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Translation of chloroplast *psbD* mRNA in *Chlamydomonas* is controlled by a secondary RNA structure blocking the AUG start codon

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ABSTRACT

Translation initiation represents a key step during regulation of gene expression in chloroplasts. Here, we report on the identification and characterization of three suppressor point mutations which overcome a translational defect caused by the deletion of a U-rich element in the 5′-untranslated region (5′-UTR) of the *psbD* mRNA in the green alga *Chlamydomonas reinhardtii*. All three suppressors affect a secondary RNA structure encompassing the *psbD* AUG initiation codon within a double-stranded region as judged by the analysis of site-directed chloroplast mutants as well as *in vitro* RNA mapping experiments using RNase H. In conclusion, the data suggest that these new element serves as a negative regulator which mediates a rapid shut-down of D2 synthesis.

INTRODUCTION

The control of mRNA translation represents a key regulatory step for gene expression in both prokaryotes and eukaryotes. Despite the fundamental differences between their translation machineries, in both types of organisms the initial steps of translation are known to play an essential role (1). These early events of protein synthesis depend on a variety of *cis*- and *trans*-acting determinants which usually mediate their functions via the 5′- and 3′-untranslated regions (5′ and 3′-UTR) of protein-coding transcripts (2–6). In chloroplasts, the typical endosymbiotic organelles of photoautotrophic plants and algae—a special situation developed during evolution. A basic translational system, which is of prokarytic phylogenetic origin, is embedded in a eukaryotic cellular context. This results in a novel hybrid apparatus for protein synthesis which is controlled by nucleus-encoded eukaryotic-type factors (7–9). During the last years, substantial work has been invested to identify these factors and to elucidate the molecular mechanisms which underlie regulatory translational processes within this specialized cellular compartment.

Genetic analyses in the green alga *Chlamydomonas reinhardtii* and vascular plants have recently led to the identification and cloning of some of the *trans*-acting translation factors involved (10–14). Moreover, the genes for RNA-binding proteins which associate with chloroplast 5′-UTRs *in vitro* [for a review see (15,16) and *in vivo* (17,18)] were identified by biochemical means. However, apart from the target regions on chloroplast mRNAs, relatively little is known about the precise molecular working mode of the respective factors.

Initially, the *cis*-acting RNA targets were mapped by analyzing reporter gene constructs carrying mutated versions of distinct chloroplast 5′-UTRs (19–22) or by site-directed mutagenesis of the endogenous chloroplast gene regions (16,23,24). Alternatively, a chloroplast *in vitro* translation system from tobacco was used to define *cis*-acting translational elements within various plastid 5′-UTRs in higher plants (25,26). These analyses revealed that the typical prokaryotic signal for translation initiation, i.e. a Shine–Dalgarno element located 4–12 nt upstream of the AUG start codon, is functional in some but not all chloroplast mRNAs (25,27–29). Furthermore, sequences surrounding the AUG start codon were shown to significantly affect translational efficiency (30–32).

More systematic mutagenesis approaches then identified additional regions within 5′-UTRs as well as downstream regions (33,34) which affect chloroplast protein synthesis. In both *C.reinhardtii* and tobacco, stem–loop structures within the *psbA* 5′-UTR have been shown to be critical for determining translational efficiency (27,35). RNA secondary structure elements within 5′-UTRs were also found to affect protein synthesis from the *psbC*, *petD* and *rps7* mRNAs in *C.reinhardtii* (20,22,24,36) and the *atpB* mRNA in tobacco (25).

We have previously demonstrated that the *psbD* 5′-UTR in *C.reinhardtii* contains the target site for the nucleus-encoded RNA stability factor Nac2 (3,37) which connects processes of *psbD* RNA stabilization and translation initiation

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[for a recent review see (38)]. Nac2 guides the RNA-binding protein RBP40 to its cognate target site which is located 15 nt upstream of the *psbD* AUG start codon. This *cis*-acting element comprises a stretch of multiple U residues whose deletion in the mutant Δ*U* completely abolished the synthesis of the *psbD* gene product, i.e. the D2 protein of the photosystem II reaction centre (23), and, furthermore, led to the loss of RBP40-binding. A *cis*-acting suppressor of the Δ*U* mutation was isolated and shown to harbour a 5 bp duplication within the mutated region which partially restored both photosynthetic growth and RNA recognition by RBP40 (39).

Here, we report on the identification and characterization of three novel, independent second-site suppressor mutations of the Δ*U* mutation which are all located further downstream of the U-element close to the AUG start codon. Site-directed mutagenesis studies demonstrated that these mutations affect a secondary RNA structure including the AUG start codon. The data suggest that this structure serves as a negative regulatory element for D2 synthesis.

MATERIALS AND METHODS

Algal strains, suppressor isolation and genetic crosses

C.reinhardtii strains were grown on tris-acetate-phosphate medium at 25°C (40). Suppressors of the Δ*U* mutation were isolated as described (39). In brief, Δ*U* cells were plated on HS medium and kept in the dark for 24 h, exposed to ultraviolet (UV)-light (7.5 mJ and 254 nm) in a stratalinker (Stratagene) and transfered to darkness for another 24 h-period to prevent photoreactivation. Suppressors were selected in bright light (100 μE m^{-2} s^{-1}) for a period of up to 6 weeks. To test whether the suppressor mutations reside within the nuclear or chloroplast genome, all three suppressor strains (mt+) were genetically crossed to the wild-type (mt−). All 4 members out of 33 (*su*Δ*U+9*), 31 (*su*Δ*U−3*) or 18 (*su*Δ*U+10*) analyzed tetrads from these crosses were able to grow photoautotrophically indicating a chloroplast localization of the respective suppressor mutations. Photoautotrophic growth rates were followed by measurement of the OD_{700} of cell cultures.

Plasmid construction and chloroplast transformation

Plasmids containing *psbD* 5′-UTR mutations were generated via mutagenesis PCR as described (23) with oligonucleotides 1963 and 1365 as well as oligonucleotides including the mutation, i.e. su2-a: 5′-gcaatgacaatttcgatcgg-3′; su2-b: 5′-ccgatcgaaattgtcattgc-3′; su4-a: 5′-gcaatgacaatggcgatcgg-3′; su4-b: 5′-ccgatcgccattgtcattgc-3′; su5-a: 5′-gagatacacacaatgacaat-3′; su5-b: 5′-attgtcattgtgtgtatctc-3′; revsu2-a: 5′-ggagatacacgaaatgacaa-3′; revsu2-b: 5′-ttgtcatttcgtgtatctcc-3′; revsu4-a: 5′-gagatacacgccatgacaat-3′; revsu4-b: 5′-attgtcatggcgtgtatctc-3′; revsu5-a: 5′-atgacaattgtgatcggtac-3′; revsu5-b: 5′-gtaccgatcacaattgtcat-3′; mutsu-a: 5′-ggagatacacgccatgacaa-3′; mutsu-b: 5′- ttgtcatggcgtgtatctcc-3′. Chloroplasts were then transformed with these plasmids using a helium-driven particle gun (41). The resultant strains were selected for photoautotrophic growth on HS medium plates. Plasmid 72.1 containing the wild-type *psbD* 5′-UTR was used as a positive control (23).

Analysis of nucleic acids and proteins

Total DNA from *C.reinhardtii* was isolated using the DNeasy Plant Kit (Qiagen, Hilden). Algal RNA was prepared with hot phenol (42). RNA secondary structures were calculated by using the RNAdraw software (43). Northern analysis, primer extension assays and western analysis were performed exactly as described (44). Radioactive labelling of RNAs and UV cross-linking with proteins were also performed as described (39).

RNase H mapping of RNA secondary structure

Templates comprising 134 bp (wt) or 127 bp (*su*Δ*U+10*, *su*Δ*U+9* and *su*Δ*U−3*) for *in vitro* synthesis of the various *psbD* RNA probes were PCR-amplified from appropriate DNAs with the oligonucleotide su3131 : 5′-tgtgcgtttctcttgatatgtaccg-3′, complementary to the coding region of *psbD* from position +39 to +15 relative to the ATG and oligonucleotide 2126: 5′-taatacgactcactatagggacacaatgattaaaattaaa-3′ spanning the *psbD* 5′ region from position −74, as well as the T7 promotor sequence (39). *In vitro* transcription reactions and radioactive labelling of the RNAs were performed as described (23). RNA probes (15 fmol) were diluted in cacodylate buffer (50 mM Na-cacodylate, 20 mM $CaCl_2$ and 10 mM KCl) and incubated with 10 pmol of the oligonucleotide RH-1: 5′-aattgtcattgcgtgtatct-3′ which is complementary to position −11 to +9 relatively to the AUG start codon. The samples were heated to 60°C for 5 min and cooled down with a rate of 1°C per min to 25°C. After addition of one volume 2× cacodylate buffer with $MgCl_2$ (50 mM Na-cacodylate, 10 mM KCl, 20 mM $CaCl_2$ and 20 mM $MgCl_2$), samples were incubated with 0.5 U RNase H (Ambion, Cambridgeshire) for 2 min or 5 min and loaded on 12% Polyacrylamide TBE gels (45). After electrophoresis, gels were sealed in plastic bags and exposed to Fuji X-ray films at −20°C.

Pulse labelling of membrane proteins

For pulse labelling of proteins, cells were grown as described (23). After measurement of chlorophyll content, cells were harvested and resuspended to 80 μg chlorophyll/ml. A total of 500 μl of the cells were incubated with cycloheximide (10 μg/ml) for 10 min. Subsequently, cells were fed with 50 μCi ^{35}S-sulphate (Amersham, Freiburg) for 20 min in bright light (100 μE m^{-2} s^{-1}). After centrifugation, sedimented cells were frozen in liquid nitrogen. Broken-cell preparation and gel electrophoresis were carried out as described (44). Signals were quantitated by using the Scion Image software and standardized to the internal Cyt*f* control.

RESULTS

Three independent point mutations suppress the ΔU mutation

Previously, it was shown that a striking U-rich element within the 5′-UTR of the chloroplast *psbD* mRNA is involved in its translation (Figure 1). This element is recognized by the RNA-binding protein RBP40, a process that is dependent on the presence of the nucleus-encoded RNA stability factor Nac2 (39). Replacement of the U-track by a BamHI restriction

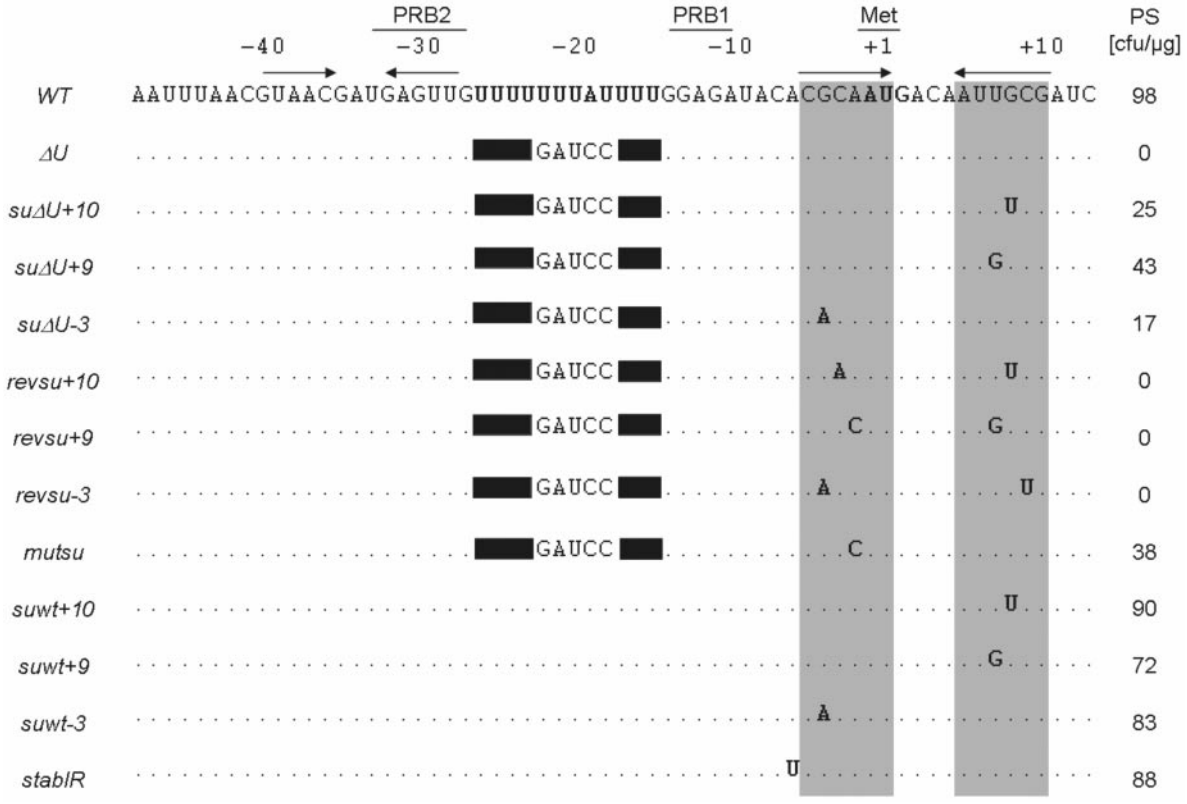

Figure 1. Sequence alignment of the *psbD* 5′ region from wild-type (WT) and analyzed mutant strains. Positions relative to the initiation codon (Met) and the formerly described PRB1 and PRB2 boxes (23) are indicated above the sequences. Dots and solid boxes mark conserved residues and deletions, respectively. The sequence of the U-rich region and the AUG start codon are given in boldface and horizontal arrows represent computer-predicted stem–loop structures of the wild-type region. PS, representative number of photoautotrophically growing colonies after transformation of the mutant Δ*U* with 1 µg of indicated DNAs.

site in the mutant Δ*U* resulted in complete loss of both D2 synthesis as well as RBP40-binding (23). Furthermore, the photosynthetic revertant *su*Δ*U* containing a 5 bp duplication immediately upstream of the BamHI site restored both D2 synthesis and RBP40-binding to almost wild-type levels. These findings together with a more comprehensive mutational analysis underlined the significance of the U-element/RBP40 interaction for translation of the *psbD* message (39).

We have now isolated three novel, independent phenotypic revertants of the Δ*U* mutation, namely *su*Δ*U+10, su*Δ*U+9* and *su*Δ*U−3*. Genetic crosses revealed that each suppressor mutation resides within the chloroplast genome (see Materials and Methods). However, in contrast to the previously characterized *su*Δ*U* strain, sequencing of the *psbD* region revealed that in the suppressors the immediate vicinity of the mutated U-tract is not altered. Instead, each suppressor strain harbours a single point muation which is localized downstream of the U-stretch at positions +10 (*su*Δ*U+10*), +9 (*su*Δ*U+9*) and −3 (*su*Δ*U−3*) with regard to the AUG start codon (Figure 1). Biolistic back-transformations of chloroplasts with constructs containing the *psbD* leader region of either *su*Δ*U+10, su*Δ*U+9* or *su*Δ*U−3* showed that all three constructs were able to complement the Δ*U* mutant verifying that indeed each mutation is sufficient to cause the suppressor phenotype on its own (Figure 1).

All three suppressors accumulated almost wild-type levels of *psbD* mRNA (Figure 2A; Table 1) indicating that the respective point mutations have no effect on RNA stabilization. The *psbD* mRNA has previously been shown to exist in two forms. The difference lies in the length of their 5′-UTR and is most likely due to a 5′ processing event (23,38). In neither of the three suppressor strains, this 5′ maturation was compromised (Figure 2B). However, D2 protein accumulation was found to be restored to varying extent. Whilst the suppressor *su*Δ*U+9* accumulated almost 50% of D2 protein as compared to the wild-type, *su*Δ*U+10* and *su*Δ*U−3* reached only levels of 20 and 15%, respectively (Figure 2C; Table 1). This was in agreement with reduced photoautotrophic doubling times of 37 h for *su*Δ*U+9*, 38 h for *su*Δ*U+10* and 45 h for *su*Δ*U−3* as compared to 35 h for the wild-type (Table 1).

Suppressor mutations do not restore RBP40-binding

As mentioned above, in the previously described *cis*-acting suppressor *su*Δ*U*, a 5 bp duplication immediately upstream of the U-rich translation element resulted in restored binding of RBP40 to the *psbD* 5′-UTR thereby explaining the observed suppressor phenotype (39). Therefore, we tested whether the suppressor mutations *su*Δ*U+10*, *+9* and *−3* also affect RBP40-binding *in vitro*. Radiolabelled RNA probes from each 5′-UTR were incubated with stromal protein extract from the wild-type, and the RBP40 signal was monitored after UV cross-linking of RNAs and proteins. As expected, a strong binding signal was observed with a wild-type *psbD*

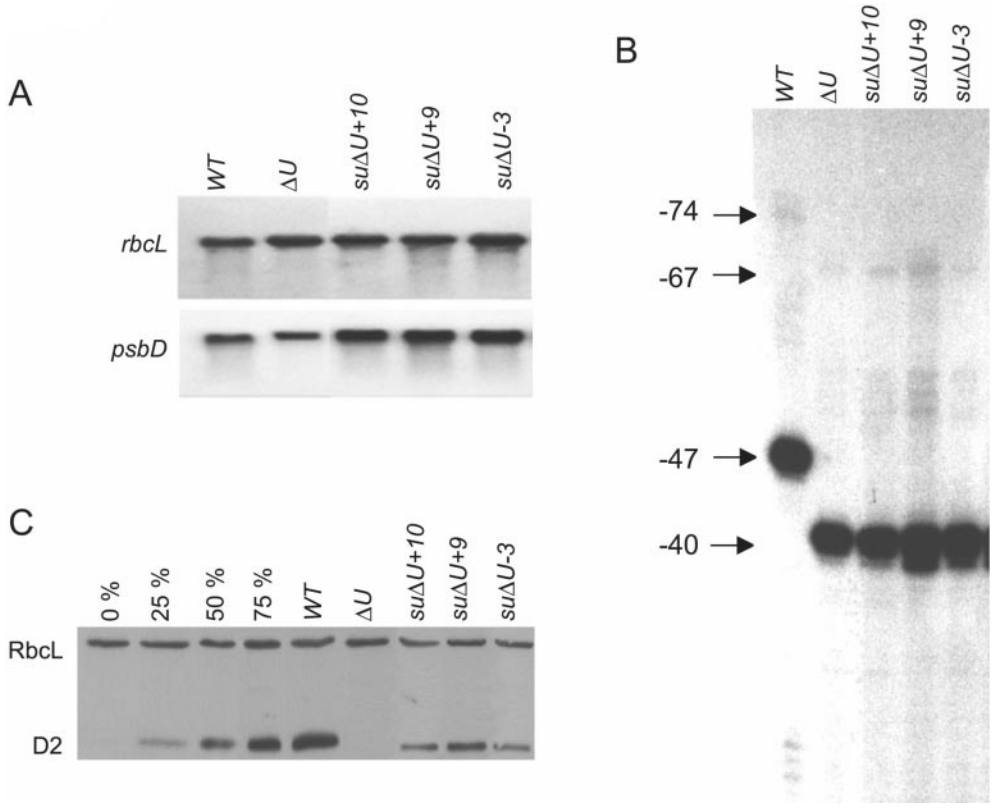

Figure 2. Molecular characterization of suppressor strains *suΔU+10*, *+9*, and *−3*. (**A**) Northern analysis of total RNA (10 μg) from the strains indicated at the top was carried out with a radiolabelled *psbD*- or, as an internal standard, a *rbcL*-specific probe. (**B**) Indicated RNAs were assayed by primer extension analysis using oligonucleotide 3131. The arrows mark the 5′ ends of the longer form starting at position −74 relative to the AUG start codon and the shorter form starting at position −47 of the *psbD* 5′-UTR from the wild-type. In the Δ*U* mutant and the suppressors, *psbD* leaders are shorter by 7 nt due to the deletion at the poly(U) region (see Figure 1). (**C**) Western analysis of total proteins (10 μg) from the same strains was performed by immunolabelling with antibodies directed against either the D2 protein or, as an internal standard, the Rubisco holoenzyme from spinach. A serial dilution of wild-type proteins (0–75%) in Δ*U* proteins was co-analyzed.

Table 1. Molecular characteristics of analyzed strains

Strains	WT	Δ*U*	*suΔU+10*	*suΔU+9*	*suΔU−3*	*mutsu*	*suwt+10*	*suwt+9*	*suwt−3*	*stabIR*
Photoautotrophic growth[a]	35 ± 2	–	38 ± 1.5	37 ± 1	45 ± 2	40 ± 2	31 ± 1.5	30 ± 2	32 ± 1	39 ± 2
RNA	100	80 ± 5	108 ± 6	112 ± 4	115 ± 3	114 ± 5	104 ± 3	99 ± 4	105 ± 4	112 ± 5
Protein[b]	100	0	20 ± 6	46 ± 5	15 ± 7	42 ± 6	117 ± 8	116 ± 9	118 ± 9	61 ± 6

Values display the mean of three independent experiments except for b.
[a]Doubling times in HS medium at 100 μE m^{-2} s^{-1}.
[b]Values display the mean of 10 independent experiments.

5′ probe but not with a mutant Δ*U* probe. The same held for the three suppressor RNAs, none of which led to a RBP40-binding signal (Figure 3A). To further confirm the different RNA-binding properties of RBP40, competition binding experiments were carried out by using radiolabelled wild-type RNA and increasing excess of now unlabelled probes. As shown in Figure 3B, only the homologous wild-type 5′-UTR efficiently competed with the wild-type probe while excess of the suppressor RNAs had only a minor effect on RBP40-binding. Taken together, these data strongly suggest that in contrast to the known suppressor *suΔU*, the suppressor mutations *suΔU+10, suΔU+9* and *suΔU−3* do not restore the binding of RBP40 to the *psbD* U-track but instead appear to act via a RBP40-independent mechanism.

The *psbD* AUG start codon is part of a secondary RNA structure

Bioinformatic inspection of the *psbD* 5′ RNA secondary structure revealed that all three supressor mutations are located in a putative stem-structure of a RNA hairpin which partly encompasses also the AUG start codon (Figure 4). To test whether this RNA secondary structure or the RNA sequence context close to the AUG codon is important for mediating suppression, compensatory mutations were introduced into the *psbD* leader by site-directed mutagenesis (23). The mutations *revsu+10*, *revsu+9* and *revsu−3* each restored the putative stem-region in the respective suppressor backgrounds by an appropriate exchange of the mismatching nucleotides on the opposite strand of the stem (Figure 4). When mutant Δ*U*

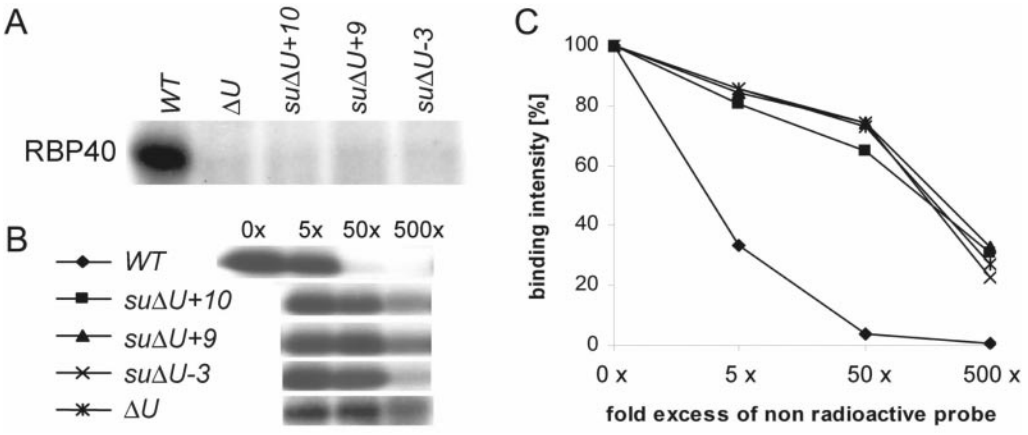

Figure 3. Binding of RBP40 to suppressor 5′-UTRs. (**A**) Radiolabelled *psbD* 5′-UTR probes from the strains indicated at the top and chloroplast stromal protein extract were analysed by UV cross-linking (39). (**B**) In competition binding experiments, radiolabelled wild-type *psbD* 5′ probe was pre-incubated with 5-, 50- or 500-fold molar excess of the marked unlabeled competitor RNAs. (**C**) The diagram displays the intensities of the RBP40-binding signal in relation to that of the signal without competitor (0×).

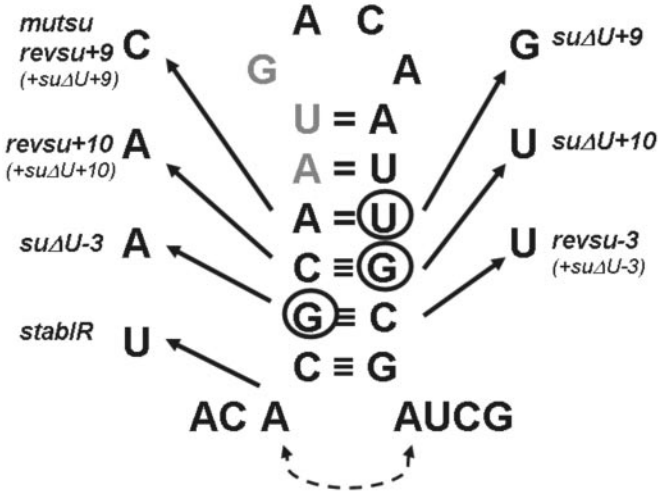

Figure 4. RNA stem–loop structure of the *psbD* 5′ region. The wild-type sequence of the *psbD* region around the initiation codon (grey) is delineated with nucleotide exchanges in the various mutants marked by arrows. All mutants harbour single point mutations in the stem-region except *revsu+10*, *+9* and *−3* which in addition contain the respective suppressor point mutations (see also Figure 1). The additional base pairing in *stabIR* is indicated by a dotted line.

chloroplasts were transformed with these constructs, no complementations were observed for neither of them (Figure 1). This suggested that indeed the restoration of the RNA stem–loop structure inhibited the suppression effect independent of the sequence context.

To further substantiate that the suppressor mutations affect a critical structural rather than a sequence element, the secondary structure of the wild-type and the suppressor *psbD* 5′-UTRs were determined by comparative RNase H mapping (45). Radiolabelled RNA probes were hybridized with oligonucleotide RH-1 which is complementary to the respective AUG regions. RNA/DNA hybrid formation is assumed to be possible only in unpaired RNA stretches which then become a substrate for RNase H. Consequently, unstructured RNA regions will cause an efficient cleavage of the RNA into defined smaller products. As shown in Figure 5, the wild-type *psbD* 5′-UTR was almost resistant against RNase H digestion over a time period of 5 min suggesting that a double-stranded RNA structure prevents stable base pairing with the oligonucleotide. In contrast, all three suppressor RNAs were efficiently cleaved into expected major fragments of 59 and 30 nt being indicative of a more or less unstructured RNA region in the vicinity of the start codon.

These findings strongly suggested that the *psbD* 5′-UTR forms a stable RNA stem–loop structure which partially includes the AUG codon in its stem-region. Furthermore, this structure appears to act as a negatively regulating element for D2 synthesis at least in a Δ*U* genetic background (Figure 1) probably by preventing access of the small ribosomal subunit to the initiation codon. If this scenario were true, then, a suppression of the Δ*U* mutation should be achievable by altering other nucleotides of the stem-region. Thus, an A to C mutation at position −1 relative to the start codon was introduced into the Δ*U* 5′-UTR to test whether this stem-destabilizing mutation, similar to the genetically selected suppressors *su*Δ*U+10*, *+9* and *−3*, would also lead to a suppression effect. A corresponding construct indeed was capable of complementing the Δ*U* mutant (Figure 1). In the resulting transformant *mutsu*, D2 accumulation was found to be restored to 42% of the wild-type level (Figure 6B; Table 1) further supporting the idea of a repressive RNA structure located at the *psbD* translation initiation codon.

Analysis of suppressor mutations in the presence of the *psbD* U-rich element

The above mentioned data provided good evidence that the *psbD* stem–loop structure is a negative determinant for *psbD* gene expression in the absence of the U-rich translation element and, as a consequence, the absence of RBP40 activity. Thus, the next question concerned the phenotype of mutants which contain both the U-element and any of the *su*Δ*U+10*, *+9* and *−3* mutations. Adequate site-directed mutant versions, named *suwt+10*, *suwt+9* and *suwt−3*, were constructed and transformed into Δ*U* chloroplasts. As expected, all three

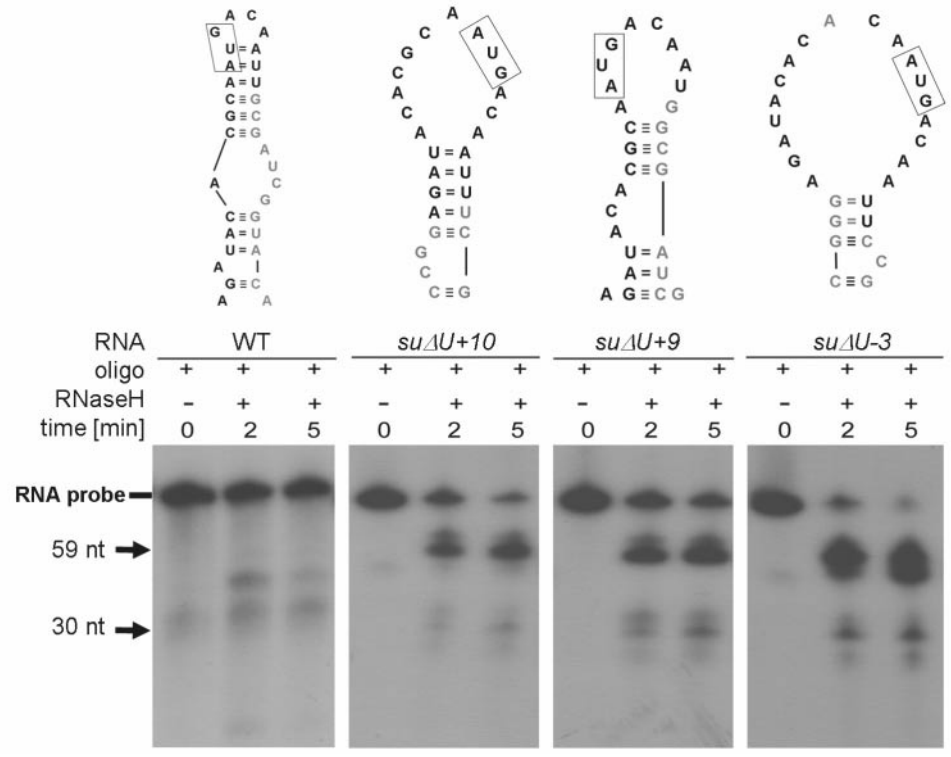

Figure 5. *In vitro* mapping of RNA secondary structure. 100 fmol of indicated radiolabelled RNA probes of 116 nt (*wt*) and 109 nt (*suΔU+10*, *suΔU+9* and *suΔU−3*) were incubated with oligonucleotide RH-1 complementary to the AUG regions and 0.5 U RNase H for 2 or 5 min. Major RNase H cleavage products of 59 and 30 nt (arrows) were visualized on autoradiograms after separation by denaturating PAGE. Computer-predicted secondary RNA structures with a boxed AUG initiation codon are given at the top. Position of oligonucleotide RH-1 is indicated by bold characters.

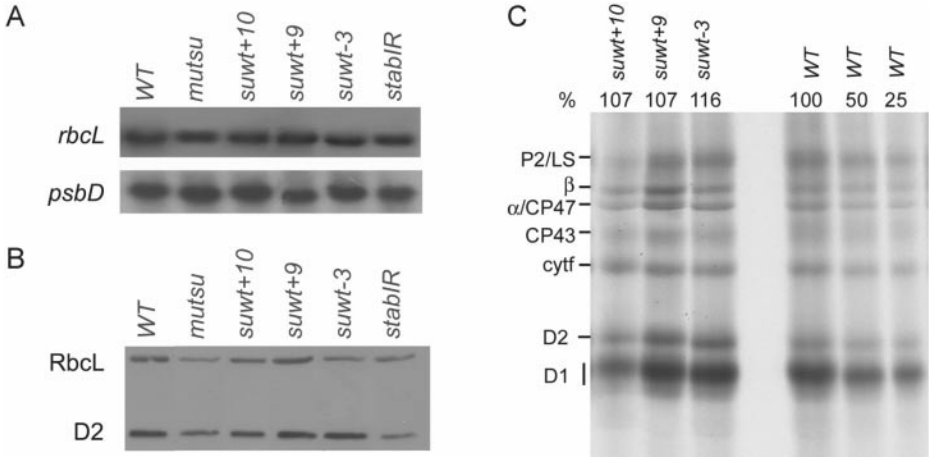

Figure 6. Molecular characterization of site-directed *psbD* mutants. For northern (**A**) and western (**B**) analyses see legend of Figure 2. (**C**) Proteins from indicated strains were pulse labelled with ^{35}S-sulphate for 20 min and separated by SDS–PAGE.

constructs complemented the U-stretch mutation (Figure 1). Interestingly, resulting transformants were shown to accumulate slightly increased levels of D2 protein (Figure 6B; Table 1). This was in agreement with a moderate enhancement of D2 synthesis in *suwt+10*, *+9* and *−3* as compared to the wild-type in protein pulse labelling experiments using radioactive sulphate (Figure 6C). This increase in D2 accumulation in *suwt+10*, *+9* and *−3* suggested that also other subunits of the PS II complex accumulated to enhanced levels because unassembled PS II proteins are rapidly degraded by a chloroplast proteolytic 'clearing system' (23). Consistently, all three strains exhibited a slightly accelerated photoautotrophic growth rate as compared to the wild-type (Table 1). These findings supported the idea that the RNA secondary structure serves as a negative element for *psbD* gene expression and, similar to the situation in cyanobacteria, D2 protein accumulation represents a key regulatory step for PS II assembly (46).

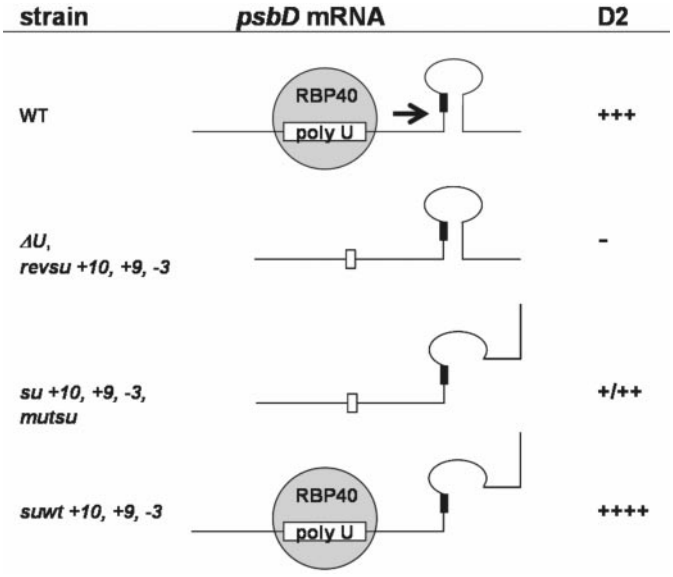

Figure 7. Model of D2 synthesis modes. Analyzed strains and their relative D2 levels are indicated at the left and right margins, respectively. The *psbD* mRNA structure is given along with RBP40. Black boxes represent the AUG initiation codon. For further explanation see text.

In conclusion, these results suggested that the RNA stem–loop structure containing the *psbD* AUG start codon negatively regulates synthesis of the D2 protein and as a consequence PS II accumulation. As an additional control for this hypothesis, the site-directed mutant *stabIR* was constructed by introducing a U residue at position −5 of the wild-type *psbD* leader (Figure 4). This alteration led to a stem-region which was extended by 1 bp and thus, a RNA structure of enhanced stability should be formed. Although this construct was still able to complement the Δ*U* mutant (Figure 1), the subsequent molecular characterization of the resulting transformant *stabIR* revealed a significant reduction of D2 accumulation to nearly 60% compared to the wild-type level (Figure 6B; Table 1). This finding provided an additional piece of evidence for the RNA conformation at the translation initiation codon playing a critical role for *psbD* gene expression.

DISCUSSION

In this report, we describe the identification and characterization of a structural RNA element which serves as a negative regulatory determinant for the synthesis of the D2 protein in *C.reinhardtii* chloroplasts. The analysis of genetically selected suppressors, various site-directed chloroplast mutants as well as *in vitro* mapping studies using RNase H showed that the AUG start codon of the *psbD* mRNA is located in a double-stranded RNA region which has to be resolved before translation initiation. It should be noted, however, that in suppressor strains *su*Δ*U+9* and *su*Δ*U+10* the amino acid sequence of the D2 protein was changed at position 3 from isoleucine to methionine and at position 4 from alanine to valine, respectively (Figure 4). These alterations might have influenced overall D2 protein stability and thus D2 accumulation. For instance, it was reported previously that the replacement of the threonine at position 2 of D2 with acidic—but not neutral—amino acid residues abolished D2 synthesis/accumulation at a post-initiation level in *C.reinhardtii* (47,48). However, similar effects are not likely to play a significant role for the D2 accumulation rates in suppressors *su*Δ*U+9* and *su*Δ*U+10* since they represent gain of function mutants. Additionally, when placed in an otherwise wild-type background, both point mutations apparently had no negative effect on PSII activity as documented by the fitness of the strains *suwt+9* and *suwt+10* (Table 1). Hence, it seems rather unlikely that the amino acid changes in *su*Δ*U+9* and *su*Δ*U+10* add a substantial effect to the suppression mechanism.

The influences of RNA secondary structure elements on chloroplast protein synthesis rates have been noticed before. In principle these structures can have two different functions (49). First, they might serve as recognition sites for *trans*-acting, translation-activating factors as has been suggested for several chloroplast 5′-UTRs (21,22,24,27,35,36). Secondly and like the element described here, secondary RNA structures might serve as negative regulatory elements which block access of the small ribosomal subunit to the translation initiation region resembling the situation which is frequently found in prokaryotes (1). In chloroplasts for instance, it has been proposed that the processing of *petD* precursor RNAs in maize results in the release of the initiation codon from base pairing within a secondary structure and only thereby allows efficient synthesis of the *petD* gene product (50). Also in barley chloroplasts, methyl jasmonate-dependent processing of the *rbcL* 5′-UTR was proposed to influence translation initiation rates (51). By using a chloroplast *in vitro* translation system from tobacco, it was recently shown that the RNA conformation around the AUG codon of the *atpB* mRNA negatively affects translation efficiency (25). Moreover, bioinformatic inspection of the close vicinity of translational initiation regions (each ranging from position −15 to +18) from all chloroplast genes in *C.reinhardtii* revealed that, potentially, nine of the respective initiation codons might be parts of double-stranded RNA structures with a thermodynamic stability in the range of the *psbD* mRNA one (ΔG = −8.53 kcal at 25°C). These regions include that of the *psbN* (−9.31 kcal), *atpB* (−7.53 kcal), *psbC* (−7.32 kcal), *ORF2971* (−6.77 kcal), *rpoA* (−6.71 kcal), *rpl2* (−6.4 kcal), *psaA* (−6.06 kcal) and *petG* (−5.3 kcal) genes. However, only additional experimental data will provide an answer to the question whether such 'prokaryotic-like', negative principles of regulation represent a more common theme of chloroplast gene expression.

Whilst the *psbD* stem–loop structure plays a crucial role for gene expression in the absence of the U-rich region and thus RBP40 activity, its destabilization in an otherwise wild-type background did not result in a dramatic but only moderate increase in D2 synthesis. This suggests that under optimal conditions the inhibition of D2 synthesis via the stem–loop is not strictly rate-limiting. This is reminiscent of the situation found in chloroplast mutants of the *petA*- or *atpB*-1 nt (31,52). In both cases nucleotide changes in the respective −1 triplets did not show any measurable effect on protein accumulation. Only if the AUG start codon was changed into the sub-optimal AUU codon, differences in protein accumulation became detectable (31,52). Under optimal conditions, i.e. the presence of the U-element, most probably the availability of positively

regulating *trans*-acting factors such as the Nac2 protein and the Nac2-dependent RNA-binding protein RBP40 mainly determine D2 synthesis rates as has recently been proposed (39,44). In the absence or reduction of Nac2/RBP40 activity however, this element might be involved in a rapid shut-down of *psbD* translation thereby allowing a fast adjustment of protein synthesis rates to changing environmental conditions.

Figure 7 summarizes the main *psbD* gene expression modes which were characterized during the course of this work. In conclusion, the fact that the isolated suppressor mutations overcome a defect in RBP40-binding without restoring the interaction between this factor and the U-rich element strongly suggests that at least one function of RBP40 is to resolve the secondary structure in the wild-type situation. We have recently identified the RBP40 gene but its putative structure does not reveal a typical helicase-like activity (I. Elles and J. Nickelsen, unpublished data). Hence, it appears more likely that the binding of RBP40 alters the *psbD* RNA conformation thereby enabling access of the small ribosomal subunit to the initiation codon. Future work is focussing on the interactions between the involved *trans*-acting factors and the characterized RNA elements and, thus, their precise molecular working modes.

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