 h and 3 h under high light.

The membrane fraction was next studied in more detail to find out whether the membrane-bound *psbAI* mRNAs are attached to ribosomes and thus under translation. To that end, we first isolated the membrane fraction and, after solubilization with 2% polyoxyethylene 10 tridecyl ether, separated the free and ribosome-attached mRNAs by centrifugation through a sucrose cushion. No free *psbAI* mRNA was detected in the membrane fraction, but instead all *psbAI* mRNA was detected in ribosome pellets (Fig. 2), suggesting that all membrane-bound *psbAI* mRNA was translationally active.

The cytosolic fraction accommodated approximately half of the *psbAI* and *psbAII/III* mRNAs both under the growth-light and high-light conditions (Fig. 1A). Since in plant chloroplasts the translation initiation of *psbA* mRNA is strictly regulated [17–19], we asked whether it is likewise regulated in *Synechococcus*. Thus, the free and ribosome-attached RNAs were separated from the cytosolic fraction and the amounts of different *psbA* mRNA species were detected by Northern blotting. All full-length *psbAI* and *psbAII/III* mRNAs were found attached to ribosomes (Fig. 3), suggesting their employment in translation.

In addition to full-length *psbA* mRNAs, we also detected 240 nt and 380 nt long *psbA* mRNA species (Fig. 3). The shortest *psbA* mRNA fragments of 240 nt were detected only free in the cytosol, while the longer 380 nt fragments were present in ribosome pellets (Fig. 3). It is therefore likely that

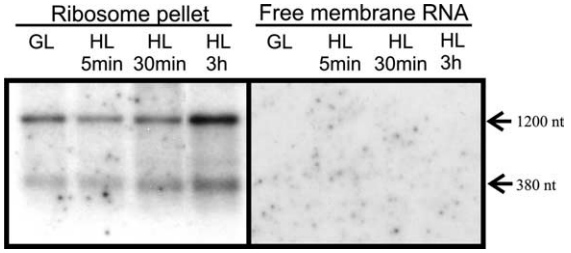

Fig. 2. Association of the membrane bound *psbAI* mRNA with ribosomes under different light conditions. The membrane fraction of *Synechococcus* cells was isolated from growth-light-acclimated cells (GL) and from cells illuminated under high light (HL) for 5 min, 30 min and 3 h. Thereafter, the ribosome-associated and free RNA species were separated and subjected to Northern blot analysis using the *psbAI* specific probe.

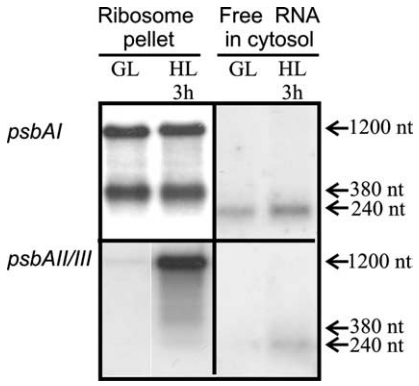

Fig. 3. Analysis of the *psbA* mRNA species in the cytosolic fraction of *Synechococcus* cells under different light conditions. *Synechococcus* cells were kept in the growth light (GL) or illuminated under high light (HL) for 3 h. After light treatments, the cytosolic fraction of the cells was isolated and the ribosome bound as well as free RNA species were separated by centrifugation through sucrose cushion. The amount of p*sbAI* and *psbAII/III* mRNAs was detected in each fraction by Northern blotting using gene-specific probes.

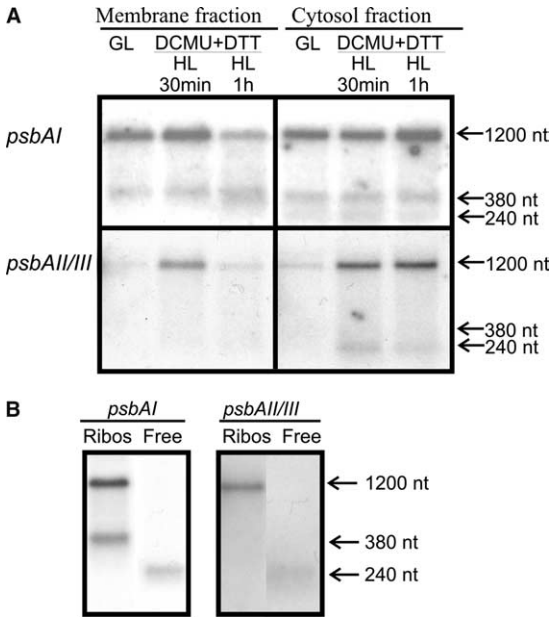

Fig. 4. Distribution of *psbA* mRNAs between the membrane and cytosolic fractions under high-light conditions in the presence of DCMU and DTT. (A) *Synechococcus* cell cultures at acclimated growth conditions were supplemented with 10 μM DCMU and 5 mM DTT upon transferring the cells to high light for 30 min and 1 h. After treatments, the membrane and cytosolic fractions were isolated, and the amount of *psbAI* and *psbAII/III* mRNAs in each fraction was measured by Northern blotting using gene specific probes. (B) *Synechococcus* culture was supplemented with 10 μM DCMU and 5 mM DTT and illuminated under high light conditions for 1 h. The cytosolic fraction was isolated and ribosome-attached (ribos) and free (free) RNAs were separated by sucrose cushion centrifugation. The amounts of *psbAI* and *psbAII/III* mRNAs were measured by Northern blot technique using gene-specific probes.

the ribosomes attach to nascent *psbAI* and *psbAII/III* mRNAs soon after the first 240 nt have been transcribed.

It was previously shown that the high-light induced exchange of the D1:1 form to D1:2 form was prevented, despite the accumulation of *psbAII/III* mRNA, if the cells were supplied with a PSII electron transfer inhibitor DCMU and a thiol-reductant DTT [10]. These conditions, leading to a net loss of the D1 protein, were used to further investigate the regulation of *psbA* mRNA translation. During 1 h treatment the amount of *psbAI* mRNA remained high but the membrane-bound fraction decreased to 10% of that measured before the treatment (Fig. 4A). High-light treatment in the presence of DCMU and DTT first induced *psbAII/III* mRNAs in membrane fraction, but after 1 h treatment hardly any *psbAII/III* mRNAs were detected in the membrane fraction (Fig. 4A). To find out whether the abundant *psbA* mRNAs in the cytosolic fraction were bound to ribosomes, we separated the free and ribosome-attached mRNAs. As demonstrated by a Northern blot (Fig. 4B), all full length *psbAI* and *psbAII/III* mRNAs, and also the 380 nt long *psbA* fragments, were attached to ribosomes and only the short 240 nt *psbA* fragments were detected free in cytosol. These results indicate that the DCMU/DTT treatment did not perturb the initiation of translation but instead the membrane targeting of the *psbA* mRNA ribosome complexes was hampered.

To find out whether the efficiency of the membrane targeting of the *psbA* mRNA ribosome complexes has any regulatory role under more physiological conditions, we measured the distribution of the *psbA* mRNAs to cytosolic and membrane fractions after treatment of cells at low temperature. Previous studies have demonstrated that the transfer of *Synechococcus* cells from acclimated growth temperature (32 °C) to 18 °C induces a severe loss of the D1 protein from the thylakoid membrane, although the total amount of *psbA* mRNAs remains high [23]. As shown by the Northern blot (Fig. 5), the transfer of *Synechococcus* cells from 32 to 18 °C downregulated the expression of *psbAI* gene and simultaneously upregulated the expression of *psbAII/III* genes. Fractionation of *Synechococcus* cells to membrane and cytosolic fractions, however, indicated that after 5 h of cold treatment only 15% of *psbAII/III* mRNAs were targeted to the membranes, and the amount of *psbAI* mRNA in the membrane fraction was below the detection limit (Fig. 5).

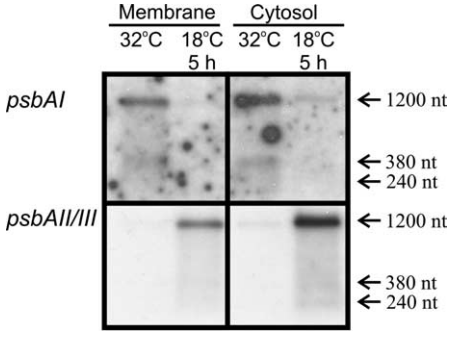

Fig. 5. Low temperature induced changes in the membrane bound and cytosolic *psbAI* and *psbAII/III* mRNAs in *Synechococcus* cells. *Synechococcus* cells were transferred from acclimated growth conditions (32 °C, 40 μmol photons m^{-2} s^{-1}) to low temperature (18 °C, 40 μmol photons m^{-2} s^{-1}) and incubated for 5 h. After treatments, the cells were fractionated to membrane and cytosolic fractions and the RNAs were isolated from each fraction. The amounts of the *psbAI* and *psbAII/III* mRNAs were measured by Northern blotting using gene-specific probes.

4. Discussion

In addition to the mature *psbAI* and *psbAII/III* mRNAs of 1200 nt, the gene-specific 5′ end *psbAI* and *psbAII/III* probes detected some shorter *psbA* mRNA species. The shortest ones, 240 nt long fragments, were detected only in the cytosolic fraction (Figs. 1,3–5) as a free RNA (Figs. 3 and 4). It is conceivable that the 240 nt fragments are transcription intermediates that are not yet associated with ribosomes. The 380 nt long *psbAI* fragments, on the contrary, were detected only in the ribosome pellets (Figs. 2–4), indicating that ribosomes are efficiently attached to the *psbA* mRNAs as soon as one-third of the *psbA* genes has been transcribed. Besides the 380 nt fragments, all mature *psbA* mRNAs were found to be associated with ribosomes (Figs. 2–4), suggesting that the formation of the translation initiation complex does not have a significant regulatory role in the expression of *psbA* genes in *Synechococcus*. We have earlier shown with another cyanobacterium, *Synechocystis* sp. PCC 6803, that the initiation of *psbA* mRNA translation is not under specific regulatory control [16]. Contrary to cyanobacteria, in plants and algae the initiation of *psbA* mRNA translation is strictly regulated [17–19]. This regulatory difference between cyanobacteria and chloroplasts can be linked to half-lives of their *psbA* mRNAs: the amount of short-lived *psbA* mRNAs in cyanobacteria varies according to environmental conditions [4,9,10], whereas the *psbA* mRNAs in chloroplasts are long-lived [29] and their amounts hardly respond to changes in environmental conditions [12,21].

Targeting of the 380 nt long transcription intermediates to the thylakoid membrane allows speculation of the domains of D1 polypeptide necessary for membrane targeting. Given the length of 380 nt for the *psbA* mRNA, and taking into consideration that the untranslated leader region of *psbA* mRNA is ca. 100 nt [4] and the RNA polymerase buries 25 nt, only 255 nt remains available for translation. This allows production of a nascent polypeptide chain of 85 amino acids. During translation, 25–40 amino acids reside inside the ribosome tunnel [30], which leaves 45–60 amino acids that can be out of the ribosome. The polytopic D1 protein contains 5 membrane spanning α-helices, the first two α-helices comprising amino acids 36–57 and 109–137 [31]. Thus, the 380 nt long *psbA* mRNA can produce a nascent D1 polypeptide in which the first α-helix has just emerged from the ribosome. In line with this interpretation, the first trans-membrane segment of the protein has been shown to function as a signal sequence for polytopic membrane proteins of the inner membrane in other bacteria [32].

Current knowledge of the mechanisms involved in membrane targeting and insertion of the D1 protein in cyanobacteria is scarce. A recent study with *E. coli* [33], showing significant variability in the biogenesis of different inner membrane proteins, suggests that mechanisms learned from the translation of the other membrane proteins might not be directly adapted to the D1 protein. In chloroplasts, both SRP [34] and the SecY translocon channel [28] have been shown to be involved in membrane targeting and insertion of the nascent D1 protein, and it is likely that those protein translocation components are also involved in cyanobacteria. In the present study, we demonstrated that the DCMU/DTT treatment (Fig. 4) hinders the membrane targeting of nascent D1:2 polypeptide ribosome complexes. In accordance with this result, *in organello* studies in chloroplasts have shown that DCMU induces almost complete inhibition of D1 polypeptide elongation that can be only partially restored by addition of DTT [15], indicating that active photosynthetic electron transfer is required for optimal translation elongation of *psbA* mRNA. Besides active photosynthetic electron transfer, a formation of trans-thylakoid proton gradient is also a prerequisite for efficient translation elongation of the *psbA* mRNA [15,35]. Studies showing the involvement of other PSII proteins as assembly partners for the nascent D1 polypeptide [14,36] demonstrate further complexity of the membrane insertion of the D1 protein.

Synechococcus cells almost completely lose their PSII activity during a 24 h incubation at 18 °C because the D1:1 protein is degraded but the cells are unable to incorporate D1:2 proteins into the PSII centers, despite high amounts of *psbAII/III* mRNAs in the cell [23]. Here, we have demonstrated that the low temperature treatment hampers the accumulation of nascent D1:2 polypeptide ribosome complexes in the thylakoid membrane (Fig. 5). Further studies, however, are required to resolve which specific steps of membrane targeting are actually malfunctioning. These steps may include the recognition of the nascent D1 polypeptide chain by translocation machinery, translation elongation of the *psbA* mRNA, and/or the co-translational insertion of the nascent D1 polypeptide chain into the thylakoid membrane. Interestingly, *Synechococcus* mutant strains capable of producing 16:2 diunsaturated fatty acids [37] were able to accumulate D1:2 proteins into PSII reaction centers also under low temperature [23]. This suggests that the membrane lipids and their level of fatty acid unsaturation have a crucial role in the co-translational insertion of polytopic membrane proteins.

After prolonged high-light illumination of *Synechococcus* cells, the D1:1 protein almost completely vanished from the thylakoid membrane although the *psbAI* mRNAs were abundant (Fig. 1). Our analysis shows that the translation initiation of *psbAI* mRNAs still occurs efficiently (Fig. 2) and the ribosome complexes containing nascent D1:1 polypeptides are targeted to the membranes (Figs. 1 and 3). Therefore, the paucity of D1:1 protein in the thylakoid membrane either results from problems in co-translational membrane-insertion of the D1:1 polypeptide or from exceptionally high susceptibility of the D1:1 protein to degradation under the high-light conditions. The measured half-lives D1:1 and D1:2 proteins [9,38] under high-light were too similar to account for the absence of the D1:1 protein in the thylakoid membrane upon high light treatment. It is therefore conceivable that the malfunctioning of the membrane insertion of the nascent D1:1 polypeptide contributes to the scarcity of the D1:1 protein under high light conditions.

In conclusion, the present study indicates that: (i) transcriptional regulation of *psbA* genes in *Synechococcus* is supplemented with the regulation at the level of translation, (ii) the *psbA* mRNAs are efficiently associated with ribosomes thus suggesting that the formation of translation initiation complexes does not have a significant regulatory role in the synthesis of different D1 protein forms, (iii) the membrane targeting of nascent D1 protein ribosome complexes is a rate limiting step for D1 protein synthesis in *Synechococcus* cells under some stress conditions and (iv) the high-light induced discrimination of the D1:1 protein occurs after the membrane targeting of the nascent D1:1 polypeptide ribosome complex.

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