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ADP-dependent phosphorylation regulates RNA-binding *in vitro*: implications in light-modulated translation

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Light-regulated translation of chloroplastic mRNAs in the green alga *Chlamydomonas reinhardtii* requires nuclear encoded factors that interact with the 5′-untranslated region (5′-UTR) of specific mRNAs to enhance their translation. We have previously identified and characterized a set of proteins that bind specifically to the 5′-UTR of the chloroplastic *psbA* mRNA. Accumulation of these proteins is similar in dark- and light-grown cells, whereas their binding activity is enhanced during growth in the light. We have identified a serine/threonine protein phosphotransferase, associated with the *psbA* mRNA-binding complex, that utilizes the β-phosphate of ADP to phosphorylate and inactivate *psbA* mRNA-binding *in vitro*. The inactivation of mRNA-binding *in vitro* is initiated at high ADP levels, levels that are attained *in vivo* only in dark-grown chloroplasts. These data suggest that the translation of *psbA* mRNA is attenuated by phosphorylation of the mRNA-binding protein complex in response to a rise in the stromal concentration of ADP upon transfer of cells to dark.

Key words: ADP-dependent phosphorylation/chloroplast/light-regulated gene expression/RNA-binding/serine-threonine protein phosphotransferase/translational regulation

Introduction

The D1 protein of photosystem II (PSII) is a reaction center protein that participates directly in photosynthetic charge separation (Prasil *et al.*, 1992). This key function of the D1 protein leads to its rapid inactivation and hence a high turnover during photosynthesis (Mattoo *et al.*, 1981; Wettern and Ohad, 1984). As such, the D1 protein has been shown to be the most highly synthesized protein in illuminated photosynthetic cells (Bottomley *et al.*, 1974; Mattoo *et al.*, 1981; Wettern and Ohad, 1984). Considering the high energy cost that is committed to the production of D1 protein in photosynthesizing cells, it is not surprising that expression of D1 protein, encoded by the *psbA* mRNA, is tightly regulated during light- and dark-growth phases of the cell. A 50- to 100-fold increase in D1 synthesis has been observed upon illumination (Fromm *et al.*, 1985; Klein *et al.*, 1988; Malnoë *et al.*, 1988; Krupinska and Apel, 1989). Interestingly, the abundance of *psbA* mRNA is invariant during light- or dark-growth in mature chloroplasts (Fromm *et al.*, 1985; Deng and Gruissem, 1988; Malnoë *et al.*, 1988; Mullet, 1988), indicating that translational regulation of *psbA* mRNA plays a major role in the observed activation of D1 synthesis. Genetic analysis in the unicellular green alga *Chlamydomonas reinhardtii* has identified nuclear encoded factors that positively regulate translation of chloroplastic mRNAs (Jensen *et al.*, 1986; Kuchka *et al.*, 1988; Rochaix *et al.*, 1989; Girard-Bascou *et al.*, 1992) and has identified the 5′-untranslated region (5′-UTR) of chloroplastic mRNAs as the target for interaction with these nuclear encoded translational activators (Rochaix *et al.*, 1989).

We have identified a protein complex that binds specifically to a stem-loop RNA structure located in the 5′-UTR of the *psbA* mRNA (Danon and Mayfield, 1991). This stem-loop RNA structure contains the ribosome-binding site which is located adjacent to and upstream of the initiation codon. Binding of this protein complex to the *psbA* mRNA correlates with the level of translation in light- and dark-grown cells and in the *y*-1 mutant cell line, which does not translate *psbA* mRNA in the dark. A minimal *psbA* mRNA-binding complex consists of two proteins, one of 47 kDa and the other of 60 kDa. The 47 kDa protein binds directly to the *psbA* mRNA while the 60 kDa protein appears to have only a minor contact with the RNA molecule. Two additional proteins were found to associate with the *psbA* mRNA-binding protein complex following isolation of proteins using *psbA* mRNA affinity chromatography. These proteins accumulated in dark-grown cells but exhibited reduced RNA affinity when compared with proteins isolated from light-grown cells (Danon and Mayfield, 1991). The observed correlation between the binding capacity of the *psbA* mRNA-binding protein complex in light- and dark-grown cells and the translation efficiency of *psbA* mRNA under the same conditions suggests that modulation of the binding of this protein complex to the 5′-UTR stem-loop RNA structure of *psbA* mRNA may enhance ribosome-binding and initiation of translation (Danon and Mayfield, 1991). To understand the mechanism by which translation is modulated in response to light, we have examined the regulation of *psbA* mRNA-protein complex formation *in vitro* to identify biochemical events within the chloroplast which may be utilized in the signal transduction from light to translational activation.

The regulation of enzymes involved in carbon dioxide assimilation ('dark reaction') has been shown to be linked to photosynthetic electron transport ('light reaction') via two main regulatory pathways. One pathway utilizes the redox potential generated by the 'light reaction' and transmitted by ferredoxin, ferredoxin-thioredoxin reductase and thioredoxin to affect regulatory sulfhydryls in target enzymes (Buchanan, 1991). The second regulatory cascade involves modulation of photosynthetic enzyme activity by protein phosphorylation (Burnell and Hatch, 1985). To test whether light-regulated translation in the chloroplast is controlled by the same mechanisms that link the 'light' and 'dark reactions', we have investigated the regulation of binding activity of the *psbA* mRNA-binding protein complex *in vitro*

by either redox potential or phosphorylation. Here, we show that phosphorylation of the 60 kDa *psbA* mRNA-binding protein, by an ADP-dependent protein phosphotransferase associated with *psbA* mRNA-binding protein complex, abolishes *psbA* mRNA-binding activity *in vitro*. The implication of the ADP-dependent protein phosphorylation to light-modulated translational regulation is discussed.

Results

ATP abolishes psbA mRNA-binding activity in vitro

To investigate the regulatory pathways by which the translation of *psbA* mRNA is modulated, we have examined the effect of products of the 'light reaction' of photosynthesis (ATP and NADPH) on *psbA* mRNA-binding activity *in vitro*. To test their effect, the different compounds were incubated with protein fraction, containing the *psbA* mRNA-binding protein complex, isolated from light-grown *C.reinhardtii* cells. Following treatment, the proteins were incubated with radioactively labeled 5′-UTR of *psbA* mRNA to form *psbA* mRNA−protein complexes. Unprotected regions of the RNA were digested with RNase T1 and the mixture was fractionated using native polyacrylamide gel electrophoresis. The presence of *psbA* mRNA-binding activity was revealed following electrophoresis by mobility shift of the protected RNA fragment caused by the formation of *psbA* mRNA−protein complex. As shown in Figure 1A, supplementing the protein fraction with 1 mM ATP (final concentration) completely abolished the *psbA* mRNA-binding activity as detected by the RNase T1 gel mobility shift (T1-GMS) assay. Incubation of the protein complex with other ribonucleoside triphosphates, such as GTP, CTP and UTP, did not affect the RNA-binding activity (Figure 1B), showing that inactivation of *psbA* mRNA-binding activity is not due to a general effect of nucleoside triphosphates. The inclusion of up to 3 mM NADPH, the second product of the 'light reaction' in photosynthesis, or its precursor NADP, in the binding assay did not affect the RNA-binding activity (Figure 1C), indicating that the inactivation process was not responsive to all products of the 'light reaction' of photosynthesis.

ATP-induced inactivation of RNA-binding is non-reversible and requires hydrolysis of a high energy phosphate bond

Inactivation of RNA-binding activity by ATP could involve the use of ATP as an enzyme substrate or could involve direct association of ATP with the RNA-binding protein complex to alter its RNA-binding capacity. To differentiate between these possibilities, we have tested both the reversibility of the inactivation upon removal of ATP from the milieu, and the requirement for hydrolysis of a high energy phosphate bond. If ATP is utilized enzymatically to modify proteins then the inactivation of RNA-binding activity should not be relieved upon removal of the ATP from the reaction mix. To test whether the inactivation by ATP is permanent, proteins were incubated with 1 mM ATP for 15 min and then dialyzed against 20 000 volumes of ATP-free buffer for 4 h. As shown in Figure 2A, inactivation of *psbA* mRNA-binding activity by ATP was found to be resistant to dialysis, indicating that the loss of RNA-binding by ATP treatment was permanent. No differences in RNA-binding activity were detected following dialysis of the untreated protein fraction, showing that dialysis *per se* did not inactivate RNA-binding.

If inactivation by ATP was due to direct association with the RNA-binding complex, then non-hydrolyzable analogs of ATP should substitute for ATP in the inactivation of the RNA-binding activity. To test the alternative requirement for the hydrolysis of a high energy phosphate bond for the inactivation of the *psbA* mRNA-binding activity, we

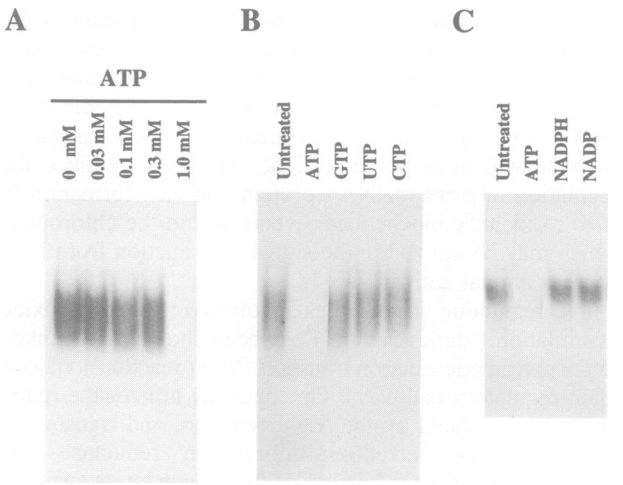

Fig. 1. ATP-mediated inactivation of *psbA* mRNA-binding activity. Protein fractions containing *psbA* mRNA-binding activity were treated with various nucleotides, incubated with ^{32}P-labeled 5′-UTR of *psbA* mRNA and subjected to T1-GMS. (**A**) Proteins incubated with increasing concentrations of ATP. (**B**) Proteins incubated with either ATP, GTP, UTP or CTP. (**C**) Proteins incubated with either ATP, NADPH or NADP. Untreated lanes contain proteins that were subjected to T1-GMS with no prior treatment.

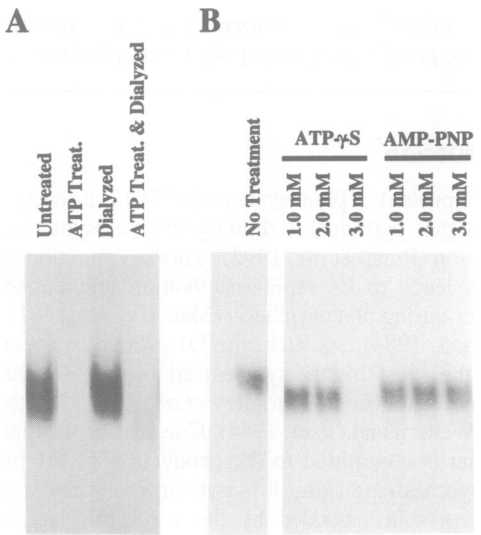

Fig. 2. Characterization of ATP-mediated inactivation of *psbA* mRNA-binding activity. Protein fractions containing *psbA* mRNA-binding activity were treated with ATP or its non-hydrolyzable analogs, ATP-γ-S or AMP-PNP, incubated with ^{32}P-labeled 5′-UTR of *psbA* mRNA, and subjected to T1-GMS. (**A**) Untreated or ATP-treated proteins were dialyzed against 20 000 volumes of ATP-free buffer for 4 h and their RNA-binding activity was analyzed and compared with that of non-dialyzed proteins. (**B**) Proteins incubated with increasing concentrations of ATP-γ-S or AMP-PNP. Untreated lanes contain proteins that were subjected to T1-GMS with no prior treatment.

challenged the protein fraction containing *psbA* mRNA-binding activity with either adenylyl-imidodiphosphate (AMP-PNP) or adenosine thiotriphosphate (ATP-γ-S) and then assayed for *psbA* mRNA-binding capacity (Figure 2B). AMP-PNP had no effect on the RNA-binding activity at any concentration tested, whereas ATP-γ-S inactivated the RNA-binding activity only at concentrations higher than are required for the inactivation by ATP. It has been shown that several enzymes, such as RecA (Yu and Egelman, 1992), phosphorylase kinase (Gratecos and Fischer, 1974), or rhodopsin kinase (Palczewski *et al.*, 1990) were capable of hydrolyzing the γ-S bond, albeit at a slower rate than the native γ-phosphate bond. Therefore, the hydrolysis of a high energy phosphate bond is required for the permanent inactivation of the *psbA* mRNA-binding activity.

The inactivation by ATP can be transiently inhibited by pre-incubation with RNA

To study the kinetics of inactivation by ATP, proteins were incubated with ATP for 1, 5, 10 or 20 min, either prior to or following the addition of saturating levels of RNA. To verify that the detected level of RNA-binding was due to the ATP treatment and was not hampered by an insufficient length of incubation with RNA, we first determined the kinetics of RNA-binding in the absence of ATP. Comparison of untreated proteins incubated with RNA for 1 min ('Untreated, 1′ RNA incubation') or 20 min ('Untreated, 20′ RNA incubation') revealed that RNA-binding was very rapid, as 1 min was sufficient for complete binding and no additional RNA was bound following an additional 20 min incubation (Figure 3A). When proteins were incubated with ATP prior to the addition of RNA, the inactivation of RNA-binding was as fast as could be measured under these conditions, and was maintained for at least 20 min (Figure 3A). Conversely, when protein lysates were incubated with RNA prior to the addition of ATP, a transient (~10 min) inhibition of the inactivation of RNA-binding was observed (Figure 3B). This inhibitory effect suggested that binding to the RNA transiently protected the RNA-binding protein complex from inactivation.

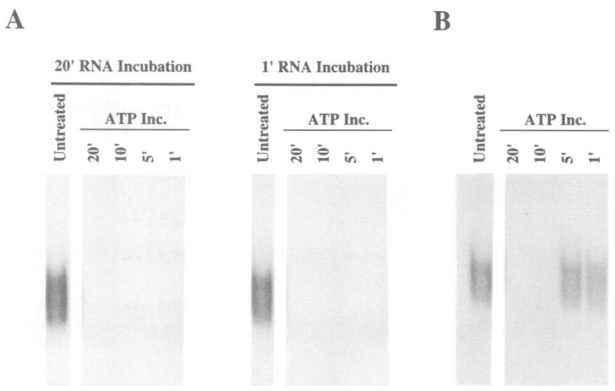

Fig. 3. Analysis of the time required for ATP-mediated inactivation of *psbA* mRNA-binding. Protein fractions containing *psbA* mRNA-binding activity were incubated with ^{32}P-labeled 5′-UTR of *psbA* mRNA and subjected to T1-GMS. (**A**) Proteins were first treated with ATP for the indicated time periods and then incubated with labeled RNA for either 20 min or for 1 min. (**B**) Proteins were incubated with labeled RNA for 5 min and then treated with ATP for the indicated time periods. Untreated lanes contain proteins that were subjected to T1-GMS with no prior treatment.

ADP also inactivates the RNA-binding activity in vitro

To test the effect of ADP, usually an inhibitor of phosphorylation, on the *psbA* mRNA-binding activity, we pre-incubated the proteins with 1 mM ADP and checked the mRNA-binding activity. Surprisingly, incubation with ADP inactivated the *psbA* mRNA-binding activity, as well (Figure 4A). AMP and the other three ribonucleoside diphosphates (CDP, GDP and UDP) did not inactivate the RNA-binding even at concentrations up to 3 mM (Figure 4A and C). To compare the concentrations of ADP that are required for the inactivation with those of ATP, we analyzed the effect of incubation with increasing levels of ATP or ADP. Interestingly, the inactivation of the RNA-binding by ADP required a lower concentration than was needed for inactivation by ATP (Figure 4B), as <0.6 mM final concentration of ADP was sufficient for complete inactivation, suggesting that ADP was utilized more efficiently than ATP in the inactivation phenomenon. The transient inhibitory effect of pre-incubation with RNA on inactivation of mRNA-binding (Figure 3A) allowed us to compare the kinetics of the ATP- and the ADP-mediated inactivation. When proteins were pre-incubated with RNA, inactivation of RNA-binding by ADP (Figure 4D) occurred

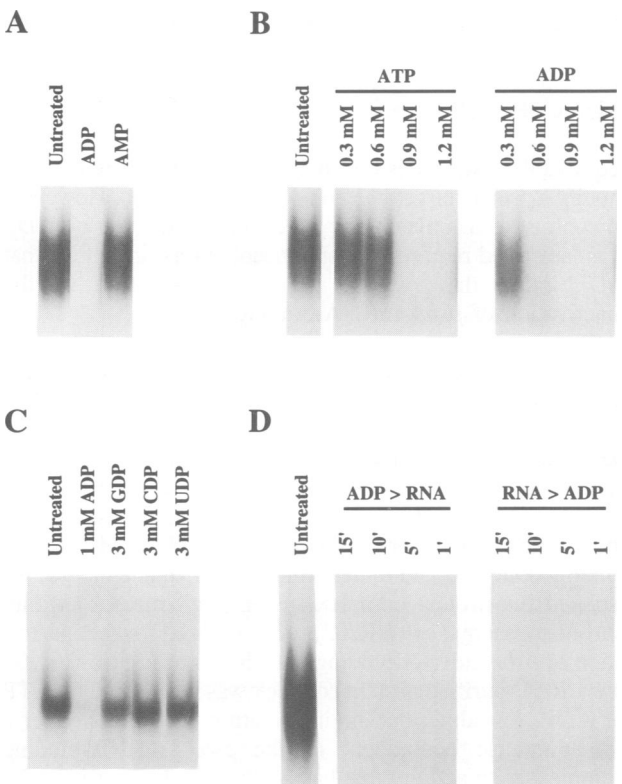

Fig. 4. Characterization of ADP-mediated inactivation of *psbA* mRNA-binding activity. Protein fractions containing *psbA* mRNA-binding activity were treated with either ADP, ATP, AMP or the other nucleoside diphosphates, incubated with ^{32}P-labeled 5′-UTR of *psbA* mRNA and subjected to T1-GMS. (**A**) Proteins treated with either 1 mM ADP or 3 mM AMP. (**B**) Proteins treated with increasing concentrations of either ATP or ADP. (**C**) Proteins incubated with 1 mM ADP, or 3 mM GDP, CDP or UDP. (**D**) Proteins were either first treated with ADP for increasing time periods and then incubated with labeled RNA for 5 min (ADP>RNA) or were first incubated with labeled RNA for 5 min and then treated with ADP for increasing time periods (RNA>ADP). Untreated lanes contain proteins that were subjected to T1-GMS with no additional treatment.

at a faster rate than the inactivation by ATP (Figure 3B). Assuming, that the rate of RNA−protein dissociation was the same for RNA−protein complexes incubated in the presence of ATP or ADP, than the faster rate of ADP-mediated inactivation again demonstrated that ADP was used more efficiently than ATP and indicated that ADP is the preferred substrate for the inactivation of *psbA* mRNA-binding. To verify that ADP was not converted to ATP by an endogenous adenylate kinase activity we performed ADP-mediated inactivation assays in the presence of Ap_5A, an inhibitor of adenylate kinase (Feldhaus *et al.*, 1975). No inhibition of inactivation by ADP was observed following co-incubation with up to 1 mM Ap_5A (data not shown), demonstrating that inactivation by ADP did not occur by conversion to ATP.

Since the nature of the inactivation of the RNA-binding activity by ATP suggested that ATP was serving as an hydrolyzable substrate for an enzymatic activity that permanently modified the RNA-binding protein complex, we tested whether the inactivation by ADP exhibited the same characteristics. When challenged with ADP-β-S, a non-hydrolyzable form of ADP, partial inactivation occurred only at concentration 10-fold higher than was required for native ADP (Figure 5A), in parallel to inactivation by ATP-γ-S (Figure 2B). Like inactivation by ATP (Figure 2A), the inactivation by ADP was found to be non-reversible, as revealed by the resistance of inactivation to dialysis (Figure 5B). These results strongly suggested that, in a fashion similar to ATP, ADP was utilized in a reaction that requires the hydrolysis of a high energy phosphate bond to modify a protein target in a non-reversible fashion. However, in contrast to inactivation by ATP, the inactivation by ADP was faster and required lower concentrations, indicating that ADP, rather than ATP, is the primary substrate for the inactivation of *psbA* mRNA-binding.

The psbA mRNA-binding protein complex contains a kinase activity

The inactivation of RNA-binding by either ADP or ATP was permanent and required the hydrolysis of a high energy phosphate bond, suggesting that protein phosphorylation was responsible for the inactivation phenomenon. To test whether the inactivating factor was associated with *psbA* mRNA-binding complex, and to determine its enzymatic nature, we isolated the protein complex, using *psbA* mRNA affinity chromatography (*psbA*-RAC), and analyzed for the inactivation of *psbA* mRNA-binding. As shown in Figure 6A, the *psbA* RAC-purified protein complex was inactivated by ATP or ADP, revealing that the inactivating factor was either a stoichiometric component of the *psbA* mRNA-binding complex or an activity that has a relatively high affinity for the protein complex. Since the characteristics of the inactivation suggested that protein phosphorylation was responsible for the permanent modification and inactivation of *psbA* mRNA-binding protein complex, we tested whether an endogenous protein phosphotransferase co-purified with the *psbA*-RAC-purified protein complex. The isolated proteins were incubated with either [α-^{32}P]ATP or [γ-^{32}P]ATP, fractionated using denaturating polyacrylamide gel electrophoresis and exposed to film to detect phosphorylated proteins. As shown in Figure 6B, the phosphorylation of proteins occurred only in reactions containing [γ-^{32}P]ATP and not in reactions containing [α-^{32}P]ATP, revealing that an endogenous protein phosphorylating activity was indeed associated with the *psbA* mRNA-binding complex.

The β-phosphate of ADP is used by the endogenous kinase to phosphorylate the psbA mRNA-binding protein complex.

If ADP inactivated the *psbA* mRNA-binding activity by serving as a substrate for a protein phosphotransferase, in a fashion similar to inactivation by ATP, then the identified

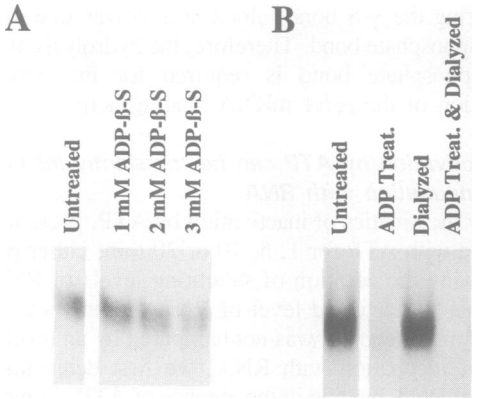

Fig. 5. Characterization of ADP-mediated inactivation of *psbA* mRNA-binding activity. Protein fractions containing *psbA* mRNA-binding activity were treated with ADP or its non-hydrolyzable analog, ADP-β-S, incubated with ^{32}P-labeled 5′-UTR of *psbA* mRNA and subjected to T1-GMS. (**A**) Proteins incubated with increasing concentrations of ADP-β-S. 'Untreated' lanes contain proteins that were subjected to T1-GMS with no additional treatment. (**B**) Untreated or ADP-treated proteins were dialyzed against 20 000 volumes of ADP-free buffer for 4 h and their RNA-binding activity was analyzed and compared with that of non-dialyzed proteins.

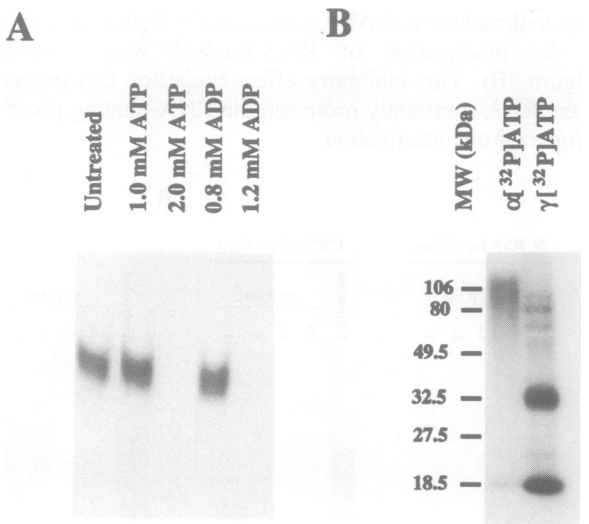

Fig. 6. (**A**) ATP- and ADP-mediated inactivation of RNA affinity purified *psbA* mRNA-binding protein complex. Affinity purified *psbA* mRNA-binding proteins were treated with either ADP or ATP, incubated with ^{32}P-labeled 5′-UTR of *psbA* mRNA analyzed, and subjected to T1-GMS. Untreated lanes contain proteins that were subjected to T1-GMS with no prior treatment. (**B**) Autoradiogram of phosphorylated proteins. Proteins were incubated with either [α-^{32}P]ATP or [γ-^{32}P]ATP for 10 min at room temperature. Assays were terminated by TCA protein precipitation. Samples were analyzed using one-dimensional reducing SDS−PAGE. M_rs of reference proteins are shown on the left.

endogenous phosphotransferase activity should also utilize the β-phosphate group of [β-^{32}P]ADP to label the target protein. To verify the existence of such activity, we used [β-^{32}P]ADP as a substrate in protein phosphorylation assays, and compared the obtained phosphorylation pattern with that when [γ-^{32}P]ATP was utilized. As predicted by the inactivation assays, the phosphotransferase activity was able to utilize the β-phosphate of ADP to phosphorylate proteins *in vitro* (Figure 7B). The co-purification of the ADP-dependent protein phosphotransferase activity and the ADP mediated inactivation of RNA-binding following the isolation of a highly purified *psbA* mRNA-binding protein complex (see stained protein profile in Figure 7A) favored the hypothesis that the ADP-dependent protein phosphotransferase activity is responsible for the inactivation of the RNA-binding complex. We have used several general phosphatases (alkaline and acid phosphatases) and protein phosphotases (types I and IIa) to dephosphorylate the inactivated RNA-binding. Since none of these treatments was successful in restoring RNA-binding activity, we assume that an additional phosphate group is required for binding RNA. This putative additional phosphate group once removed by the phosphatase treatment would disallow binding to RNA. Alternatively, the dephosphorylation of the inactivating phosphate group may be accessible only to a specific chloroplastic phosphatase.

Protein kinases generally require MgATP as a substrate and do not phosphorylate proteins in the presence of excess EDTA. When purified *psbA* mRNA-binding proteins were incubated with either [γ-^{32}P]ATP or [β-^{32}P]ADP in the presence of 6 mM EDTA a complete inhibition of protein phosphorylation was observed (Figure 7B). If protein phosphorylation was responsible for the observed RNA-binding inactivation then the ADP-mediated inactivation of RNA-binding should also be sensitive to the presence of excess EDTA. The binding of the protein complex to *psbA* mRNA was found to be independent of Mg^{2+}, as complete binding of RNA was observed in the presence of either 3 mM $MgCl_2$ or 6 mM EDTA (Figure 7C). As shown before, supplementing the reaction with 0.5 mM MgADP resulted in complete inactivation of RNA-binding while the addition of 6 mM EDTA prior to the addition of ADP resulted in a complete inhibition of ADP-mediated inactivation (Figure 7C). These data and the co-purification of the ADP-dependent protein phosphotransferase activity and the ADP-mediated inactivation of RNA-binding following the isolation of a highly purified *psbA* mRNA-binding protein complex strongly support the hypothesis that

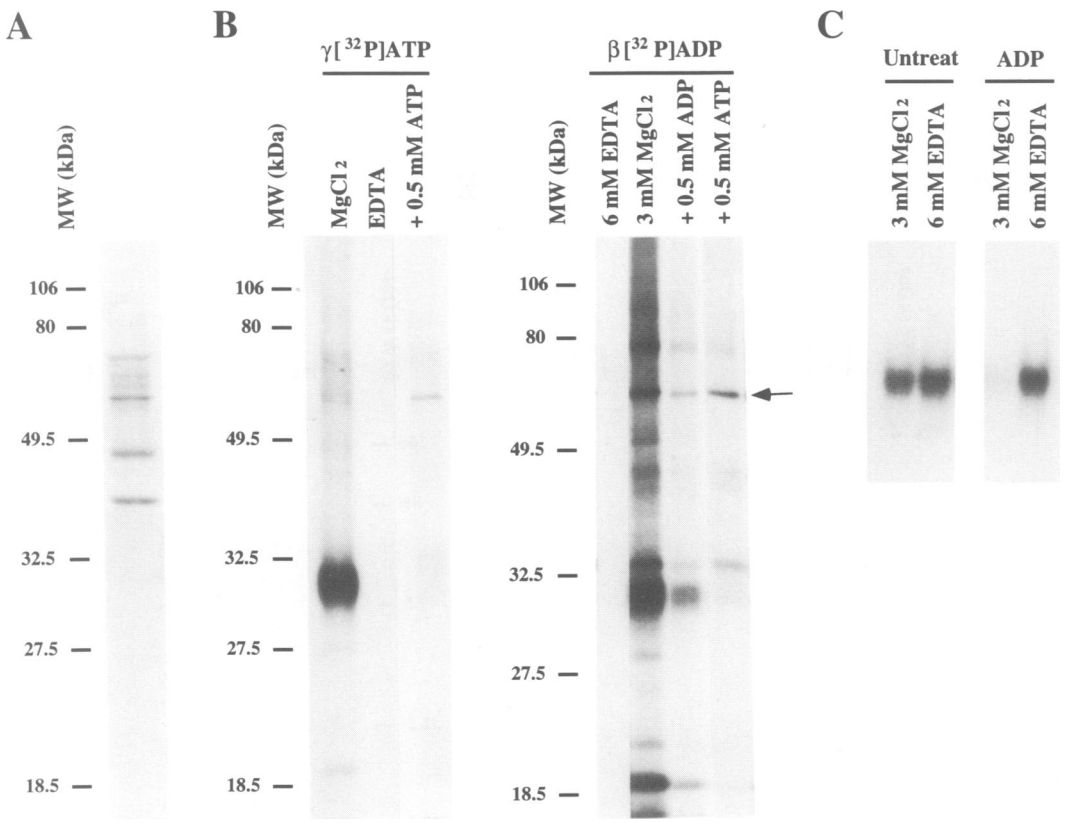

Fig. 7. (**A**) Coomassie stained protein profile of *psbA* mRNA affinity purified protein complex. The *psbA* mRNA-binding protein complex was isolated using RNA affinity chromatography (RAC) made from the 5′-UTR of *psbA* mRNA coupled to an amino-gel matrix. Isolated protein complex was denatured and fractionated using one-dimensional reducing SDS−PAGE. M_rs of reference proteins are shown on the left. (**B**) Autoradiogram of 0.5 μg RAC-purified proteins incubated with either [γ-^{32}P]ATP (10 μM, 150 Ci/mmol) or [β-^{32}P]ADP (10 μM, 150 Ci/mmol) for 10 min at room temperature, in the presence of either $MgCl_2$ or EDTA. Where indicated, an additional 0.5 mM unlabeled ATP or ADP was added with the labeled substrate. Assays were terminated by TCA protein precipitation. Samples were analyzed using one-dimensional reducing SDS−PAGE. M_rs of reference proteins are shown on the left. (**C**) *psbA* RAC-purified proteins were reacted with ADP in the presence of either $MgCl_2$ or EDTA, incubated with ^{32}P-labeled 5′-UTR of *psbA* mRNA and subjected to T1-GMS. Untreated lanes contains proteins that were subjected to T1-GMS following incubation with either $MgCl_2$ or EDTA but with no ADP treatment.

the β-phosphate of ADP was utilized for phosphorylation of the *psbA* mRNA-binding protein complex resulting in an inactivation of RNA-binding.

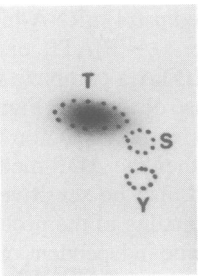

Fig. 8. Autoradiogram of two-dimensional electrophoretic analysis of phosphoamino acids. About 0.5 μg (5 μl) of *psbA* RAC-purified proteins were incubated with 2 μl of high specific activity [γ-^{32}P]ATP (7000 Ci/mmol) in the presence of 10 μM ATP and 3 mM $MgCl_2$, for 10 min at room temperature. Reactions were stopped by dilution into 20% TCA, proteins were precipitated for 20 min on ice, washed with 80% acetone, 10 mM β-mercaptoethanol, resuspended in 25 μl of SDS-sample buffer and fractionated using SDS–PAGE. Following electrophoresis proteins were transferred onto PVDF membrane by electroblotting, phosphorylated proteins were identified by autoradiography, and the labeled band containing the 60 kDa protein was cut out. The protein was hydrolyzed by incubating in 6 M HCl for 1 h at 110°C and released amino acids were fractionated, with unlabeled phosphothreonine (T), phosphoserine (S) and phosphotyrosine (Y), using two-dimensional electrophoresis on thin-layer cellulose plate (Boyle *et al.*, 1991). The migration of the unlabeled phosphoamino acids was determined by staining with ninhydrin (marked by the dotted line) and the migration of the released labeled amino acid was visualized by autoradiography.

The 60 kDa protein of the psbA mRNA-binding protein complex is phosphorylated during inactivation of RNA-binding

To determine which member of the *psbA* mRNA-binding protein complex was the target for phosphorylation, at ADP concentrations that are required for inactivation of RNA-binding, we incubated the purified protein complex with [β-^{32}P]ADP in the presence of 0.5 mM unlabeled ADP or ATP. As shown in Figure 7B, four or five proteins were phosphorylated under these conditions. Of these phosphorylated proteins only the 60 kDa protein was a stoichiometric component of the purified *psbA* mRNA-binding complex, shown in Figure 7A. In addition, the 60 kDa protein, which was the main phosphorylated protein in reactions that included 0.5 mM unlabeled ADP or ATP, was the only protein phosphorylated at 20- to 30-fold increased rate relative to reactions that included only 10 μM unlabeled ADP or ATP. Labeling of all other proteins decreased dramatically when unlabeled ADP or ATP was added, which is expected as the specific activity of [γ-^{32}P]ATP or [β-^{32}P]ADP was reduced 50-fold under these conditions. The fact that ADP and ATP exhibited the same characteristics of inactivation, the non-reversibility, the requirement for the hydrolysis of high energy phosphate bond, etc., suggested that they shared the same mechanism for inactivation. If so, both [β-^{32}P]ADP and [γ-^{32}P]ATP should cause the phosphorylation of the same target protein. As shown in Figure 7B, the 60 kDa protein was also phosphorylated when the *psbA* mRNA-binding complex was incubated with [γ-^{32}P]ATP in the presence of 0.5 mM

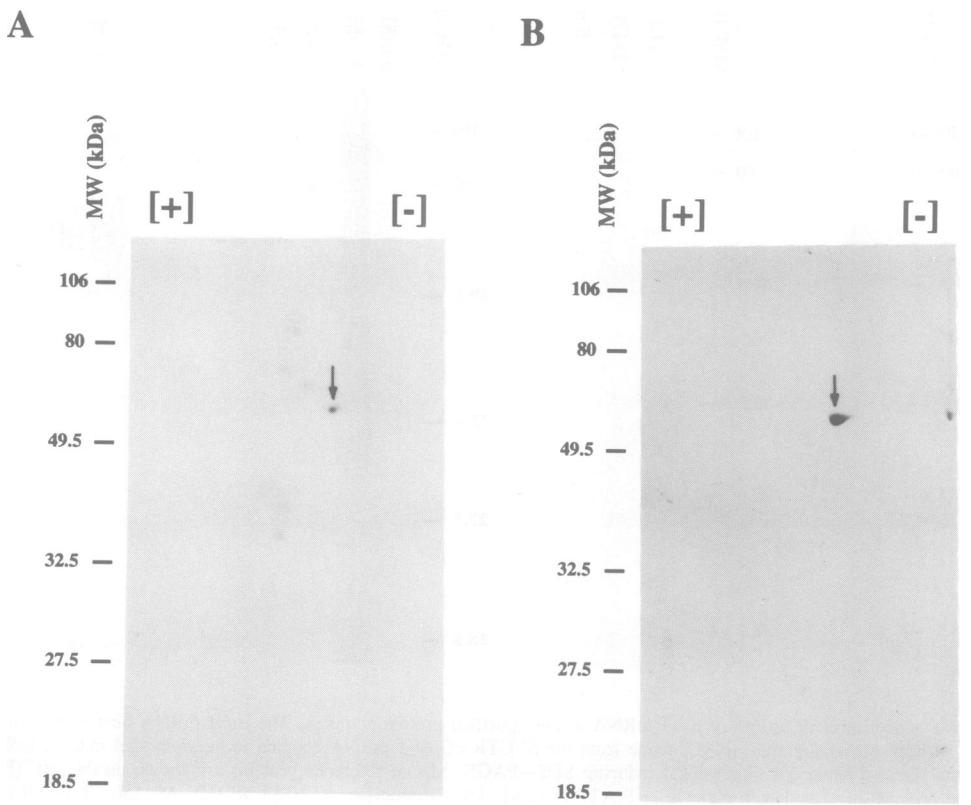

Fig. 9. (**A**) Autoradiogram of the two-dimensional gel electrophoresis of *psbA* mRNA affinity purified protein complex phosphorylated in the presence of [γ-^{32}P]ATP and 1 mM unlabeled ATP. (**B**) Immunoblotting assay of an identical two-dimensional gel electrophoresis of *psbA*-RAC-purified proteins decorated with anti-60 kDa polyclonal sera. The arrow indicates the migration of the 60 kDa protein on both gels. M_rs of reference proteins are shown on the left.

ATP. In addition, the 60 kDa protein was the main substrate for phosphorylation in the reaction containing [β-^{32}P]ADP and 0.5 mM ATP as a competitor (Figure 7B), demonstrating enhanced phosphorylation by ADP as compared with ATP. These results are consistent with ADP being the preferred substrate for the phosphorylation of the 60 kDa protein and the inactivation of *psbA* mRNA-binding.

To verify that the 60 kDa protein is labeled by phosphorylation of an amino acid, and to identify which amino acid serves as the substrate for phosphorylation, we hydrolyzed the ^{32}P-labeled 60 kDa protein with 6 M HCl, for 1 h at 110°C and fractionated the released amino acids using two-dimensional electrophoresis on thin-layer cellulose plates (Boyle *et al.*, 1991). As shown in Figure 8, ^{32}P-labeled threonine was released from the hydrolyzed ^{32}P-labeled 60 kDa protein, confirming that a serine/threonine phosphotransferase, associated with the RNA-binding complex, utilized the β-phosphate of ADP or the γ-phosphate of ATP to phosphorylate the 60 kDa protein.

When the RNA affinity purified proteins were incubated with [γ-^{32}P]ATP in the presence of 1.0 mM unlabeled ATP (concentrations that are sufficient for inactivation), and fractionated using two-dimensional gel electrophoresis, the main substrate for phosphorylation was the 60 kDa protein (Figure 9A), supporting the hypothesis that the 60 kDa protein of the *psbA* mRNA-binding protein complex is the target for the phosphorylation that causes the inactivation of the RNA-binding activity of the protein complex. To verify the authenticity of the phosphorylated 60 kDa protein as a component of the *psbA* mRNA-binding complex (Danon and Mayfield, 1991), we fractionated the phosphorylated proteins using two-dimensional gel electrophoresis. Following fractionation, phosphorylated proteins were detected either by autoradiography (Figure 9A) or by immunoblotting using a polyclonal serum that was raised against *psbA*-RAC purified 60 kDa protein (Figure 9B). The same protein was detected by both autoradiography and immunoblotting, verifying the 60 kDa protein as the stoichiometric member of *psbA* mRNA-binding protein complex that serves as a target for phosphorylation, resulting in inactivation of binding to the *psbA* mRNA.

Discussion

Translation of chloroplastic mRNAs, coding for photosynthetic proteins, is light-regulated in higher plants and algae (Fromm *et al.*, 1985; Malnoë *et al.*, 1988; Mullet, 1988) and requires nuclear encoded factors that interact with the 5′-UTR of the mRNAs (Jensen *et al.*, 1986; Kuchka *et al.*, 1988; Rochaix *et al.*, 1989; Girard-Bascou *et al.*, 1992). Previously, we have identified a set of proteins that specifically binds to the 5′-UTR of the *psbA* mRNA, and have shown that formation of the 5′-UTR−protein complex is light-regulated and correlates with the level of translation of the *psbA* mRNA *in vivo* (Danon and Mayfield, 1991). To understand better the light-regulated translation of *psbA* mRNA, we have investigated the mechanisms by which *psbA* mRNA-binding is modulated *in vitro*. We have shown that ADP-dependent phosphorylation of the *psbA* mRNA-binding protein complex regulates its binding activity *in vitro*, and present a model for the light-regulated translation of *psbA* mRNA in the chloroplast (Figure 10). As described in Figure 10, binding of the protein complex to the 5′-UTR of *psbA* mRNA is required for translation of its protein product. The RNA-binding activity of the protein complex is regulated by an ADP-dependent phosphorylation of at least one of its protein components (the 60 kDa protein). Phosphorylation of this protein inhibits the mRNA-binding activity of the protein complex. RNA-bound proteins are protected from inactivation but because bound and unbound proteins exchange rapidly, modifications to the unbound proteins quickly affect the bound population. The protein phosphotransferase, utilizing the β-phosphate of ADP to phosphorylate its protein substrate, is associated with the *psbA* mRNA-binding protein complex. The threshold level of ADP (~0.4 mM) that is required for the phosphorylation and inactivation of RNA-binding may act as the signal for inactivation of translation.

The hypothesis that the level of ADP is the regulatory signal for the inactivation of RNA-binding and inhibition of translation *in vivo* is consistent with the rise in the stromal concentrations of ADP observed in chloroplasts after transfer to the dark (Stitt *et al.*, 1980). Moreover, the concentrations of ADP that are required for the inactivation of *psbA* mRNA-binding *in vitro* (Figure 4B) are in close agreement with the *in vivo* concentrations of ADP found in the stroma of dark-grown chloroplasts (0.3−0.6 mM) (Heber and Santarius, 1965; Stitt *et al.*, 1980; Hampp *et al.*, 1982). The ratio of ADP to ATP approximately doubles upon transfer to dark (Stitt *et al.*, 1980; Hampp *et al.*, 1982), presenting a narrow range of fluctuating concentrations. The inactivation of RNA-binding *in vitro* requires a threshold level of ADP below which no inactivation is observed even after a 20 min incubation (data not shown). Such a threshold effect would be a requirement for regulation that is responsive to such a narrow increase in ADP concentration upon transfer to

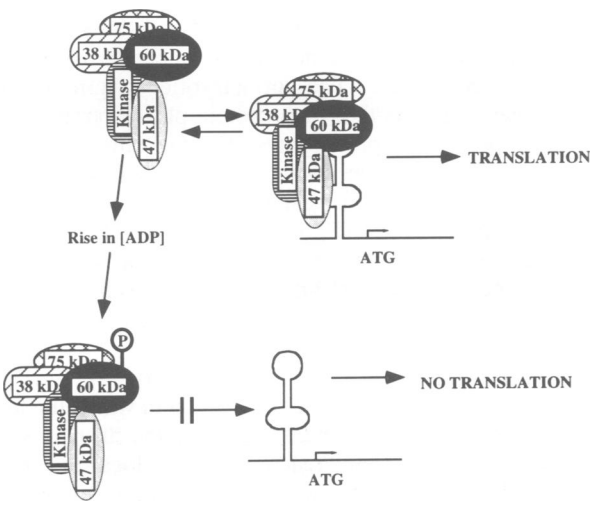

Fig. 10. Model for the regulation of chloroplastic mRNA translation by phosphorylation of the *psbA* mRNA-binding protein complex. Binding of the protein complex to the 5′-UTR is required for the translation of *psbA* mRNA, perhaps by exposing a sequestered ribosome-binding site located within the stem−loop of the 5′-UTR. High ADP concentrations, resulting from shutdown of photosynthesis upon transfer to dark, activates an ADP-dependent phosphotransferase that phosphorylates the 60 kDa protein of *psbA*-mRNA-binding complex on a threonine residue and inactivates its RNA-binding capacity. Although only the non-bound *psbA* mRNA-binding protein complex is phosphorylated, it exchanges rapidly with the bound protein complex, thus any modification to the non-bound protein complex would quickly affect the bound protein complex.

dark. Thus, the characteristics of the *in vitro* inactivation of *psbA* mRNA-binding describe a mechanism for regulating the translation of the D1 protein that is consistent with the physiology within chloroplast during light- and dark-growth.

Interestingly, the protein phosphotransferase that is responsible for the inactivation of RNA-binding can use the β-phosphate of ADP to phosphorylate its substrate. Light-activated protein phosphorylation in the chloroplast has been observed for both the thylakoid (Bennett, 1977; Lin *et al.*, 1982; Mullet, 1983; Coughlan and Hind, 1986; Michel *et al.*, 1988) and the stromal compartments (Guitton *et al.*, 1984; Guitton and Mache, 1987; Sun *et al.*, 1989). The light-regulated enzyme pyruvate P_i dikinase is inactivated by threonine phosphorylation, mediated by a 90 kDa protein phosphotransferase which also utilizes the β-phosphate of ADP (Burnell and Hatch, 1983; Budde *et al.*, 1986). Here, we show the presence of an ADP-dependent protein phosphotransferase that is associated with the *psbA* mRNA-binding protein complex. The co-purification of the ADP-dependent protein phosphotransferase with the highly purified *psbA* mRNA-binding protein complex indicates that the phosphotransferase may be a stoichiometric component of the complex or has high affinity for the RNA-binding protein complex. It will be interesting to determine whether the ADP-dependent phosphotransferase which associates with an mRNA-binding complex is the 90 kDa protein phosphotransferase that phosphorylates the soluble PPDK enzyme. The presence of a common protein phosphotransferase for both reactions would suggest the existence of a general mechanism in the chloroplast that uses an ADP-dependent protein phosphotransferase both to inactivate enzymes of the 'dark reaction' and to inhibit translation of specific 'light reaction' mRNAs, all in response to the rise in levels of ADP upon transfer into dark. The isolation of the chloroplastic protein phosphotransferase that uses the β-phosphate of ADP to phosphorylate its substrate may lead to the identification of a new class of regulatory protein phosphotransferases. The prokaryotic origin of the chloroplast suggests that such protein phosphotransferases may be present in prokaryotes. On the other hand, the chloroplast is a unique organelle, whose main symbiotic function is in capturing light-energy and converting it to chemical energy (i.e. ATP), resulting in relatively high levels of ADP that are uncommon to cytoplasmic protein kinases. It is also possible that ADP-dependent kinases have evolved under such particular conditions as a unique type of protein kinases.

The light-modulated translation of chloroplastic mRNAs presents an interesting regulatory system in which the level of protein synthesis, which takes place in the stroma, is tied to the level of the 'light reaction' that is localized in the thylakoids. We have found that a regulatory signal that mediates the responsiveness of translation is keyed to the energy status of the chloroplast, the gauge being elevated ADP concentration. These high ADP concentrations are the result of diminishing levels of ATP production by the 'light reaction' triggered by a decrease in available light energy and the continued consumption of ATP molecules by the 'dark-reaction'.

Materials and methods

Cell growth conditions, isolation of heparin–actigel purified fractions containing the *psbA* mRNA-binding protein complex and *in vitro* transcription of the 5′-UTR of *psbA* mRNA were performed as previously described (Danon and Mayfield, 1991). RNase T1 protection gel mobility shift (T1-GMS) assays were usually performed according to Danon and Mayfield (1991), except for minor modifications in specific experiments as indicated in the text.

Purification of *psbA* mRNA-binding protein complex using RNA affinity chromatography generally followed the procedure as previously described (Danon and Mayfield, 1991) except for an additional 10 ml wash that included binding buffer + 0.1 M KOAc, 0.6 mg/ml wheat germ tRNA and 0.02 mg/ml total RNA extracted from *Fud7*, a *C.reinhardtii* mutant strain that is missing the *psbA* gene. Following this final wash, a highly purified *psbA* mRNA-binding complex (Figure 7A) was eluted with binding buffer + 0.55 M KOAc.

Phosphorylation *in vitro* was performed by incubating the fractions containing the *psbA* mRNA-binding activity with previously mixed unlabeled nucleotides and [γ-^{32}P]ATP or [β-^{32}P]ADP, in the presence of 3 mM $MgCl_2$, for 10 min at room temperature. [γ-^{32}P]ATP was purchased from Amersham and [β-^{32}P]ADP was custom synthesized by ICN. The reactions were terminated by adding TCA to 20% final concentration and incubating for 5 min on ice. Precipitated proteins were washed with an 80% ice-cold acetone solution, containing 10 mM β-mercaptoethanol, and resuspended with SDS sample buffer. Phosphorylated proteins were fractionated using 12% polyacrylamide gel electrophoresis according to Laemmli (1970). Two-dimensional gel electrophoresis was performed according to O'Farrell *et al.* (1977). First dimension isoelectric focusing and NEPHGE gels contained 3-10 broad range Pharmalyte (Pharmacia) and the second dimension (SDS–PAGE) was a 12% polyacrylamide gel.

Anti-60 kDa protein polyclonal serum was raised following the protocol that was used to obtain the anti-47 kDa protein (Danon and Mayfield, 1991), using a *psbA* mRNA affinity chromatography purified protein complex.

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