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Cis-acting elements and *trans*-acting factors for accurate translation of chloroplast *psbA* mRNAs: development of an *in vitro* translation system from tobacco chloroplasts

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Translational regulation is an important step of gene expression in chloroplasts. To analyze biochemical mechanisms of translational regulation unique to higher plant chloroplasts, an *in vitro* translation system has been developed from tobacco chloroplasts. Conditions for chloroplast extraction and the *in vitro* translation reaction have been optimized with a tobacco *psbA–lacZ* fusion mRNA. The *in vitro* system supports accurate translation of a variety of chloroplast mRNAs. Using a series of mutant *psbA* mRNAs, we showed that three elements within the 5′-untranslated region of the mRNA are required for translation. Two of them are complementary to the 3′-terminus of chloroplast 16S rRNA (termed RBS1 and RBS2) and the other is an AU-rich sequence (UAAAUAAA) located between RBS1 and RBS2 and is termed the AU box. mRNA competition experiments using the *in vitro* translation reaction and gel mobility shift assays revealed the existence of a *trans*-acting factor(s) for translation and its possible interaction with the AU box. We propose a model for the initiation of *psbA* translation whereby RBS1 and RBS2 bind cooperatively to the 3′-end of 16S rRNA resulting in looping out of the AU box, which facilitates the interaction of a *trans*-acting factor(s).

Keywords: chloroplast/*cis-acting* element/*in vitro* translation/*psbA*/*trans*-acting factor

Introduction

Chloroplasts have their own translation system, consisting of 70S-type ribosomes, ~30 tRNA species, initiation/elongation factors (e.g. IF-1, EF-Tu and EF-G) and aminoacyl-tRNA synthetases which are highly homologous to those in prokaryotes (Sugiura, 1992; Subramanian, 1993; Gillham *et al.*, 1994; Harris *et al.*, 1994). Despite these structural similarities, the chloroplast translation system has several features distinct from those in prokaryotes. Prokaryotic 30S ribosomal subunits primarily enter into internal translation initiation sites via Shine–Dalgarno (SD) sequences (McCarthy and Brimacombe, 1994). SD sequences, which are commonly found 7 ± 2 nucleotides upstream from initiation codons in 5′-untranslated regions (5′-UTRs) of prokaryotic mRNAs, are critical for ribosome binding and accurate translation initiation. Over 90% of higher plant chloroplast genes encoding polypeptides possess an upstream sequence similar to the bacterial SD sequence. Spacing of these chloroplast SD-like sequences is less conserved, ranging from –2 to –29 (Ruf and Kössel, 1988; Bonham-Smith and Bourque, 1989; Gillham *et al.*, 1994). In the tobacco chloroplast genome 28 of 73 polypeptide genes [including conserved open reading frames (ORFs)] contain no SD-like sequences (GGA, AGG, GAGG, GGAG or GGAGG) within the region 25 nucleotides upstream of the initiation codon.

Most chloroplast mRNAs in higher plants are primarily transcribed as polycistronic forms and endonucleolytically processed into monocistronic mRNAs, during which some of the transcripts are edited and/or spliced (Herrmann *et al.*, 1992; Rochaix, 1992; Sugiura, 1992; Gruissem and Tonkyn, 1993; Freyer *et al.*, 1993; Hirose *et al.*, 1994; Ruf *et al.*, 1994). It has been reported that pre-mRNA and processed mRNAs from the maize *psbB–psbH–petB–petD* operon are all translated (Barkan, 1988) and recently the monocistronic *petD* mRNA was suggested to be more efficient in translation than its precursors (Barkan *et al.*, 1994). A methyl jasmonate-induced change in the length of the 5′-UTR was reported to impair translation of the plastid *rbcL* transcript in barley (Reinbothe *et al.*, 1993). These observations raise the possibility that mRNA processing may often affect translational efficiency. However, experimental evidence for this hypothesis has been lacking.

Translation of several chloroplast mRNAs is also regulated in response to light or developmental signals at the steps of both initiation and elongation (Gruissem and Tonkyn, 1993; Staub and Maliga, 1993; Kim and Mullet, 1994). The D1 protein of photosystem II encoded by *psbA* is most actively synthesized in light-grown chloroplasts, but not in chloroplasts in the dark nor in non-photosynthetic plastids. However, *psbA* transcript accumulates in the dark, in roots and in non-photosynthetic cultured cells, but it is not incorporated into polysomes in non-photosynthetic roots (amyloplasts) and cultured cells (proplastids) (Deng and Gruissem, 1988; Hirose *et al.*, 1995). Illumination of etiolated barley seedlings causes an increase in polysome-associated *psbA* mRNA and the abundance of initiation complexes bound to *psbA* mRNA (Kim and Mullet, 1994) and light-induced translation of tobacco *psbA* mRNA is regulated via its 5′-UTR (Staub and Maliga, 1993, 1994). In the green alga *Chlamydomonas* a set of nuclear-encoded proteins have been reported to bind specifically to the 5′-UTR of *psbA* mRNA (Danon and Mayfield, 1991) and their binding is modulated by light through ADP-dependent phosphorylation and the redox potential (Danon and Mayfield, 1994a,b). Two RNA elements contained within the 5′-UTR of *psbA* mRNA have also been reported to be required for translation; an SD-like sequence necessary for ribosome association and an upstream sequence of the message-specific protein binding site required for high level translation (Mayfield *et al.*, 1994). An *in organello* assay using isolated pea chloroplasts indicated that D1 protein is not synthesized in the dark, while its translational intermediates accumulate in the presence of externally

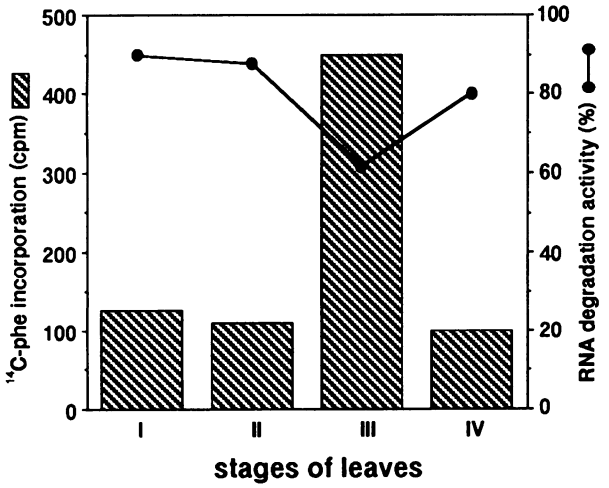

Fig. 1. Poly(U)-dependent [^{14}C]phenylalanine incorporation activity and RNA degradation activity in chloroplast S30 fractions prepared from tobacco leaves of various sizes (stage I, 1–2 cm; II, 3–5 cm; III, 5–10 cm; IV, 10–15 cm). Both activities were assayed under conditions similar to *in vitro* translation using either [^{14}C]phenylalanine (1.85 kBq, 12.5 MBq/mmol) or ^{32}P-labeled *psbA–lacZ* mRNA (5 fmol). TCA-insoluble fractions were counted.

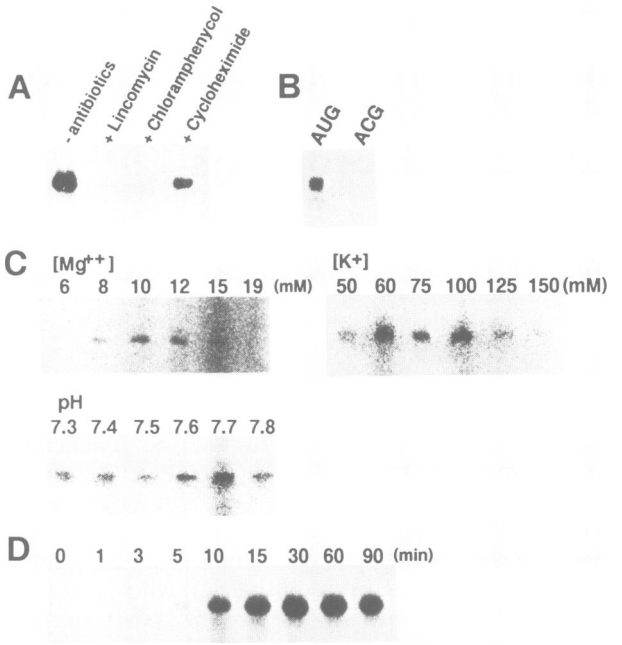

Fig. 2. (**A**) Effects of antibiotics on *in vitro* translation of *psbA–lacZ* mRNA. Lincomycin (15.6 μM), chloramphenicol (500 μg/ml) or cycloheximide (50 μg/ml) was added. (**B**) Initiation codon dependency of *in vitro* translation of *psbA–lacZ* mRNA. Wild-type *psbA–lacZ* mRNA with the AUG start codon (lane AUG) or mutant *psbA–lacZ* mRNA with ACG at the start site (lane ACG) was used. (**C**) [Mg^+], [K^+] and pH dependencies of *in vitro* translation of *psbA–lacZ* mRNA. (**D**) Kinetic analysis of *in vitro* translation of *psbA–lacZ* mRNA. *In vitro* translation was carried out under the conditions indicated above and products (15.1 kDa) were separated by SDS–PAGE.

added ATP in the dark (Taniguchi *et al.*, 1993). This observation suggests that light also regulates *psbA* translation at the elongation step. In addition, the 5′-UTRs of a variety of *Chlamydomonas* chloroplast mRNAs have been shown to be involved in translational control and mRNA stability (Rochaix, 1992; Sakamoto *et al.*, 1993, 1994; Nickelsen *et al.*, 1994; Zerges and Rochaix, 1994).

In order to analyze biochemical mechanisms of translational regulation unique to higher plant chloroplasts we have developed an *in vitro* translation system from tobacco chloroplasts. Tobacco is a suitable material for this purpose, because the chloroplast genome has been completely sequenced (Shinozaki *et al.*, 1986), a detailed transcription map has been constructed, enough isolated chloroplasts can be routinely obtained for biochemical analyses and tobacco is the only higher plant available for chloroplast transformation (Svab *et al.*, 1990; Svab and Maliga, 1993). Our *in vitro* system reproducibly supports accurate initiation of translation from a variety of chloroplast mRNAs, produces discrete translation products of the expected sizes and therefore provides a good system for the functional analysis of possible regulatory factors. Using this *in vitro* system we have identified a novel *cis*-acting element in tobacco *psbA* mRNA. We also present evidence for the existence of a *trans*-acting factor(s), possibly interacting with this *cis*-element.

Results

Development of a chloroplast in vitro translation system

In order to determine the best tobacco leaf stage for our purpose we carried out poly(U)-dependent [^{14}C]phenylalanine incorporation assays using chloroplast S30 fractions prepared from four groups of tobacco leaves of different sizes. S30 fractions were prepared from intact chloroplasts isolated by Percoll gradient centrifugation. As shown in Figure 1, [^{14}C]phenylalanine incorporation was highest while RNA degradation activity was lowest in the S30 fraction from stage III leaves (5–10 cm). Aminoacyl-tRNA synthetase activity as assayed by [^{14}C]leucine charging was apparently constant during leaf growth (data not shown). We therefore used stage III leaves for further analysis.

To elaborate S30 fractions and optimize the translation reaction accurate initiation of translation *in vitro* was monitored using a *psbA–lacZ* fusion mRNA as a model template (see Figure 3). *psbA* mRNA is the most actively translated chloroplast transcript *in vivo* under light-grown conditions and fusion to *lacZ* mRNA renders the translation product soluble and eliminates possible effects of the 3′-UTR of *psbA* mRNA on translation initiation. Translation activity of S30 fractions was quantified by counting ^{35}S-incorporation into the expected product. Chloroplast S30 fractions were pre-incubated with unlabeled amino acids and then treated with micrococcal nuclease to digest endogenous transcripts. This step is critical to reduce non-specific translation. Extensive dialysis was also found necessary to remove EGTA added to inactivate micrococcal nuclease and to standardize reaction constituents. The S30 fraction thus obtained produced a single product of the expected size (15.1 kDa) from the *psbA–lacZ* mRNA. The present *in vitro* system is sensitive to chloramphenicol and lincomycin but resistant to cycloheximide, indicating that chloroplast ribosomes with prokaryotic features, but not cytoplasmic ribosomes, are involved in translation (Figure 2A). The apprarent reduction in

translation product with cycloheximide was probably caused by non-specific inhibition due to the relatively high dose (50 μg/ml). *In vitro* translation was not observed using a mutagenized *psbA–lacZ* mRNA in which the AUG initiation codon was substituted by ACG, confirming that our S30 fraction supports accurate translation from the authentic initiation site (Figure 2B).

Optimal buffer conditions for the translation reaction *in vitro* were 10 mM Mg^{2+}, 50–100 mM K^+, with a peak at 60 mM, pH 7.7 (Figure 2C). Translation activity was also dependent on total protein concentration (optimal at 7.5 mg/ml), mRNA concentration (optimal at 0.1 mg/ml), tRNA (from *Escherichia coli*, 0.4 mg/ml) and the ATP generating system (data not shown). In addition, RNase inhibitor, leupepcin (thiol proteinase inhibitor) and *E.coli* $tRNA^{fmet}$ were added, which caused a slight enhancement of translation activity (data not shown). The translation product was detected after a time lag of 5 min and reached a plateau at 30 min (Figure 2D), hence we adopted a reaction time of 30 min for further analysis.

We carried out *in vitro* translation using chloroplast mRNAs from the photosynthetic genes *atpB*, encoding the β subunit of H^+-ATP synthetase CF_1, and *rbcL*, encoding the large subunit of ribulose-1,5-bisphophate carboxylase/oxygenase, and a housekeeping gene *rps4*, encoding ribosomal protein S4, which were also fused to *lacZ* mRNA at their 3′-ends. Chimeric mRNAs were used to normalize the effects of the elongation step, 3′-UTRs and product stability. As shown in Figure 3, translation products of the expected sizes were detected in all cases. Thus our *in vitro* system supports correct translation of a variety of chloroplast mRNAs and translational efficiency *in vitro* varies from mRNA to mRNA (*psbA* > *rbcL* > *atpB* > *rps4*), probably reflecting structural differences in the 5′-UTRs of the mRNAs. Minor bands observed probably represented immature or degradation products.

Assessment of potential ribosome binding sites in psbA mRNA

We first examined the effect on translation of RNA elements possibly involved in ribosome binding using our *in vitro* translation system. In the 5′-UTR of tobacco *psbA* mRNA we found three possible ribosome binding sites (RBSs) which can base pair with the 3′-terminal region of tobacco chloroplast 16S rRNA (Tohdoh and Sugiura, 1982; Figure 4A). The proximal AAG (termed RBS1), located 9–11 bp upstream of the AUG codon, was first proposed as a putative ribosome binding site because it is complementary to the 3′-end of 16S rRNA ($UCCUUU_{OH}$) (Sugita and Sugiura, 1984), but it is not similar to the *E.coli* SD sequence. The distal GGAG (termed RBS3) is a typical SD sequence, but is located far upstream (–36 to –33) as compared with the position found in *E.coli* mRNAs (–7 ± 2). A short ORF of three amino acids (AUG AUU AAA UAA) is also present downstream of RBS3. The third sequence, UGAUGAU (termed RBS2), is located between –28 and –22, which resembles the sequence proposed as an additional ribosome binding site, UGAUCC, existing in mRNAs of highly expressed *E.coli* genes and located further upstream of SD sequences (Thanaraj and Pandit, 1989). This sequence consists of two overlapping UGAU which are complementary to the 5′-region adjacent to the anti-SD sequence (CCUCC) at

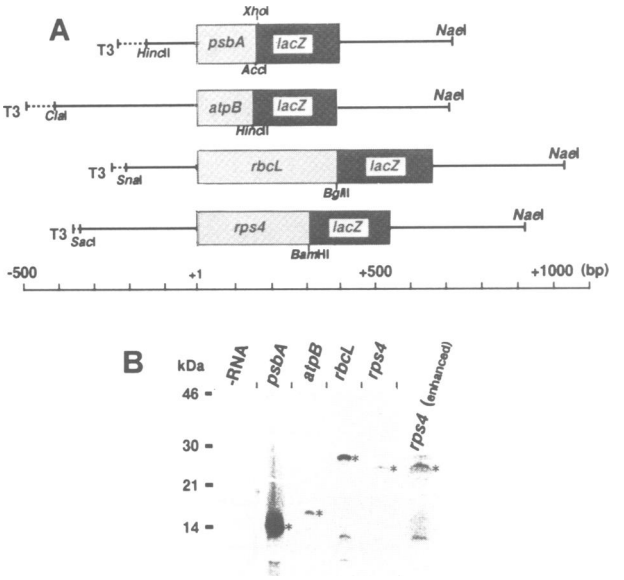

Fig. 3. *In vitro* translation of four tobacco chloroplast mRNAs. (**A**) Schematic representation of plasmid constructs used for template mRNA synthesis. mRNAs start from the initiation site with T3 RNA polymerase (the left terminus) and end at the right-most *Nae*I site. (**B**) SDS–PAGE patterns of *in vitro* translation products. Size markers are shown on the left and major products are indicated by asterisks. An enhanced *rps4* signal is on the right.

the 3′-end of chloroplast 16S rRNA (5′-GAUCACCUC-$CUUU_{OH}$-3′; Ruf and Kössel, 1988).

We constructed a series of *psbA* mRNA constructs mutated at the above three RBS (Figure 4A) and assayed their translation *in vitro*. As shown in Figure 5, disruption of either RBS1 or RBS2 reduced translation (M-RBS1, 33%; M-RBS2, 38%), but that of RBS3 showed little effect on translation (M-RBS3, 88%). We next constructed double mutants with respect to the three RBS (see Figure 4A). Disruption of both RBS1 and RBS2 (M-RBS1/2) resulted in a drastic decrease in translation, while the other two double mutants (M-RBS1/3 and M-RBS2/3) were translated at a level similar to a single mutation of either RBS1 or RBS2. Deletion of the stem–loop structure (see below) did not abolish translation of RBS-mutated mRNA [M-RBS(ΔS/L)]. These observations indicate that RBS1 and RBS2 are cooperatively involved in translation of *psbA* mRNA, possibly via mRNA–16S rRNA interaction, and that RBS3, although a typical SD sequence, and the following short ORF are not essential for translation. In addition, mutation of the GUGUG sequence (Figure 4A, M1), located 5 nucleotides upstream of RBS3, which is highly conserved among higher plant *psbA* mRNAs, had no influence on translation (data not shown).

Additional RNA elements required for psbA translation

To search for additional sequence elements in the 5′-UTR of *psbA* mRNA required for translation a series of 5′ deletion mRNAs were constructed (see Figure 4B) and their translation was assayed *in vitro*. As shown in Figure 6A, deletion of the stem–loop structure (positions –72 to –49) did not significantly affect translation, however, deletion to –22 or more completely abolished translation. This result suggests that Δ–22 mRNA possessing RBS1 is not enough, but the sequence located between the stem–

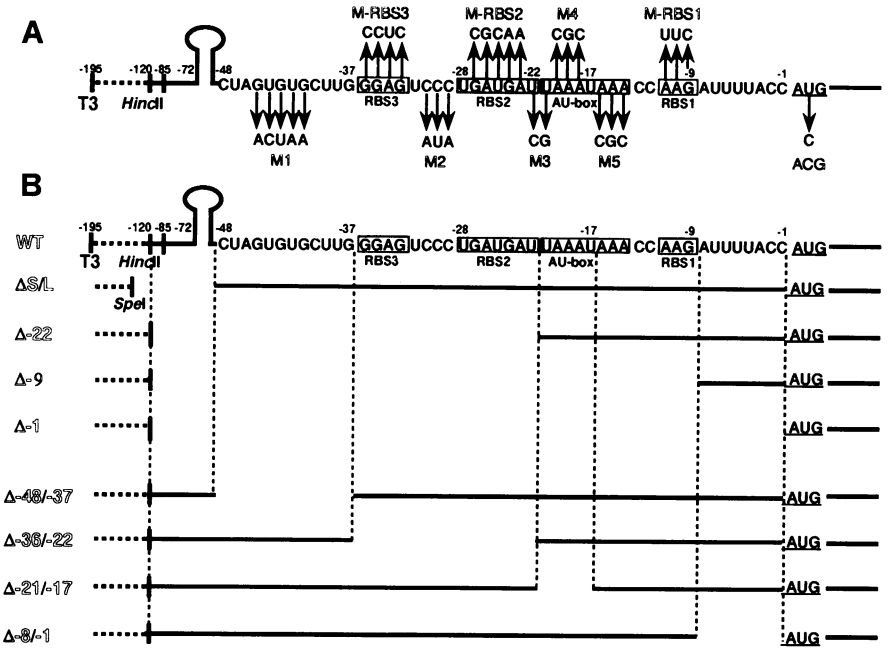

Fig. 4. (**A**) Partial sequence of the 5′-UTR of *psbA* mRNA showing site-directed mutations. Arrows indicate mutated nucleotides with mutant names. (**B**) Diagrams of 5′ and internal deletion mutants of the *psbA* 5′-UTR. Vector sequences are represented by dashed lines and T3 indicates the initiation site of T3 RNA polymerase transcription.

loop and RBS2 (positions –48 to –22) is obligatory for translation of *psbA* mRNA. We next examined internal deletion mutants of the *psbA* 5′-UTR (see Figure 4B). As shown in Figure 6B, deletion of the spacer between the stem–loop and RBS3 (Δ–48/–37) enhanced translation, while deletion from RBS3 to RBS2 (Δ–36/–22) led to a drastic reduction in translation. Furthermore, deletion of an AU-rich region (Δ–21/–17) after RBS2 completely abolished translation. Severe inhibition of translation was also observed when the spacer between RBS1 and AUG was deleted (Δ–8/–1), probably by impairing mRNA–ribosome binding. Since double mutations of RBS3 and RBS2 did not drastically affect translation (Figure 5), an additional sequence element(s) is considered to be located outside these RBS.

We introduced site-directed mutations in four regions between RBS3 and RBS1 (see Figure 4A). As shown in Figure 6C, translation was inhibited more when the mutated positions where closer to AUG and mutations immediately upstream of RBS1 abolished translation. Consistent with the results using deletion mutants, this observation suggests that the AU-rich sequence (UAAAU-AAA), which we have designated the AU box, located between RBS1 and RBS2 is an additional element critical for *psbA* translation. As no complementary regions are found in chloroplast 16S rRNA, this AU box is suggested as a target sequence for a potential *trans*-acting translational factor(s).

Existence of a protein factor(s) interacting with the novel RNA element in psbA mRNA

To functionally detect the essential factor(s) which interacts with the 5′-UTR of *psbA* mRNA and enhances its translation we carried out *in vitro* translation of *psbA* mRNA with 2.5- to 10-fold concentrations of competitor mRNAs. *psbA* mRNA lacking the entire 5′-UTR (Δ–1,

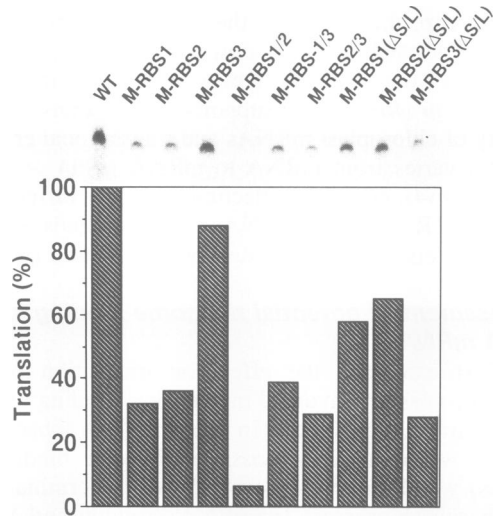

Fig. 5. Effect of putative ribosome binding sites (RBS1–3) in the *psbA* 5′-UTR on *in vitro* translation. Mutant mRNAs used are shown in Figure 4. Translation products were separated by SDS–PAGE. The translational efficiency of each mutant mRNA was calculated from band densities (wild-type as 100%) and is shown below.

Figure 4B) and *psbA* mRNA with ACG in place of the initiation codon (ACG, Figure 4A), both of which lack template activity, were used as competitors. As shown in Figure 7, ACG mRNA inhibited translation of wild-type *psbA* mRNA, while Δ–1 mRNA did not affect or rather enhanced translation. This result suggests that a putative *trans*-acting factor(s) is trapped by excess ACG mRNA and is no longer available to functional *psbA* mRNA.

In order to confirm the above result gel mobility shift assays were carried out using ^{32}P-labeled *psbA* mRNA and the chloroplast S30 fraction. Incubation of *psbA* mRNA with the S30 fraction without an energy sources,

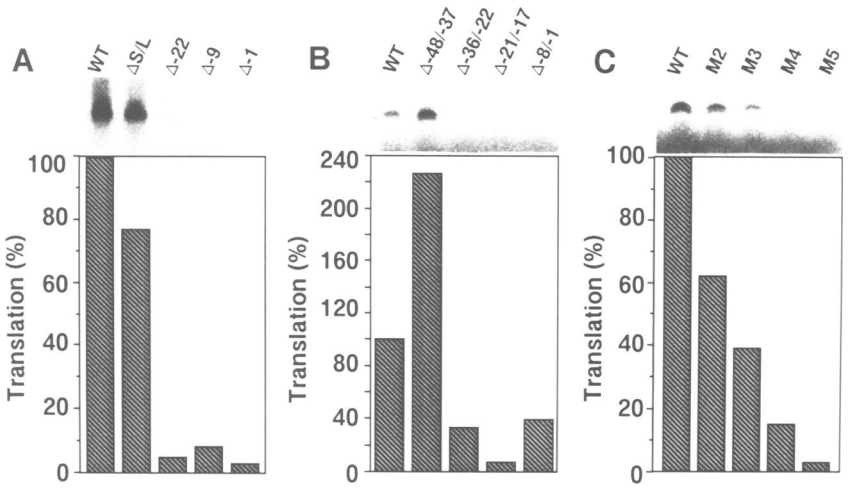

Fig. 6. Identification of an additional *cis*-acting sequence in the *psbA* 5′-UTR. *In vitro* translation was carried out using the 5′ deletion mRNAs (**A**), internal deletion mRNAs (**B**) and site-directed mutagenized mRNAs (**C**) shown in Figure 4B. Translation products were separated by SDS–PAGE and translational efficiencies are shown below.

tRNAs and amino acids (inactive for ribosome binding to mRNA) caused strong retardation of the mRNA on a native gel (Figure 7B), indicating that a chloroplast protein(s) binds to *psbA* mRNA to form a RNA–protein complex (lane 2). Formation of the RNA–protein complex is inhibited by excess unlabeled competitor mRNA possessing the 5′-UTR (ACG, lanes 3–5), while being relatively resistant to excess competitor lacking the 5′-UTR (Δ–1, lanes 6–8). The results obtained from gel mobility shift and functional assays indicate the existence of a specific protein factor(s) binding to the 5′-UTR of *psbA* mRNA.

In the 5′-UTR of *psbA* mRNA the AU box required for its translation is a candidate as the target sequence of the protein factor(s). To examine this possibility *in vitro* translation of *psbA* mRNA was performed with additional competitor mRNAs. When Δ–21/–17, M4 and M5 mRNAs (see Figure 4) were used as competitors *psbA* mRNA translation was unaffected or slightly enhanced, as in the case of Δ–1 used as competitor (Figure 7A). These results indicate that only the AU box in the 5′-UTR competes with *psbA* mRNA and inhibits its translation. Therefore, this AU box is likely to be the target sequence of the *trans*-acting factor(s).

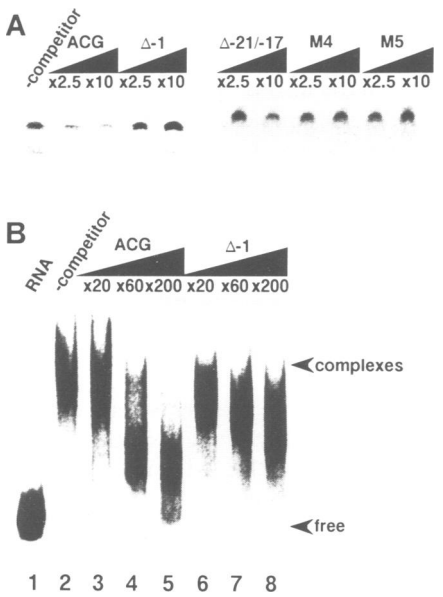

Fig. 7. (**A**) *In vitro* translation of *psbA–lacZ* mRNA in the presence of competitor mRNAs. Competitor mRNAs used are those possessing the whole 5′-UTR but with either the AUG initiation codon replaced by ACG (ACG), lacking the entire 5′-UTR (Δ–1) or with the 5′-UTR with a mutagenized AU box (Δ–21/–17, M4 and M5), as shown in Figure 4. Competitor mRNA concentrations of 2.5- and 10-fold were added (×2.5 and ×10 respectively). (**B**) Gel retardation assay of *psbA* mRNA by chloroplast proteins. ^{32}P-Labeled *psbA* mRNAs was incubated with the S30 fraction under the conditions of *in vitro* translation except for the energy sources, amino acids and tRNAs. mRNA–protein complexes were separated by native PAGE. ACG and Δ–1 mRNAs were used as competitors. 'complexes' represent shifted mRNA–protein complexes and 'free' the mRNA probe used.

Discussion

Development of a chloroplast in vitro translation system

Chloroplast translation has several unique features. Transformation of chloroplasts has recently been developed in the unicellular alga *Chlamydomonas reinhardtii* (Boynton *et al.*, 1988) and the land plant tobacco (*Nicotiana tabacum*; Svab *et al.*, 1990) and has been successfully applied to address questions of chloroplast translation (Chen *et al.*, 1993; Staub and Maliga, 1993; Sakamoto *et al.*, 1994; Zerges and Rochaix, 1994). However, there are still limitations to elucidating the biochemical mechanism of complex translational regulation using the above systems, particularly in higher plants. A variety of prokaryotic and eukaryotic cell-free systems have been used to translate chloroplast mRNAs (reviewed in Steinmetz and Weil, 1989); *rbcL* mRNA from spinach, *Euglena* and maize chloroplasts has been translated in extracts from *E.coli*, wheat germ and rabbit reticulocytes respectively (Hartley *et al.*, 1975; Sagher *et al.*, 1976; Bedbrook *et al.*, 1978; Driesel *et al.*, 1980; Westhoff *et al.*, 1981). However, it was difficult to analyze detailed translational regulation

unique to chloroplasts using heterologous *in vitro* systems. Chloroplast DNA and its DNA fragments were shown to direct *in vitro* synthesis of chloroplast proteins in a cell-free transcription/translation system from tobacco chloroplasts (Bard *et al.*, 1985) as well as from *E.coli* (Bottomley and Whitfeld, 1978) and in a mixed transcription/translation (*E.coli* RNA polymerase + rabbit reticulocyte lysate) system (Bogorad *et al.*, 1978). However, no further analysis using these systems have been reported. Recently an *in vitro* system was developed that allowed the formation of translation initiation complexes with *Euglena* chloroplast 30S ribosomal subunits and natural mRNAs (Wang *et al.*, 1989) and using this system the translational initiation region of *Euglena rbcL* mRNA was analyzed (Koo and Spremulli, 1994a,b).

In order to analyze detailed molecular mechanisms of chloroplast translation and its regulation we have developed a genuine *in vitro* translation system from isolated tobacco chloroplasts. We carefully selected the leaf stage and optimized reaction conditions to obtain S30 fractions with high translation activity. Pre-incubation of S30 fractions with unlabeled amino acids followed by micrococcal nuclease treatment and extensive dialysis was critical for exogenous mRNA-dependent translation. The time course experiments revealed a 5 min lag phase (see Figure 2D). Pre-incubation of mRNA without an energy source, tRNAs and amino acids did not alter the length of the lag phase (data not shown), suggesting that the lag phase might be the time required for tRNA aminoacylation and ribosome assembly onto mRNAs. Using wild-type and initiation codon-mutated (AUG→AUG) *psbA* mRNA we have also shown that our *in vitro* system utilized authentic *in vivo* sites for translation.

RNA elements required for translation of psbA mRNA

In order to define RNA elements required for efficient translation of *psbA* mRNA we examined three possible ribosome binding sites, RBS1–3, and found that RBS1 and RBS2 are cooperatively required for efficient translation of *psbA* mRNA (see Figures 4A and 5). RBS1 (AAG) is located at the appropriate position (–11 to –9) but the interaction with 16S rRNA is thought to be weaker than a typical SD sequence (GGAGG). RBS2 (UGAUGAU) is similar to the translation promoting sequence (UGAUCC) found in the 5′-UTR of highly expressed gene transcripts in *E.coli* and is complementary to an upstream region of the anti-SD sequence (CCUCC). Taken together, we propose the hypothesis that the two RNA elements RBS1 and RBS2 interact cooperatively with the 3′-end of 16S rRNA resulting in looping out of the AU box, as shown in Figure 8.

Based on toeprint analysis of *in vivo* polysome-associated *psbA* mRNA and of that bound with *E.coli* ribosomes *in vitro*, Kim and Mullet (1994) proposed a model of translation initiation on *psbA* mRNA involving transient binding of chloroplast ribosomes to the upstream SD-like sequence RBS3, followed by scanning to localize the initiator AUG. Our functional analysis with an *in vitro* system has shown that RBS3 (GGAG) did not affect the translational efficiency of *psbA* mRNA, suggesting that transient binding of ribosomes to a distant SD-like element is not required, at least for translation of tobacco *psbA*

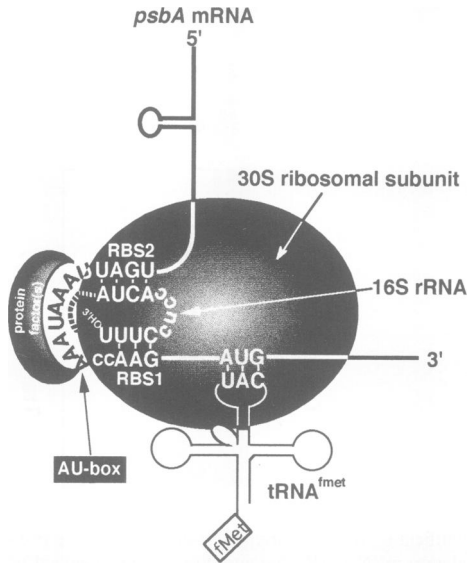

Fig. 8. Model of possible interactions between RBS1, the AU box, RBS2, a *trans*-acting protein factor(s) and 16S rRNA in the 30S ribosomal subunit at the step of translation initiation.

mRNA. The upstream stem–loop structure was reported to be important for translation of *psbA* mRNA in the unicellular green alga *Chlamydomonas* (Mayfield *et al.*, 1994). Deletion of the stem–loop structure itself did not significantly affect translation of tobacco *psbA* mRNA (see Figure 6A), but it slightly relieved the lowered translation of RBS1 and RBS2 mutants and further reduced translation of the RBS3 mutant (see Figure 5). These observations suggest the occurrence of some interaction between the stem–loop structure and RBS elements which to some extent affects *psbA* translation, but not critically.

A novel AU-rich element in the 5′-UTR of psbA mRNA

Evidence obtained from *in vivo* experiments suggests that a chloroplast protein factor(s) interacts with a distinct region(s) within the 5′-UTR of *psbA* mRNA and modulates its translation in a light-dependent and/or tissue-specific manner (Danon and Mayfield, 1991; Staub and Maliga, 1994). We have identified a critical region for translation of *psbA* mRNA, in addition to RBS1 and RBS2, by *in vitro* translation experiments using mutant mRNAs. The sequence UAAAUAAA (the AU box between RBS2 and RBS1) has been found to be essential for translation of *psbA* mRNA, as mutation or deletion of it abolishes translation. AU-rich sequences can be found in the 5′-UTR of various chloroplast mRNAs. The S1-like protein (CS1) in spinach chloroplast ribosomes has been reported to exhibit specific poly(A) binding activity and is also engaged in chloroplast mRNA binding during initiation of translation (Franzetti *et al.*, 1992). In *E.coli* the sequence preceding the SD domain was found to be the target for S1, which acts as an enhancer of translation (Boni *et al.*, 1991; Zhang and Deutscher, 1992). Therefore, ribosomal protein S1 might be one of the protein factors involved in efficient translation of *psbA* mRNA. It seems unlikely, however, that S1 regulates *psbA* translation in a light-dependent and tissue-specific manner.

A trans-acting factor(s) for psbA translation

We have shown here that a specific protein factor(s) is required for efficient translation of *psbA* mRNA by competition experiments in an *in vitro* translation reaction. Furthermore, we have confirmed the existence of a protein factor(s) specifically binding to the AU box of the 5′-UTR of *psbA* mRNA by gel retardation experiments. From these observations we favor the idea that a *trans*-acting factor(s) interacts with the AU box (Figure 8) and modulates *psbA* translation dependent on light and/or developmental signals. As reported in *Chlamydomonas* chloroplasts (Danon and Mayfield, 1994a,b), it is possible that the interaction between the *trans*-acting factor(s) and the AU box is controlled through the ATP/ADP ratio and/or the redox potential in higher plant chloroplasts.

Using the newly developed *in vitro* translation system from tobacco chloroplasts we have identified three RNA elements, RBS1, RBS2 and the AU box, required for efficient translation of *psbA* mRNA and present evidence for the involvement of a *trans*-acting factor(s) in chloroplast translation. Thus this *in vitro* system is useful for the functional analysis of *cis*- and *trans*-acting elements in the translation of various chloroplast transcripts and this line of work is in progress in our laboratory. A combination of the *in vitro* system and reverse genetics will allow us to analyze unique features of chloroplast translation in greater detail

Materials and methods

Preparation of mRNA templates

Unless otherwise indicated all techniques for manipulating DNA and RNA were as described by Sambrook *et al.* (1989). The 5′-UTRs and 5′-coding regions of *psbA* (*Hin*cII–*Acc*I, 175 bp protein coding region), *atpB* (*Cla*I–*Hin*cII, 169 bp protein coding region), *rbcL* (*Sna*I–*Bgl*II, 412 bp protein coding region) and *rps4* (*Sac*I–*Bam*HI, 304 bp protein coding region) were prepared from the clone bank of tobacco chloroplast DNA (Sugiura *et al.*, 1986) and cloned into pBluescriptII SK$^+$ (Stratagene). Resultant plasmid DNAs were cut with the enzyme indicated on the right above, blunted and fused to the reading frame of *lacZ* (α-peptide gene) in pBluescript SK$^+$. The stem–loop of the *psbA* 5′-UTR (ΔS/L) was removed by digestion with *Spe*I followed by self-ligation (see Figure 4B). The other 5′ deletion mutants were prepared by inverse PCR using proper primer pairs. Internal deletions and site-directed mutants of *psbA* were constructed using the Transformer™ Site Directed Mutagenesis Kit version 2 (Clontech). All mutants were verified by sequence analysis. Plasmids were amplified in *E.coli* XL-1 Blue, isolated by the alkaline lysis method and further purified by CsCl gradient centrifugation. Plasmids were linearized with *Nae*I, extracted with phenol/chloroform and precipitated with ethanol. mRNA templates were synthesized using T3 MEGASCRIPT™ (Ambion).

Preparation of intact chloroplasts

Tobacco (*Nicotiana tabacum* var. Blight Yellow 4) was grown in a growth chamber (28°C, 16 h light and 8 h dark) for 4 weeks. Intact chloroplasts were prepared by the method of Bartlett *et al.* (1982) with modifications. Briefly, 200 g expanded leaves (stage III, 5–10 cm) in 600 ml MCB1 (0.3 M mannitol, 50 mM HEPES–NaOH, pH 8.0, 2 mM EDTA, 5 mM β-mercaptoethanol) with 0.1% bovine serum albumin (BSA) and 0.6% polyvinylpyrrolidone were ground with a Polytron homogenizer and filtered through four layers of cotton gauze. The filtrate was centrifuged at 1000 *g* for 10 s. The green pellet was resuspended in 12 ml MCB1 with 0.1% BSA and loaded onto four 10–80% Percoll gradients (40 ml each) in MCB1. After centrifugation at 6000 *g* at 4°C for 30 min the lower green band was collected (~10 ml) and mixed with 3–5 vol MCB2 (0.32 M mannitol, 50 mM HEPES–NaOH, pH 8.0, 2 mM EDTA). Intact chloroplasts were collected by centrifugation at 700 *g* for 30 s at 4°C and washed once with 10 ml MCB2.

Preparation of chloroplast S30 fractions

The chloroplast pellet (~1 ml) was suspended by pipetting into 400 μl Ex buffer (30 mM HEPES–KOH, pH 7.7, 10 mM magnesium acetate, 60 mM potassium acetate, 2 mM dithiothreitol), disrupted by sonication twice for 10 s at volume seven using a Handy Sonic (Tomy UR-20P) and centrifuged at 30 000 *g* for 30 min at 2°C. The supernatant was centrifuged again as above and the resultant non-green supernatant was saved as the S30 fraction (~500 μl). The S30 fraction was incubated at 30°C for 15 min with 40 μM each of 20 non-radioactive amino acids and then treated with 0.26 U/μl micrococcal nuclease (Pharmacia) at 30°C for 15 min in the presence of 1 mM $CaCl_2$. The nuclease reaction was stopped by adding 250 mM EGTA to 3 mM and S30 fractions were dialyzed twice against 300 ml Ex buffer at 4°C for 3 h using an Oscillatory Microdialysis system (molecular weight cut-off 12 000; Bio-Tech International Inc.). The dialyzed S30 fraction (~500 μl, ~15 mg/ml protein) was divided into 52 μl aliqots and stored at –70°C for up to 3 months.

In vitro translation reaction

The *in vitro* translation reaction was carried out at 30°C for 30 min in a 50 μl solution containing 30 mM HEPES–KOH, pH 7.7, 10 mM magnesium acetate, 60 mM potassium acetate, 60 mM NH_4Cl, 1% polyethyleneglycol 6000, 1 mM ATP, 0.1 mM GTP, 8 mM creatine phosphate, 0.4 mg/ml creatine phosphokinase (Type I; Sigma), 2 mM dithiothreitol, 0.4 mg/ml *E.coli* tRNAs (Boehringer), 0.4 mg/ml *E.coli* $tRNA^{fmet}$ (Sigma), 1 μg/ml leupepcin, 130 U RNase inhibitor (TaKaRa), 0.37 MBq [^{35}S]L-methionine (>37 TBq/mmol; Amersham), 20 pmol mRNA and 24 μl (360 μg protein) S30 fraction. After the reaction proteins were precipitated with 50 μl 100% cold acetone and rinsed once with 50% cold acetone. The precipitated proteins were suspended in loading buffer, heated at 95°C for 3 min and separated by 0.1% SDS–15% or 18% PAGE. The separated products were visualized and quantified with a BAS2000 Bio-imaging Analyzer (Fuji Photo Film Co).

Gel mobility shifts

^{32}P-Labeled *psbA* mRNA was synthesized using a *psbA* plasmid construct linearized with *Xho*I (see Figure 3) and an *in vitro* transcription kit (Stratagene) with [α-^{32}P]UTP (>110 Tbq/mmol; Amersham) and purified by 6% PAGE containing 7 M urea. Non-labeled competitor mRNAs were synthesized as above. The mRNA–chloroplast protein binding reaction was performed in a 50 μl volume containing the same components as the *in vitro* translation reaction (except for the energy compounds, amino acids, tRNAs and mRNA) with indicated amounts of a competitor mRNA for 3 min at 30°C and for an additional 10 min at 30°C after adding 1.5 fmol ^{32}P-labeled *psbA* mRNA. The incubated mixture was electrophoresed on a native 5% polyacrylamide gel at 100 V for 1 h. Gel patterns were visualized with a BAS2000 Bio-imaging Analyzer.

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