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Determinants for stability of the chloroplast *psbD* RNA are located within its short leader region in *Chlamydomonas reinhardtii*

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Stability of the chloroplast *psbD* mRNA encoding the D2 protein of the photosystem II reaction center is drastically decreased in the nuclear photosynthetic mutant *nac2-26* of *Chlamydomonas reinhardtii*. Using biolistic transformation and genetic crosses we have introduced chimeric genes consisting of the *psbD* leader fused to a reporter gene into the chloroplast in both wild-type and mutant nuclear backgrounds. The chimeric message is destabilized in the latter, but not in the former case, indicating that the 74 nt *psbD* leader includes one of the target sites for *psbD* RNA degradation in the absence of wild-type NAC2 function. Increased instability of the *psbD* leader in mutant versus wild-type chloroplast lysates is also demonstrated *in vitro* and the primary cleavage sites have been mapped. The instability of the *psbD* RNA in the mutant correlates with the loss of binding of a 47 kDa protein to the *psbD* leader RNA, suggesting that this factor acts as message stabilizer in wild-type.

Key words: Chlamydomonas/chloroplast transformation/leader region/*psbD* RNA stability/RNA binding protein

Introduction

The biosynthesis of the photosynthetic apparatus in eukaryotic algae and higher plants depends on the concerted action of the chloroplast and nuclear genetic systems. Several subunits of the photosynthetic complexes are encoded by chloroplast genes and translated on chloroplast 70S ribosomes, while other subunits are encoded by the nuclear genome translated as precursors on cytosolic 80S ribosomes and imported into the chloroplast where they assemble with the chloroplast-encoded subunits to form functional complexes. Analysis of numerous nuclear photosynthetic mutants of *Chlamydomonas reinhardtii* and higher plants has revealed the existence of nuclear-encoded factors that are required for the expression of chloroplast genes (for a review see Rochaix, 1992). These factors appear to act mostly post-transcriptionally, either at the level of RNA stability, RNA processing or translation. Striking features of these factors, at least in *C.reinhardtii*, are that they act in a gene-specific manner and that they exist in large numbers. As an example, the *trans*-splicing of the chloroplast *psaA* gene of *C.reinhardtii* requires at least 14 of these factors (Goldschmidt-Clermont *et al.*, 1990).

The levels of many plastid RNAs vary considerably during chloroplast development and plastid differentiation (for review see Gruissem, 1989). Most of these changes have been attributed to altered RNA stability. The 3′ untranslated regions of several chloroplast mRNAs contain inverted repeats, some of which are required for message stability both *in vitro* and *in vivo* (Stern and Gruissem, 1987; Stern *et al.*, 1989, 1991; Adams and Stern, 1990; Blowers *et al.*, 1993). Several proteins that bind to these regions have been identified (Nickelsen and Link, 1989, 1991; Stern *et al.*, 1989; Hsu-Ching and Stern, 1991). In at least one case the corresponding gene has been isolated and shown to encode a protein with typical RNA binding domains (Schuster and Gruissem, 1991). cDNAs of several chloroplast RNA binding proteins have been isolated and characterized, although their exact role is not clear (Li and Sugiura, 1990, 1991; Mieszczak *et al.*, 1992). It appears, therefore, that chloroplast gene expression requires numerous nuclear-encoded factors. Little is known, however, about how they act at the molecular level.

We have described previously a nuclear mutant of *C.reinhardtii*, *nac2-26*, that is deficient in photosystem II activity (Kuchka *et al.*, 1989). This mutant lacks *psbD* RNA and appears to be specifically affected in the stabilization of this RNA since the *psbD* gene is transcribed and other chloroplast RNAs accumulate to wild-type levels. Transcripts of *psbD* with extensions at their 3′ end are also destabilized in a *nac2-26* nuclear background indicating that a free 3′ end of *psbD* RNA is not required for this effect. Here, we have further examined the stability of the *psbD* transcript and we show that the 74 nt 5′ untranslated region of *psbD* acts as a target site for RNA degradation both *in vivo* and *in vitro* in *nac2-26* background. This instability of the *psbD* RNA can also be correlated with the loss of binding of a 47 kDa protein to the *psbD* leader RNA in the mutant. This protein may mask a critical endonucleolytic cleavage site in wild-type.

Results

Determination of the 5′ ends of the psbD transcripts

Previous S1 nuclease mapping of the 5′ end of the *psbD* transcript revealed two different 5′ ends located around positions –70 and –40 relative to the AUG initiation codon (Erickson *et al.*, 1986). Because the precise location of these sites is important for the experiments described below, primer extension with an oligonucleotide complementary to the *psbD* translation initiation region was performed. The –74 distal site was confirmed (Figure 1A, lane 6). However, the proximal site mapped at –47, further upstream than the earlier estimation which indicated multiple S1 nuclease-resistant fragments around –40, most likely caused by artefactual S1 nuclease cleavage within

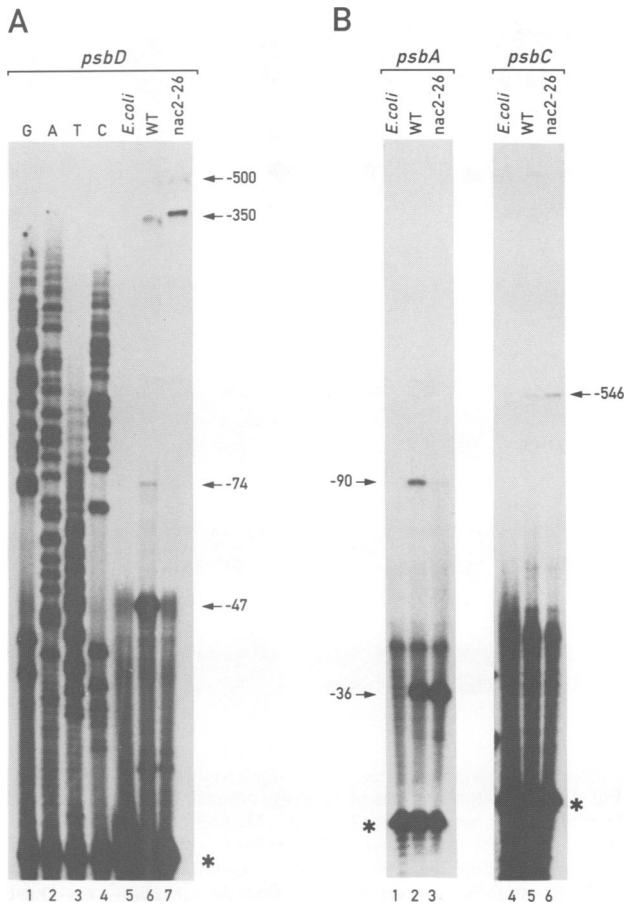

Fig. 1. Analysis of *in vivo* 5′ ends of the *psbD*, *psbA* and *psbC* transcripts in wild-type and *nac2-26*. Labeled oligonucleotides 799 (*psbD*), 2054 (*psbA*) and 2118 (*psbC*) were hybridized to 60 μg of *E.coli* tRNA (lanes A5, B1 and B4), wild-type RNA (lanes A6, B2 and B5) and *nac2-26* RNA (lanes A7, B3 and B6) and reverse transcription products were separated on 10% sequencing gels. In lanes 1–4, sequencing reactions using oligo 799 as primer were coelectrophoresed. Arrows point to detected 5′ ends (see also Figure 8) and asterisks mark labeled oligos.

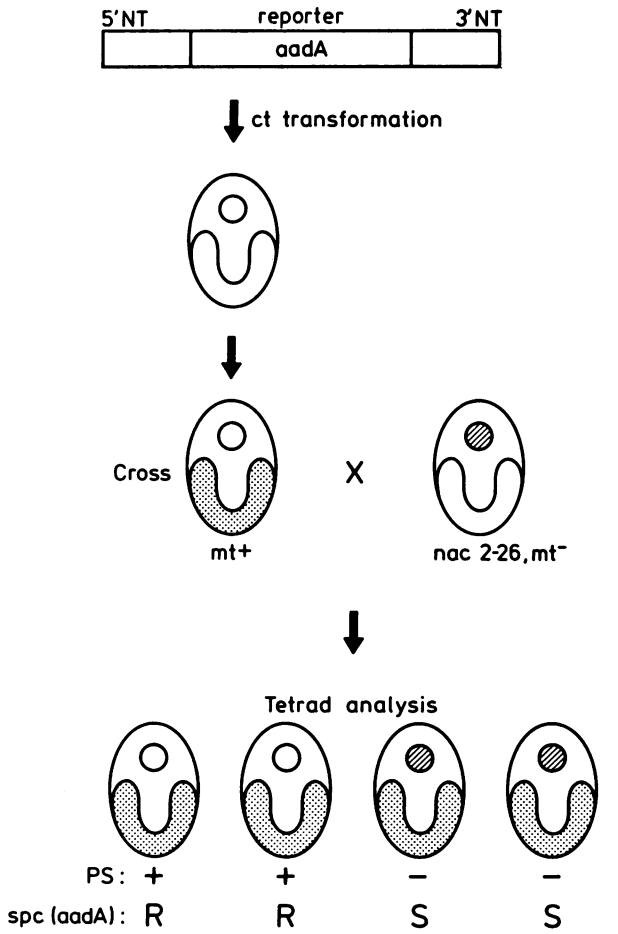

Fig. 2. Strategy for identifying target sites for degradation on the chimeric *psbD* message in the *nac2-26* nuclear background. 5′NT, 3′NT, 5′/3′ untranslated region of *psbD*; PS, phototrophic growth; R, resistance to spectinomycin; S, sensitivity to spectinomycin (see text for further details).

this AU-rich region. As expected, both primer extension products were absent or drastically reduced in *nac2-26* (Figure 1A, lane 7). In addition, two high molecular weight primer extension products, of ~500 and 350 nt, were detected in both wild-type and *nac2*-26 that had not been observed previously. These might originate from read-through transcription of genes located further upstream of *psbD*. The 5′ ends of these fragments are located upstream of the 210 bp *psbD* 5′ region which is sufficient for promoting *psbD* gene expression (see below).

Two different 5′ ends of the *psbA* transcript encoding the photosystem II D1 reaction center polypeptide have been localized at –90 and –36 (Erickson *et al.*, 1984). Primer extension of this RNA revealed a 4-fold decrease of the longer transcript in *nac2-26* but no significant difference in the level of the shorter mature *psbA* RNA between wild-type and mutant (Figure 1B, lanes 2 and 3). Similar primer extensions with the *psbC*, *psbB* and *rbcL* mRNAs did not reveal a significant difference between wild-type and mutant (Figure 1B and data not shown).

Strategy for identifying the target site of trans-acting factors involved in psbD RNA stability

To identify the target site on the *psbD* RNA responsible for its specific degradation in the *nac2-26* nuclear background, the approach depicted in Figure 2 was used. The 5′ and 3′ untranslated regions of *psbD*, which are 74 and 200 nt in length, respectively, were fused separately to a reporter gene and inserted into the chloroplast genome using the biolistic transformation procedure. The reporter genes used were either *aadA* (aminoglycoside adenyltransferase), a bacterial gene that confers spectinomycin resistance when expressed in the chloroplast (Goldschmidt-Clermont, 1991), or *atpB*, encoding the β subunit of ATP synthase. The transformants containing the chimeric gene of mating type plus (mt+) were crossed to the nuclear *nac2-26* mutant of mating-type minus (mt–). Since chloroplast DNA of the mt+ parent is usually transmitted uniparentally in such a cross (Harris, 1989), all four members of the tetrads will inherit the chimeric chloroplast gene. In contrast, the nuclear mutation will segregate 2:2. If the 5′ untranslated region of *psbD* includes the target site for RNA degradation, the two progeny carrying the *nac2-26* mutation will be unable to accumulate the chimeric transcript and therefore be spectinomycin-sensitive when *aadA* is used as reporter.

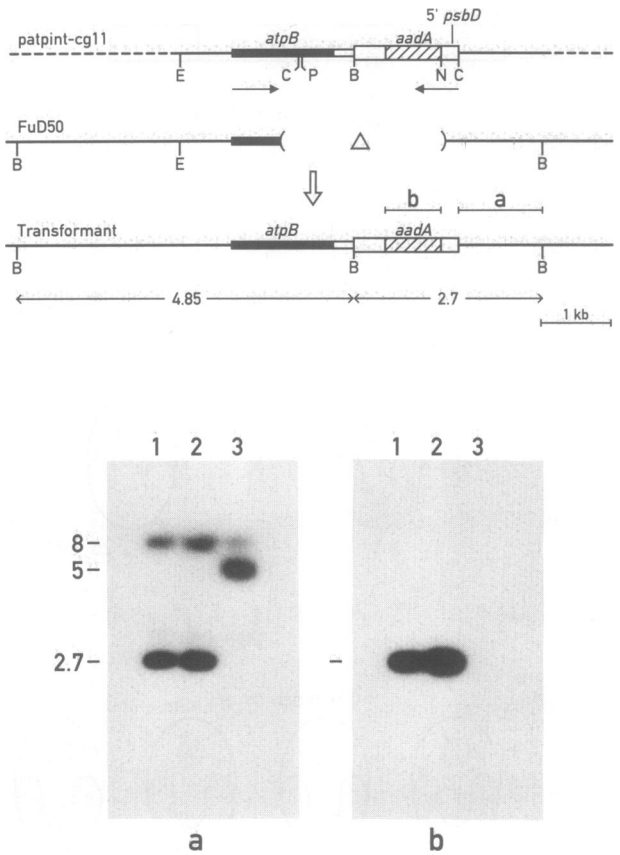

Fig. 3. Construction and analysis of transformants containing a chimeric gene consisting of the 5′ untranslated *psbD* region fused to *aadA*. (Top) Physical map of patpint-cg11 plasmid containing the chimeric construct. Arrows indicate direction of transcription. The corresponding chloroplast DNA region in the *atpB* deletion mutant *Fud50* and in the transformant are shown with relevant restriction sites: E, *Eco*RI; B, *Bam*HI; C, *Cla*I; P, *Pst*I; N, *Nco*I. (Bottom) Southern analysis of *Bam*HI-digested DNA of two transformants (lanes 1 and 2) and *Fud50* (lane 3). The probes a and b are indicated.

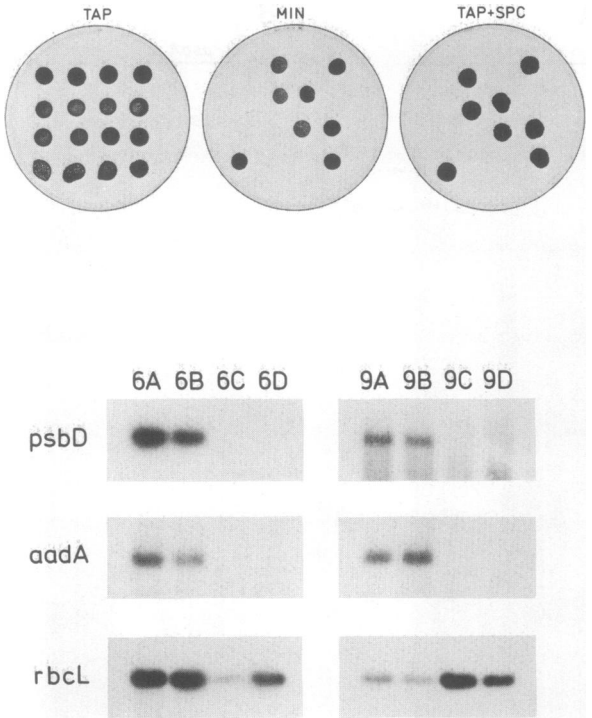

Fig. 4. (Top) Tetrad analysis of the cross between transformants *Fud50* (5′*psbD–aadA*), mt+ and *nac2-26*, mt–. Members of four tetrads (horizontal rows) were plated on TAP, minimal (MIN) and on TAP medium containing spectinomycin (SPC). (Bottom) Northern analysis of the RNAs from members of tetrads from the same cross. 6A–D and 9A–D refer to the four members of each tetrad. Probes specific for *psbD*, *aadA* and *rbcL* were used.

Hence, cosegregation between photosynthetic deficiency and spectinomycin sensitivity would be expected. If, however, the 5′ untranslated region of *psbD* is not sufficient to promote degradation of the chimeric RNA in *nac2-26*, all members of the tetrads will be spectinomycin-resistant.

Chloroplast transformation and genetic analysis of the transformants

To construct the chimeric *psbD–aadA* gene, a 210 bp fragment that contains the region upstream of the *psbD* initiation codon was inserted upstream of the *aadA* coding sequence within the patpB-int vector (Goldschmidt-Clermont *et al.*, 1991). This plasmid contains the *atpB* gene and its flanking regions and can be used for transforming the chloroplast mutant *Fud50*, which has a deletion covering part of *atpB* (Woessner *et al.*, 1984). The new plasmid containing the chimeric *psbD–aadA* gene, called patpint-cg11, was used to transform *Fud50* with the particle gun by selecting for phototrophic growth (Figure 3). To test whether the plasmid had integrated as expected, DNA from two transformants was isolated, digested with *Bam*HI and subjected to Southern blot analysis with a probe specific for *aadA* (probe b in Figure 3) or with the 1.2 kb flanking chloroplast DNA fragment (probe a in Figure 3). As expected, probe b hybridizes exclusively to a 2.7 kb *Bam*HI fragment in the transformants but does not hybridize to the DNA of *Fud50*. Probe a hybridizes to the same fragment and to an 8 kb fragment in the transformants. The latter fragment corresponds to Ba6, which spans the other chloroplast ribosomal inverted repeat (Rochaix, 1978). The same probe also hybridizes to a 5 kb fragment in *Fud50* which corresponds to Ba5 with a 2 kb deletion in this mutant. This fragment is missing in the transformants, indicating that they are homoplasmic with respect to the insertion of the chimeric gene. Hybridization with an *atpB*-specific probe revealed a fragment of 4.8 kb in the transformants but not in *Fud50*, as expected (data not shown).

Table I. Tetrad analysis from crosses of *psbD–aadA* transformants of *Fud50* with *nac2-26*

Transformant	Number of tetrads	PSII$^-$, spc^R	PSII$^-$, spc^S	PSII$^+$, spc^R	PSII$^+$, spc^S
Transformant *Fud50*; *psbD–aadA* mt+ × *nac2-26* mt–					
1	14	0	2	2	0
3	7	0	2	2	0
5	6	0	2	2	0
7	11	0	2	2	0
Transformant *Fud50*; *psbD–aadA* mt– × *nac2-26* mt+					
9B	4	0	2	0	2
3C	11	0	2	0	2

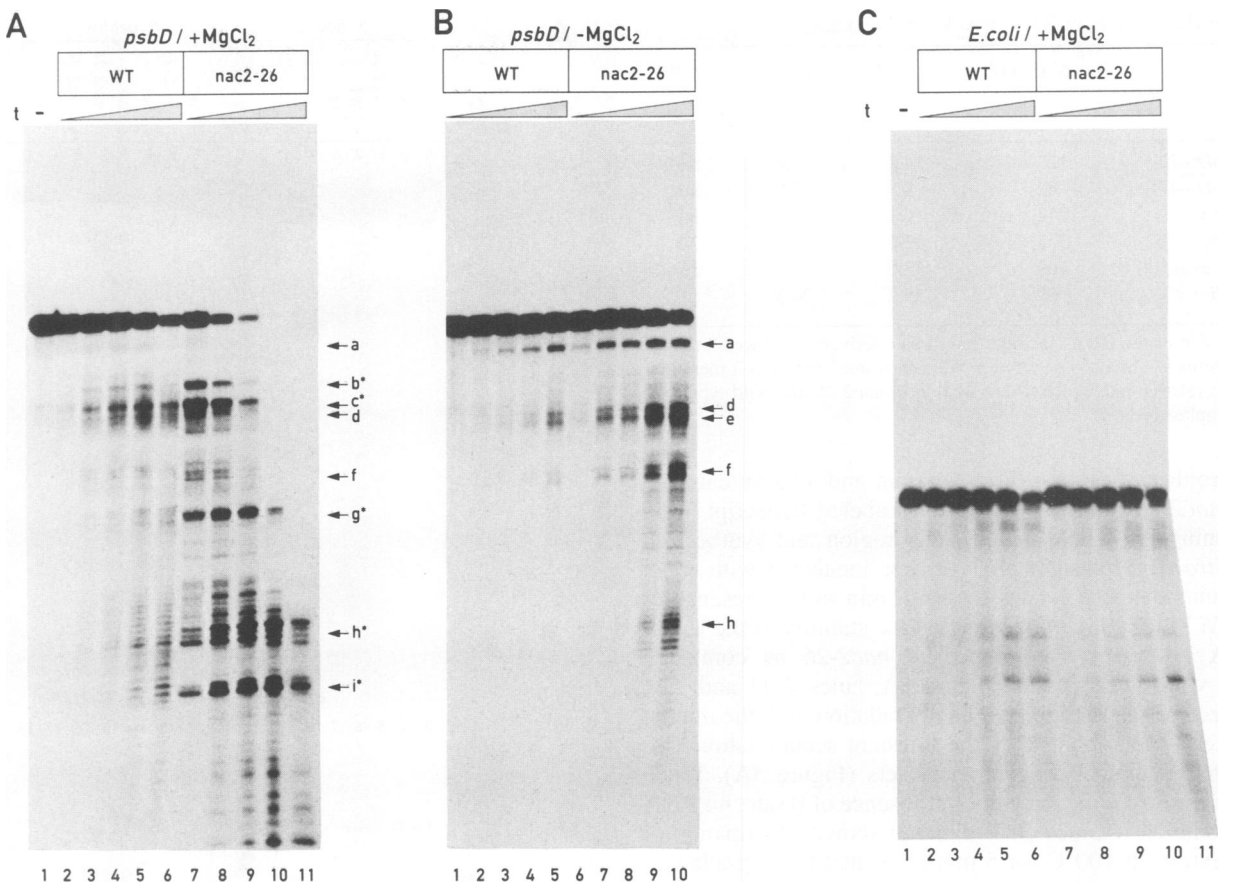

Fig. 5. *In vitro* degradation of RNA in wild-type and *nac2-26* lysates. ^{32}P-labeled transcripts spanning either the *psbD* leader (**A**) or Bluescript polylinker region (**C**) were incubated with wild-type (lanes 2–6) or *nac2-26* (lanes 7–11) chloroplast lysates for 1, 3, 5, 10 and 20 min in the presence of 5 mM $MgCl_2$. Lane 1 in (A) and (C) shows the probe alone. (**B**) As in (A) but in the absence of $MgCl_2$. Arrows indicate degradation products of the *psbD* leader probe, and those that are induced in *nac2-26* are marked by an asterisk.

Several transformants of mt+ were crossed to the *nac2-26* mutant of mt–. Tetrads were analyzed and the individual members were plated on TAP medium (containing acetate as a carbon source), minimal medium (restrictive for phototrophic growth) or TAP medium containing spectinomycin. As an example, Figure 4 displays four tetrads. It can be seen that in all cases colonies unable to grow phototrophically are sensitive to spectinomycin. This cosegregation between photosystem II deficiency (also measured by fluorescence induction transients) and spectinomycin sensitivity was confirmed in 38 tetrads obtained from four independent transformants (Table I). A reciprocal cross was performed in which the transformants carried the mt– and the *nac2-26* parent the mt+ allele. In this cross, all members of the 15 tetrads examined were spectinomycin-sensitive, as expected, since the antibiotic resistance marker was carried by the mt– chloroplast DNA and, therefore, was not transmitted to the progeny.

To test whether the sensitivity to spectinomycin was due to the absence of the chimeric *aadA* transcript, RNA was isolated from the members of four tetrads and hybridized with probes specific to *psbD* and *aadA*. It can be seen in Figure 4 that the two members of each tetrad which lack *psbD* RNA also lack the chimeric *aadA* RNA and correspond to those that are deficient in PSII activity and that are spectinomycin-sensitive. Degradation is specific for transcripts containing the 5′ untranslated region of *psbD*, as another chloroplast transcript, *rbcL* RNA, accumulates stably in all four members of the tetrads. The same results were obtained with two other tetrads (data not shown). When *atpB* instead of *aadA* was used as reporter in similar chimeric constructs, the chimeric *atpB* RNA was also found to be degraded in the *nac2-26* nuclear background (data not shown).

The role of the 3′ untranslated region of *psbD* in RNA stability was examined using the same approach as outlined in Figure 2, except that the *atpB* structural gene was used as reporter. The 3′ untranslated region of *atpB* was replaced with the 3′ untranslated region of *psbD*, thus generating plasmid patpB-D (see Materials and methods for details). This plasmid was used to transform *Fud50* cells by selecting for phototrophic growth. Transformants of mt+ were crossed to wild-type mt– and RNA isolated from the members of the tetrads was examined by Northern hybridization. In contrast to the results obtained with the chimeric *psbD* leader construct, chimeric RNA accumulated in all members of the tetrads (data not shown) indicating that the 3′ untranslated region of *psbD* is not sufficient to promote RNA degradation in the *nac2-26* nuclear background.

In vitro stability of psbD leader RNA

To analyze the mechanisms of RNA stabilization at the molecular level, an *in vitro* system with lysates of

Table II. Half-lives of RNAs in chloroplast lysates

	Size (nt)[a]	$t_{\frac{1}{2}}$ WT (min)	$t_{\frac{1}{2}}$ *nac2* (min)	$\frac{t_{\frac{1}{2}}\text{ WT}}{t_{\frac{1}{2}}\text{ nac2}}$
5′*psbD* –79	127	14.2	1.6	8.9
5′*psbD* –47	62	8.8	2.8	3.1
5′*psbA*	242	6.1	3.5	1.7
5′*psbC*	337	3.0	1.3	2.3
psaA exon 1	269	13.0	5.1	2.5
E.coli	69	25.2	24.3	1.0

[a]The size of the RNAs includes polylinker-derived sequences; nt, nucleotides. The ratio between $t_{\frac{1}{2}}$ WT and $t_{\frac{1}{2}}$ *nac2* provides a measure of the relative rate of RNA degradation in *nac2-26* and wild-type chloroplast lysates.

chloroplasts from a wild-type strain and a strain carrying the *nac2-26* mutation was used. Labeled transcript T-79, spanning the whole *psbD* leader region and synthesized *in vitro* from plasmid pbD-79, was incubated with equal amounts of these lysates for 0–20 min in the presence of 5 mM $MgCl_2$. It can be seen that the stability of the leader RNA is drastically reduced in *nac2-26* as compared with wild-type extracts (Figure 5A, lanes 7–11 and 2–6, respectively). The rapid RNA degradation with the mutant lysate is accompanied by the transient accumulation of a discrete set of degradation products (Figure 5A). When T-79 RNA was incubated in the absence of lysate, no RNA degradation was detected (data not shown). Incubation of the extract at 100°C for 5 min abolished the degradation, indicating that this reaction is most probably mediated by proteins. The reaction also appears to be Mg^{2+}-dependent because the difference in degradation rates detected with wild-type and mutant lysates was considerably diminished when $MgCl_2$ was omitted (Figure 5B). Increasing the $MgCl_2$ concentration to 10 mM had no further effects on RNA stability. No significant difference in stability in the two lysates was observed with an unrelated RNA probe derived from the Bluescript polylinker (Figure 5C), indicating that the degradation observed with *nac2-26* lysates is specific for the *psbD* leader RNA. To further confirm this specificity, other *in vitro* synthesized chloroplast RNA probes, including the 5′ leaders of *psbA* and *psbC* and exon 1 of *psaA*, were tested. The results of these RNA degradation studies are summarized in Table II which shows the *in vitro* RNA half-lives with wild-type and *nac2-26* lysates estimated from scanning autoradiograms similar to those of Figure 5 corresponding to the different RNA probes. Since the stabilities of these RNAs in wild-type extracts can differ considerably from each other, we have used the ratio of the half-lives in wild-type and mutant lysates to obtain a measure of the relative rate of RNA degradation *in vitro*. It can be seen that for the long *psbD* T-79 RNA this ratio is 9, while for the Bluescript polylinker probe this ratio is 1. Values between 1.7 (*psbA*) and 2.5 (*psaA* exon 1) were obtained with the other RNA probes which are significantly lower than for the long *psbD* RNA. It is noteworthy that the increase in the rate of degradation of the short *psbD* T-47 RNA (see Figure 6B) in the mutant relative to the wild-type lysate is considerably less than for the long *psbD* RNA, suggesting that *in vitro* the larger putative *psbD* precursor transcript is the main target for NAC2 function.

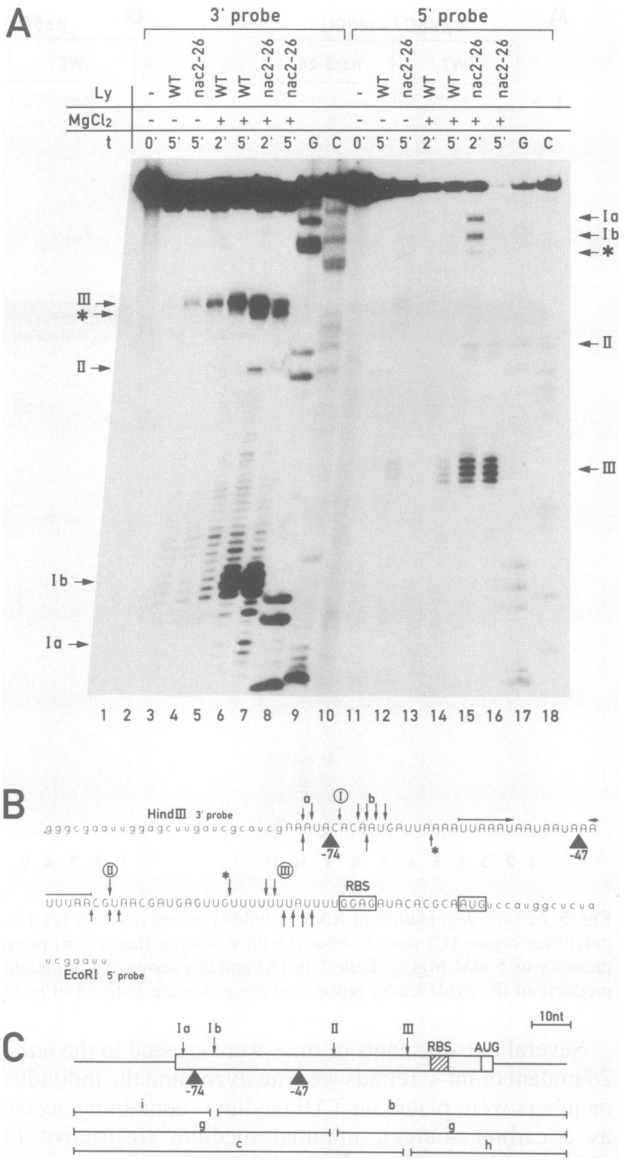

Fig. 6. S1 mapping of degradation products from the *psbD* leader. (**A**) Unlabeled products of T-79 degradation reactions carried out for times (*t*) indicated in minutes at the top were hybridized with either 3′ or 5′ end-labeled cDNA probe –79 and subsequently digested with nuclease S1 (lanes 2–7 and 10–16, respectively). The lysates used (Ly) and the addition of $MgCl_2$ (5 mM) are also indicated at the top. S1-resistant fragments are marked by arrows and the roman numbers refer to defined cleavage sites. G- and C-specific chemical sequencing reactions were coelectrophoresed (lanes 8 and 9, and 17 and 18). (**B**) Summary of S1 mapping results. The nucleotide sequence of T-79 is presented, with lower-case letters representing polylinker portions. Vertical arrows above the sequence mark the determined 5′ ends of S1-resistant fragments labeled at the *Hin*dIII site, while arrows beneath point to 3′ ends of S1-resistant fragments 5′ end-labeled at the *Eco*RI site. Both the ribosomal binding site (RBS) and the AUG start codon are boxed. A potential stem-loop structure is overlined by horizontal arrows. Triangles indicate the *in vivo* 5′ ends of the *psbD* transcript (see Figure 1) and asterisks mark sites mapped only by either the 5′ or 3′ probe. (**C**) Scheme of endonucleolytic T-79 degradation in *nac2-26*. Sites I–III are indicated and the resulting cleavage products are given (see Figure 5). The two *in vivo* 5′ ends at –74 and –47 are also shown.

The formation of distinct degradation products in the presence of *nac2-26* chloroplast lysates suggests that the degradation of the *psbD* leader occurs at defined sites

rather than randomly. To map these sites and to distinguish between endonucleolytic and exonucleolytic degradation events, nuclease S1 protection experiments were performed with the unlabeled T-79 RNA incubated previously with chloroplast lysates from wild-type or *nac2-26*, and a T-79 antisense DNA probe labeled either at its 5′ or 3′ end. The 3′ probe mapped four S1 nuclease-resistant fragments, labeled Ia, Ib, II and III (Figure 6A, lanes 6 and 7). Appearance of these fragments is Mg^{2+}-dependent, as expected (Figure 6A, lanes 2 and 3). Comparison of the S1-resistant signals obtained with wild-type and *nac2-26* lysates reveals that those at positions Ia, Ib and III are drastically enhanced in the mutant and that the signal at position II is unique to *nac2-26* (Figure 6A, lanes 4–6). The fragment corresponding to this signal is rapidly processed (Figure 6A, lanes 6 and 7). Analysis of the RNA products obtained with the 5′ end-labeled probe also revealed four S1-resistant fragments when $MgCl_2$ was present. They accumulate to a much higher level with mutant than with wild-type lysates (Figure 6A, lanes 12–16). As shown in Figure 6B, these fragments define four sites (Ia, Ib, II and III) coinciding with those mapped by the 3′ end-labeled probe, indicating that they represent endonucleolytic cleavage sites rather than endpoints of successive exonucleolytic degradation events. Endpoints of this type would be defined by only one set of S1 nuclease-resistant fragments (such as those indicated by asterisks in Figure 6B).

The site Ia is located 3 nt upstream of the mapped principal *in vivo psbD* RNA 5′ end at position –74. The significance of this site is not clear, especially because of the presence of larger transcripts in the cells (Figure 1). However, cleavage at position –70 (Ib), –38 (II) and –20 (III), with respect to the AUG start codon, could explain the formation of the degradation products that accumulate after RNA processing in *nac2-26* lysates (Figures 5A and 6C). These results suggest that the destabilization of the *psbD* leader in *nac2-26* is due to enhanced endonucleolytic cleavage within this region.

In vitro complementation of psbD leader RNA stability

The *nac2-26* mutation has been shown to be recessive suggesting a loss of function (Kuchka *et al.*, 1989). One would expect, therefore, that in the mutant a factor protecting the RNA from degradation is defective rather than that a ribonuclease is activated. To test this possibility, an *in vitro* complementation test was performed: the stability of *psbD* leader RNA was examined after adding increasing amounts of wild-type lysate (5, 10 and 20 μg proteins) to 10 μg of proteins from mutant lysate. As shown in Figure 7 (lanes 1–4 and 8–11), this resulted in increased stability of the *psbD* probe as determined after 2 or 6 min incubation. To ensure that the stabilization was not due to a protein concentration effect, equivalent amounts of *nac2-26* lysate were assayed similarly. No effect on RNA stability was observed (Figure 7, lanes 5–7 and 12–14). These results indicate that a protective activity is indeed present in wild-type, but not in mutant, chloroplasts.

Binding activities to the psbD leader RNA

After the establishment of an *in vitro* system in which *psbD* leader RNA is preferentially degraded in mutant

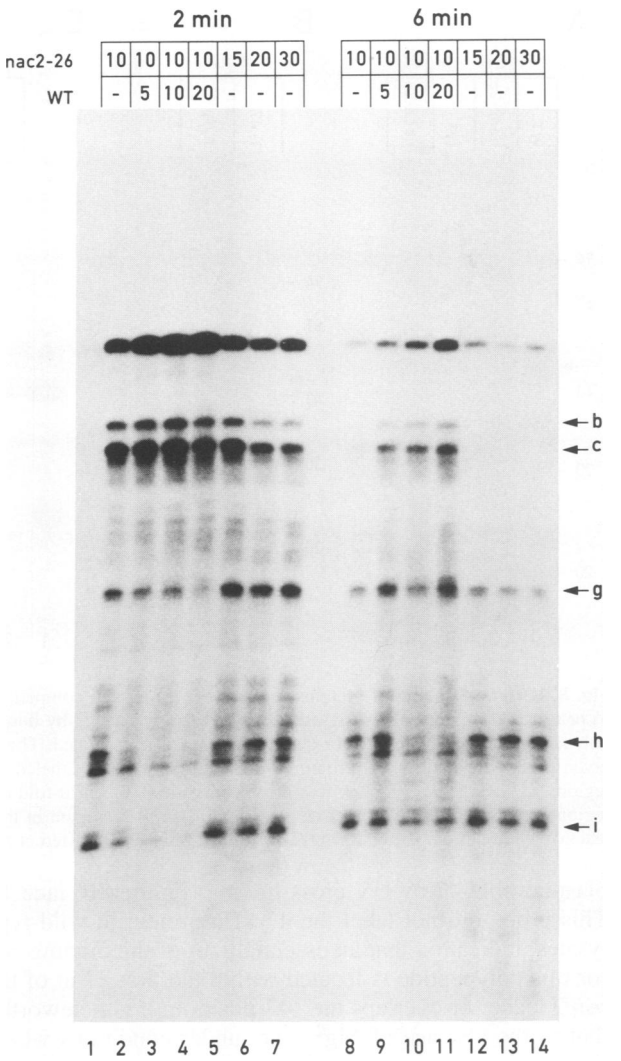

Fig. 7. *In vitro* complementation of *psbD* leader RNA stability. Mixtures of different amounts (μg protein, as indicated at the top) of *nac2-26* and wild-type lysates were incubated with labeled T-79 RNA in the presence of 5 mM $MgCl_2$ for 2 and 6 min (lanes 1–7 and 8–14, respectively). Arrows mark degradation products.

relative to wild-type extracts, attempts were made to identify factors binding this RNA using UV cross-linking (Nickelsen and Link, 1989). After adding radiolabeled T-79 RNA to either wild-type or mutant lysates, samples were irradiated with UV light as a cross-linking agent. To avoid rapid degradation of the RNA probe, especially with a *nac2-26* lysate, all manipulations were performed on ice. Under these conditions, no degradation was detectable (data not shown). RNase-treated RNA–protein complexes were then separated by SDS–PAGE. Proteins from wild-type lysates with an apparent molecular weight of 95, 47, 40, 29 and 18–16 kDa were labeled by T-79 RNA in the presence of Mg^{2+}. In contrast, the 47 kDa signal was absent and that of 29 kDa was reduced with mutant lysate (Figure 8A, lanes 1 and 2). The 29 kDa signal could not be detected reproducibly with different preparations and further analysis was, therefore, limited to the 47 kDa protein. To test whether the 47 kDa protein also binds to the second *psbD* transcript starting at position –47 (Figure 6B), we analyzed the T-47 transcript synthesized from

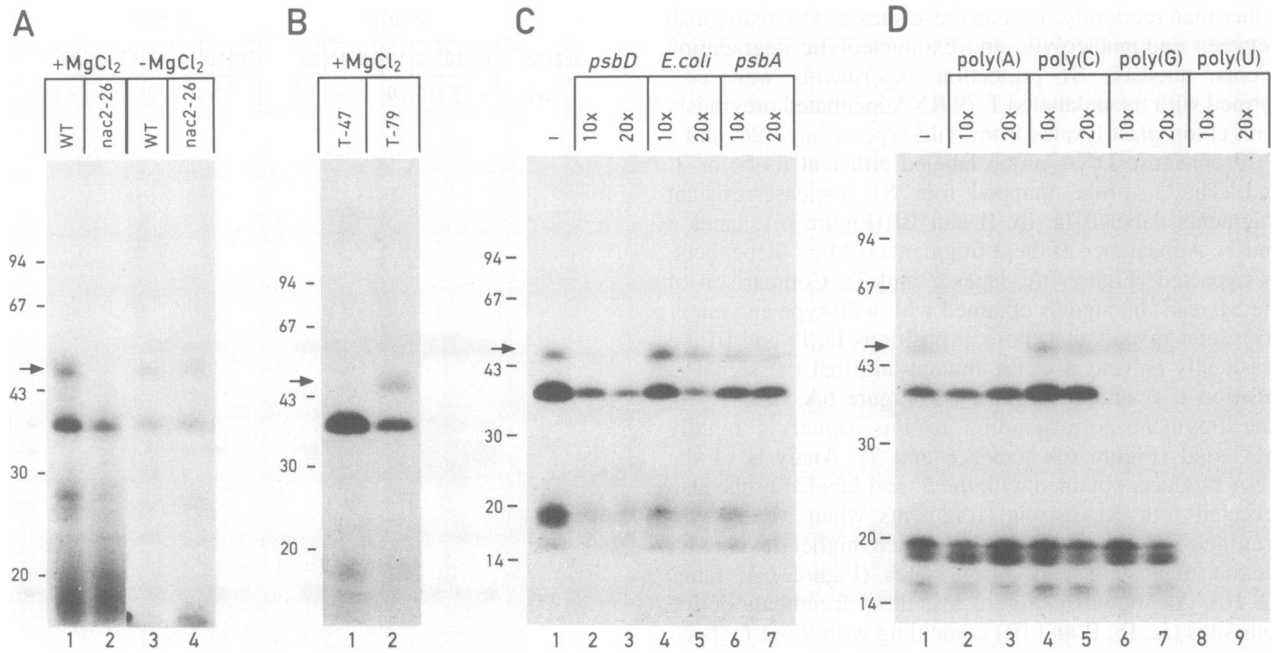

Fig. 8. UV cross-linking of proteins to the *psbD* leader and competition binding experiments. (**A**) Chloroplast proteins from wild-type (lanes 1 and 3) or *nac2-26* (lanes 2 and 4) lysates were covalently labeled by bound T-79 using UV light, digested with RNase A and separated on 12% SDS–polyacrylamide gels. Presence or absence of $MgCl_2$ is indicated. The arrow marks the 47 kDa protein. Molecular sizes (kDa) of marker proteins are given in the left margin. (**B**) Proteins from wild-type lysates labeled by either the RNA probes T-47 (lane 1) or T-79 (lane 2) from the *psbD* leader region. (**C**) Labeled T-79 RNA was incubated with a 10- or 20-fold excess of the given unlabeled competitor RNAs corresponding to the *psbD* 5′ region, the Bluescript polylinker (*E.coli*) and the *psbA* 5′ region in the presence of $MgCl_2$. The arrow marks the 47 kDa protein. Lane 1, control reaction without competitor RNA. (**D**) As in (C) but using different ribohomopolymers as competitors.

plasmid pbD-47 by UV cross-linking (Figure 8B, lane 1). This probe did not label the 47 kDa protein in wild-type lysates, indicating that an essential part of the binding site for this polypeptide is located within the first 27 nt of the *psbD* leader or overlaps the –47 position. It is noteworthy that in the absence of Mg^{2+}, i.e. under conditions where the *psbD* leader RNA is stable with mutant extract, a binding activity at 47 kDa is also detectable in the mutant lysate with the T-79 RNA (Figure 8A, lane 4) but not with T-47 RNA (data not shown). These similar RNA binding specificities suggest that the 47 kDa signal detected in the absence of Mg^{2+} may represent the same 47 kDa RNA binding protein seen exclusively in wild-type extracts in the presence of Mg^{2+}. In this case, the differential RNA binding activity observed between wild-type and mutant extracts would be dependent on Mg^{2+}. It is important to note that the 47 kDa signal, as well as the difference in *psbD* leader stability between wild-type and *nac2-26*, is not detected when the chloroplast lysates are prepared without adding Triton X-100, suggesting that the 47 kDa protein is associated with chloroplast membranes.

To further test the specificity of the interaction between the 47 kDa protein and the labeled *psbD* leader, competition experiments were performed with a 10- and 20-fold excess of various unlabeled RNAs. Addition of unlabeled T-79 RNA resulted in almost complete disappearance of the 47 kDa signal, whereas RNA made from the Bluescript polylinker did not interfere with the RNA–protein interaction (Figure 8C, lanes 2–5). A *psbA* leader probe competed to a limited extent, suggesting a weak interaction of the 47 kDa protein with this RNA *in vitro*. This is further supported by the finding that labeled *psbA* 5′ RNA did not significantly detect the 47 kDa signal in UV cross-linking experiments (data not shown). Amongst ribohomopolymers tested, poly(A) competed selectively for binding of the 47 kDa protein (Figure 8D, lanes 2 and 3) and had little effect on the binding of the other RNA binding proteins. The crucial role of A residues in this RNA–protein interaction is also apparent from the fact that the distal 27 nt region of the *psbD* leader required for binding of the 47 kDa protein (see Figure 6B) is very A-rich (59%). Poly(U) interfered with all detected RNA binding activities, whereas poly(C) and poly(G) did not compete significantly, with the exception of the 40 kDa protein whose binding was competed efficiently by poly(G) (Figure 8D, lanes 6 and 7). It is apparent in Figure 8C (lanes 2 and 3) that in addition to the 47 kDa protein binding, other signals are competed. This may be due to the fact that the *psbD* leader contains U-rich tracts and that poly(U) has a strong quenching effect (Figure 8D, lanes 8 and 9).

Discussion

One structural determinant for psbD mRNA stability is localized within the psbD leader

The *nac2-26* mutant is specifically affected in the accumulation of *psbD* RNA (Kuchka *et al.*, 1989). Other nuclear mutants of *C.reinhardtii* deficient in the accumulation of *psbB* (Sieburth *et al.*, 1991; Monod *et al.*, 1992), *psbC* (Sieburth *et al.*, 1991), *atpA* or *atpB* (Drapier *et al.*, 1992) have been isolated. In each case, a single chloroplast mRNA appears to be affected, suggesting the existence of gene-specific nuclear-encoded chloroplast factors that are involved in RNA stabilization.

While previous studies on chloroplast transcript stability

have concentrated mostly on the role of the RNA 3′ untranslated regions (Stern and Gruissem, 1987; Stern *et al.*, 1989, 1991; Adams and Stern, 1990; Blowers *et al.*, 1993), our work reveals the presence of structural determinants for RNA stability in the 5′ untranslated region of the chloroplast *psbD* transcript in *C.reinhardtii*. Using biolistic transformation and genetic crosses, we have introduced a chimeric gene consisting of the *psbD* 5′ untranslated region fused to the *aadA* coding sequence into the chloroplast genome in both wild-type and *nac2-26* nuclear backgrounds. The chimeric message was shown to be destabilized in the latter, but not in the former case, indicating that the *psbD* leader which consists of 74 nt includes one of the determinants for *psbD* RNA degradation. An important role for a chloroplast 5′ untranslated region in transcript stability has also been shown by Salvador *et al.* (1993). In this study, chimeric chloroplast RNAs derived from the *rbcL* leader fused to a reporter are highly unstable upon illumination. This destabilization can be overcome when 5′ sequences of the *rbcL* coding region are inserted in the chimeric transcript.

Nuclear mutants affected in mitochondrial RNA stability have also been characterized in yeast. Mutants affected at the *CBP1* locus are unable to accumulate cytochrome *b* mRNA (Dieckmann *et al.*, 1984). The effect of these mutations can be suppressed by mitochondrial genome rearrangements in which the 5′ leader region of the cytochrome *b* gene is replaced by the leader sequence of the ATPase subunit 9 gene. From these studies it appears that the *CBP1* product interacts with the distal portion of the cytochrome *b* leader and that this interaction is required for the stabilization of the mRNA (Dieckmann and Mittelmeier, 1987).

The 5′ regions of several prokaryotic mRNAs have also been shown to be important for mRNA stability (Gorski *et al.*, 1985; Belasco *et al.*, 1986). Fusions of these leader sequences to unrelated mRNAs confer stability properties to the chimeric RNA that are largely determined by the new leader.

In vitro instability of psbD RNA in chloroplast lysates of nac2-26

We have used lysates from purified chloroplasts to examine the stability of the *psbD* leader RNA *in vitro*. The results show that *psbD* leader RNA is degraded nine times more rapidly in *nac2-26* than in wild-type extracts. The rapid degradation in *nac2-26* chloroplast lysates appears to be mediated by site-specific endonucleolytic cleavages upstream of the ribosomal binding site.

A similar situation has been found in bacterial systems where endonucleolytic cleavage within the 5′ untranslated region of several messages is followed by rapid RNA degradation (reviewed in Petersen, 1992). While it is clear that there is no significant difference in stability of *E.coli* RNA *in vitro* in wild-type versus *nac2-26* chloroplast extracts, there is a slight (~2.5-fold) enhancement in plastid RNA decay with mutant extract. This effect can be correlated with a decrease in the level of the *psbA* precursor RNA in *nac*2-26 *in vivo* (Figure 1B), although the accumulation of *psbA* mature RNA and other RNAs such as *psbC* transcripts is not altered in the mutant (Figure 1C). These differences between *in vivo* and *in vitro* data could be due to the fact that not all *cis*-acting RNA stability determinants were included in the analyzed RNA probes.

We have been unable to detect the processing in wild-type extracts of the –74 into the –47 form of the *psbD* leader. Most probably this activity and/or essential co-factors are lost during the preparation of the lysate. It is unlikely that position –47 corresponds to a transcription initiation site since repeated attempts to cap the 5′ end of the corresponding abundant *psbD* RNA failed.

A role for a 47 kDa RNA binding protein in psbD mRNA stability is suggested by its inactivity in nac2-26

Analysis of proteins binding to the *psbD* leader has revealed the specific activity of a 47 kDa protein in wild-type that is absent in *nac2-26*. It is therefore possible that this protein plays some role in *psbD* RNA stability. This is further supported by the finding that in eight phototrophic transformants obtained after complementation of *nac2-26* with a cosmid library from wild-type DNA that have been examined, the binding activity of the 47 kDa protein is restored (J.Nickelsen and J.-D.Rochaix, unpublished results). The 47 kDa protein could be involved in the protection of the 5′ end of the *psbD* RNA against the endonuclease whose activity is readily detectable in *nac2-26* lysates. In contrast to bacterial systems where the accessibility of the internal cleavage sites to endonucleases such as RNase K appears to be restricted by secondary structure elements at or near the 5′ end (Emory *et al.*, 1992), no conspicuous stem-loop structure is apparent in the *psbD* leader of *C.reinhardtii*. The possibility that the 47 kDa protein may provide a 5′ mRNA stabilizer function is supported by the fact that essential parts of the binding region of the 47 kDa protein are located within the first 27 nt of the A-rich *psbD* leader starting at position –79. In this context, the absence of binding of the 47 kDa protein to the short *psbD* T-47 probe, which corresponds to the 5′ end of the mature *psbD* RNA, is surprising. However, it can be correlated with increased *in vitro* stability of this probe relative to the longer *psbD* T-79 probe in a *nac2-26* lysate (Table II). These data are compatible with the idea that the *psbD* RNA precursor (starting at position –79) is the target for *NAC-2* function and that this interaction, mediated by the 47 kDa protein, leads to the stabilization of the precursor *psbD* mRNA. In the *nac2-26* mutant, rapid degradation of the *psbD* precursor transcript would result in loss of the mature *psbD* RNA.

Two proteins of 47 and 60 kDa have been shown to interact with the *psbA* leader of *C.reinhardtii* (Danon and Mayfield, 1991). These proteins appear to be involved in the light-induced activation of *psbA* translation. Although a weak effect on *psbA* RNA can be detected *in vivo* as well as in *vitro* in *nac2-26*, it is unlikely that the two 47 kDa proteins binding to the *psbA* and *psbD* leaders, respectively, are the same because the latter but not the former is only detectable with chloroplast extracts solubilized with non-ionic detergent. On the other hand, if the 47 kDa signals represent the same polypeptide, it appears, based on our competition experiments, that its affinity for the *psbD* leader is much higher than for the *psbA* 5′ region, thereby explaining the *psbD*-specific phenotype of *nac2-26*.

The detergent-dependence for *psbD* leader binding suggests that the 47 kDa protein is associated with chloroplast membranes. In yeast, some translational activators of mitochondrial membrane proteins, which presumably interact directly or indirectly with the corresponding leader regions, are localized within mitochondrial membranes. (Costanzo and Fox, 1990; Michaelis *et al.*, 1991). These factors have been proposed to mediate targeting of messages of membrane proteins to membrane-bound ribosomes, thereby enhancing mRNA translation at the final destination of the proteins (Michaelis *et al.*, 1991). It is possible that the 47 kDa protein could have a related function besides its role in *psbD* mRNA stabilization.

Although it is not yet clear whether the *nac2-26* product is the 47 kDa protein or whether it interacts directly or indirectly with this protein, the isolation and characterization of the gene affected in *nac2-26* is likely to provide further insights into the functional role of its product and more generally into mechanisms of chloroplast mRNA stabilization.

Materials and methods

Strains and media

The *nac2-26* and *Fud50* mutants have been described previously (Woessner *et al.*, 1984; Kuchka *et al.*, 1989). Genetic crosses were performed as described (Harris, 1989) except that the agar was washed repeatedly with distilled water and dried before use. Each member of the tetrads was grown on non-selective Tris–acetate–phosphate medium (TAP; Gorman and Levine, 1965) and tested for growth on high salt minimum (HSM; Rochaix *et al.*, 1988) which selects for photosynthesis, and on TAP medium containing 100 μg/ml spectinomycin to test for resistance to the drug. The fluorescence induction transients of individual members of these tetrads were examined to confirm their genotypes.

Nucleic acid analysis and plasmid constructions

Nucleic acids were analyzed and manipulated using standard techniques (Sambrook *et al.*, 1989). PCR was used to amplify DNA fragments corresponding to the *psbD* promoter and 5′ *psbD* untranslated region (from −160 to the ATG initiation codon) from the cloned *Eco*RI fragment R3. The two oligonucleotides used were oligo 800 (5′-CGCATCGATGACTATGCACAAAGC) and oligo 799 (5′-GAGCCATGGACATTGCGTGTATCTCCA). These DNA fragments were cloned into the Bluescript vector SK(−) from which they were excised by digestion with *Cla*I and *Nco*I, which cleave at sites engineered into the 5′ and 3′ ends of these fragments, respectively. The *Nco*I site was positioned immediately 3′ to the ATG initiation codon of *psbD*. These fragments were inserted into the cg20 plasmid which had been cut with *Nco*I and partially digested with *Cla*I. The cg20 plasmid (W.Zerges and J.D.Rochaix, unpublished results) consists of the chloroplast transformation vector patp-int (Goldschmidt-Clermont *et al.*, 1991), containing between its *Xba*I and *Bam*HI sites the *psbC* promoter and leader region fused to the *aadA* structural gene which is terminated with the 3′ end of *rbcL* (Goldschmidt-Clermont, 1991). The resulting plasmid, cg11, had the *psbD* promoter and leader fused to *aadA* within the patp-int vector.

Plasmid pbD-79, containing the *psbD* 5′ region from position −79 to the ATG start codon, was constructed by inserting a PCR fragment amplified by using oligos 799 and 1393 (5′-CGCATCGAAATACACAATGATTAAA) into the *Eco*RI site of the Bluescript SK(−) vector. This intermediate was digested with *Hin*dIII and *Kpn*I, blunt-ended by T4 DNA polymerase treatment and religated to minimize polylinker portions in the final *in vitro* transcript T-79. Plasmid pbD-47, containing the *psbD* leader region from position −47 to the ATG start codon, contains the PCR fragment amplified by using oligos 799 and 2125 (5′-TAATACGACTCACTATAGGGCGAAATTTAACGTAACGATG). The latter contains the T7 promoter sequence and thus allows transcription of this region after insertion of the fragment into the *Sma*I site of pUC19. Plasmid pbA-140 contains the *psbA* 5′ region from position −140 to the ATG start codon (Erickson *et al.* 1984) and was generously provided by W.Zerges.

The chimeric gene containing the structural gene of *atpB* as a reporter fused to the *psbD* 3′ untranslated region was constructed as follows. Plasmid BR5, consisting of the *Bam*HI–*Eco*RI subfragment of Ba5 cloned into pUC19, was cut with *Kpn*I, digested to a limited extent with *Bal*31 and cut with *Eco*RI. A plasmid containing the 354 bp *Eco*RI–*Ava*II fragment of the chloroplast *Eco*RI fragment R06 was cut with *Eco*RI, digested to a limited extent with *Bal*31 and cut with *Bam*HI. The smaller fragment was cloned into the *Bal*31-treated plasmid containing *atpB* and resulted in the plasmid patpBD-18. This plasmid contains the structural gene of *atpB* and the first 35 bases of the *atpB* 5′ untranslated region fused to the 100 3′ terminal bp of *psbD* with 225 bp of 3′ untranslated region.

Primer extensions on *psbA* and *psbC* RNA were performed with oligos 2054 (5′-GATCCATGGTCATATGTTAATTTTTTTAAAG) and 2118 (5′-CCATGGACATGACAACGTTCCTACTAAT), respectively.

Preparation of RNA probes and chloroplast lysates

In vitro RNA probes were prepared from linearized plasmid DNA, as described (Nickelsen and Link, 1989).

For isolation of chloroplasts, 1.5 l cultures of cell wall-deficient *C.reinhardtii* cells were grown at 25°C in dim light (250 lux) in TAP to a density of 2×10^6 cells/ml. After washing with 20 mM HEPES–KOH pH 7.5, cells were resuspended in 40 ml of breaking buffer (300 mM sorbitol, 50 mM HEPES–KOH pH 7.2, 2 mM EDTA pH 8.0, 1 mM $MgCl_2$) and cooled on ice for 3 min. Cell disruptions were performed by passing the suspension through a BioNebulizer (Col gas) at 20 p.s.i. Chloroplasts were sedimented at 4°C at 5000 r.p.m. in an HB4 rotor with fast stopping. The pellets were resuspended in 3 ml of cold breaking buffer and layered on Percoll step gradients (10 ml 75% Percoll and 10 ml 45% Percoll in 300 mM sorbitol, 50 mM Tris–HCl pH 7.9, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol) that were centrifuged for 20 min at 7000 r.p.m. in an HB4 rotor. Chloroplasts were collected from the 75–45% Percoll interphase, sedimented and lysed on ice in 0.5 ml lysis buffer (30 mM Tris–HCl pH 7.9, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, and 0.2% Triton X-100). The lysate was dialyzed against 2 l of dialysis buffer (30 mM Tris–HCl pH 7.9, 0.5 mM EDTA, 100 mM NaCl, 5 mM 2-mercaptoethanol, 25% glycerol, 0.1% Triton X-100), quickly frozen and stored at −70°C. Protein concentrations were determined using the Bio-Rad protein quantification assay.

In vitro degradation of RNA

Radioactively labeled RNA probes (10 ng) were incubated with chloroplast lysate (20 μg protein) at room temperature in degradation buffer [30 mM Tris–HCl pH 7.0, 100 mM NaCl, 5 mM 2-mercaptoethanol, 25 μg/ml poly(C)] in a final volume of 40 μl. $MgCl_2$ concentrations were as indicated. 7.5 μl aliquots were removed at various time points, mixed with 100 μl 1% SDS solution containing 2 μg of *Escherichia coli* tRNA, and phenol-extracted. After ethanol precipitation the RNA was analyzed on 8% sequencing gels.

Nuclease S1 mapping of degradation products of the *psbD* leader was as described (Nickelsen and Link, 1993) using the *psbD* DNA −79 probe, 3′ end-labeled at the *Hin*dIII site or 5′ end-labeled at the *Eco*RI site. Primer extension analysis of whole cell RNA was performed by following the protocol for RNA sequencing of Johanningmeier *et al.* (1987), except that addition of ddNTPs was omitted.

UV cross-linking of RNA with proteins

20 μl reactions contained 40 ng ^{32}P-labeled *in vitro* transcript and chloroplast lysate (40 μg protein) in degradation buffer. Samples were incubated for 5 min on ice and then irradiated with UV light (1 J) in a Stratalinker (Stratagene). After RNase A digestion (3 mg/ml) for 30 min at 37°C, proteins were separated electrophoretically in 12% SDS–polyacrylamide gels (Laemmli, 1970).

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