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A Nuclear Mutation That Affects the 3′ Processing of Several mRNAs in Chlamydomonas Chloroplasts

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We previously created and analyzed a *Chlamydomonas reinhardtii* strain, Δ26, in which an inverted repeat in the 3′ untranslated region of the chloroplast *atpB* gene was deleted. In this strain, *atpB* transcripts are unstable and heterogeneous in size, and growth is poor under conditions in which photosynthesis is required. Spontaneous suppressor mutations that allow rapid photosynthetic growth have been identified. One strain, Δ26S, retains the *atpB* deletion yet accumulates a discrete and stable *atpB* transcript as a consequence of a recessive nuclear mutation. Unlike previously isolated Chlamydomonas nuclear mutations that affect chloroplast mRNA accumulation, the mutation in Δ26S affects several chloroplast transcripts. For example, in the *atpA* gene cluster, the relative abundance of several messages was altered in a manner consistent with inefficient mRNA 3′ end processing. Furthermore, Δ26S cells accumulated novel transcripts with 3′ termini in the *petD–trnR* intergenic region. These transcripts are potential intermediates in 3′ end processing. In contrast, no alterations were detected for *petD*, *atpA*, or *atpB* mRNA 5′ ends; neither were there gross alterations detected for several other mRNAs, including the wild-type *atpB* transcript. We suggest that the gene identified by the suppressor mutation encodes a product involved in the processing of monocistronic and polycistronic messages.

INTRODUCTION

Post-transcriptional events play important roles in prokaryotic and eukaryotic gene expression. In chloroplasts of vascular plants and the green alga *Chlamydomonas reinhardtii*, RNA maturation and stabilization are highly regulated processes that govern mRNA accumulation. RNA structure plays a major role; the 3′ untranslated regions (UTRs) of chloroplast mRNAs usually contain an inverted repeat sequence that potentially can fold into a stem–loop structure. Both in vitro (Stern and Gruissem, 1987) and in vivo (Stern et al., 1991; Blowers et al., 1993; Lee et al., 1996) studies have shown that a stable secondary structure is required for mRNA stability and for generating a discrete 3′ end. In addition, in vitro assays have shown that 3′ UTRs are able to bind soluble chloroplast proteins, including several that bind to the stem–loop structure. Some recognize specific sequences or structures (Chen and Stern, 1991; Yang et al., 1996), whereas others bind nonspecifically (Lisitsky et al., 1995). These proteins may regulate 3′ end maturation and/or stability; a 28-kD spinach protein was shown to be required for RNA processing (Schuster and Gruissem, 1991), and both 41- (Yang et al., 1996) and 100-kD (Hayes et al., 1996) spinach proteins and a 54-kD mustard protein (Nickelsen and Link, 1993) were reported to have ribonuclease homology or activity. Because chloroplast inverted repeats are inefficient transcription terminators (Stern and Kindle, 1993; Rott et al., 1996), 3′ ends are generated by processing events in which the 3′ UTR plays an essential role.

Several mutants with defects in chloroplast mRNA processing and stability have been described. In vascular plants, nuclear mutations in maize (Barkan et al., 1994) and Arabidopsis (Meurer et al., 1996) have been shown to alter transcript accumulation patterns. In Chlamydomonas, numerous nuclear mutations that affect splicing and mRNA accumulation have been isolated (reviewed in Rochaix, 1996). In contrast to the pleiotropic effects seen in the vascular plant mutants, the Chlamydomonas mutations appear to affect the accumulation of a single chloroplast transcript.

Using biolistic transformation to introduce mutations into chloroplast genes, we have studied the *cis*-acting sequences that regulate chloroplast mRNA processing and stability. Much of this work has focused on the *atpB* gene, which encodes the β subunit of the CF_1 complex of ATP synthase and which is transcribed into a monocistronic 1.9-kb mRNA ending in an AU-rich putative stem–loop structure (Woessner et al., 1986). Deletions in the stem–loop structure caused a dramatic decrease in mRNA stability and prevented the accumulation of a discrete transcript in vivo (Stern et al., 1991). Strain Δ26, which lacks the entire stem–loop structure, accumulates ~10% of the wild-type level of mRNA, which is heterogeneous in size. Because it accumulates only ~20% that of the wild-type level of the β subunit, Δ26 grows slowly under

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conditions requiring photosynthesis and is more sensitive to elevated temperature or light intensity than are wild-type cells.

After bombardment of an *atpB* deletion recipient strain (CC-373) with plasmid pΔ26, we noticed some larger colonies among the small, light-sensitive transformants that were selected on minimal media. We reasoned that these colonies might harbor mutations that circumvented or suppressed the defect in *atpB* mRNA stability. We have shown previously that the majority of such robust photosynthetic transformants contain amplified copies of the transforming DNA and consequently synthesize and accumulate increased amounts of *atpB* mRNA relative to single-copy transformants, although the mRNA remains unstable (Kindle et al., 1994; Suzuki et al., 1997). Here, we describe the isolation and characterization of a nuclear suppressor mutation that allows the accumulation of a discrete *atpB* transcript in Δ26. Somewhat surprisingly, this mutation also affects the accumulation of transcripts from other wild-type chloroplast genes. We propose that this locus encodes a product required for the maturation of multiple chloroplast mRNAs.

RESULTS

A Suppressor of Strain Δ26 Accumulates a Discrete *atpB* Transcript

Strain Δ26S was selected as a robust, photosynthetic, non-light-sensitive transformant after bombardment of the *atpB* deletion strain CC-373 with plasmid pΔ26, an *atpB* construct in which the entire 3′ stem–loop structure had been deleted (Stern et al., 1991). To determine the molecular basis for the increased photosynthetic growth rate, RNA filter hybridizations were performed, as shown in Figure 1A. An *atpB* coding region probe revealed three transcripts, the larger two of which were less abundant and also present in Δ26 (indicated by arrowheads). The major transcript in Δ26S, which was almost undetectable in Δ26, was slightly larger than the wild-type *atpB* mRNA and approximately half as abundant based on measurements using a PhosphorImager and normalization to the *psbA* transcript; *psbA* abundance was unaffected by the suppressor mutation. However, because Δ26S contains a 2-kb deletion in the 3′ region of the *atpB* gene, the 3′ end of this major transcript is different from that of the wild type. The level of the ATP synthase β subunit in Δ26S was equivalent to that of wild-type cells (data not shown), suggesting that the improved growth phenotype resulted from an increase in the amount of ATP synthase.

To determine whether the *atpB* gene had been altered, we performed DNA filter hybridization analysis. No gross rearrangements were observed between Δ26 and Δ26S in the 10-kb chloroplast Bam10 fragment, which contains the *atpB* gene (data not shown). The *atpB* 3′ region was amplified from Δ26S total DNA by polymerase chain reaction (PCR) and sequenced, revealing exactly the same sequence as that seen for Δ26. Together, these results suggested that the *atpB* gene had not been altered and that an extragenic suppressor mutation was responsible for the increase in *atpB* mRNA abundance.

A possible mechanism for generating a new, discrete *atpB* transcript in Δ26S would be to generate and/or stabilize an alternative 3′ end. Therefore, we compared the 5′ and 3′ ends of the *atpB* transcripts in Δ26 and Δ26S. The transcript termini were mapped by RNase protection, using an antisense RNA probe that had been uniformly labeled with ^{32}P-UTP (Figure 1B). As a control and size marker, an in vitro–synthesized probe complementary to the wild-type *atpB* 3′ region was hybridized with total wild-type RNA. Finally, a *petD* 3′ end probe was used to control for RNA quantity. Figure 1C shows that a single 170-nucleotide species was protected from wild-type RNA when the wild-type *atpB* 3′ probe (second lane from left) was used. In Δ26S, two *atpB* 3′ termini were protected by the Δ26 probe, and their abundance relative to the wild type correlated well with the accumulation of the most abundant Δ26S transcript seen on RNA filter blots (Figure 1A). Figure 1C shows that at least one of the same *atpB* 3′ termini was also present in Δ26 but at a 10-fold-lower level. Therefore, we conclude that the suppression mechanism most likely acts at the level of mRNA metabolism.

The approximate location of these 3′ ends within the downstream DNA sequence was determined by using RNA and DNA size markers and corresponds well with the size predicted for the shorter, more abundant Δ26S *atpB* transcript (Figures 1A and 1B). Examination of the sequence in the approximate location of these 3′ ends revealed no obvious alternative stem–loop structures. No fully protected probe was detected that would have been predicted if the longer *atpB* transcripts in Δ26 and Δ26S were extended at the 3′ end. Therefore, we considered the possibility that these transcripts contained new 5′ termini or that they were derived from the other DNA strand. 5′ End mapping was performed by RNase protection, but no differences were seen among wild-type, Δ26, and Δ26S *atpB* mRNA 5′ ends (data not shown). Furthermore, no protected fragments were detected by RNase protection, using a uniformly labeled sense RNA probe corresponding to the 3′ region of the *atpB* gene, which indicates that the large transcript probably is not an antisense transcript. Therefore, the most likely explanation for our inability to map these longer *atpB* transcripts is that their lower quantity and/or inability to form a stable duplex under our hybridization conditions precludes their detection.

The Suppressor Mutation Affects RNA Processing in the *petD* 3′ Region

Δ26 and Δ26S contain a modified chloroplast *atpB* gene, but to the best of our knowledge, the rest of the chloroplast genome is wild type. Therefore, we were intrigued when RNA filter hybridizations using a *petD* probe revealed a low-abundance, higher molecular weight transcript in the suppressor strain in addition to the normal 0.9-kb RNA (Figure

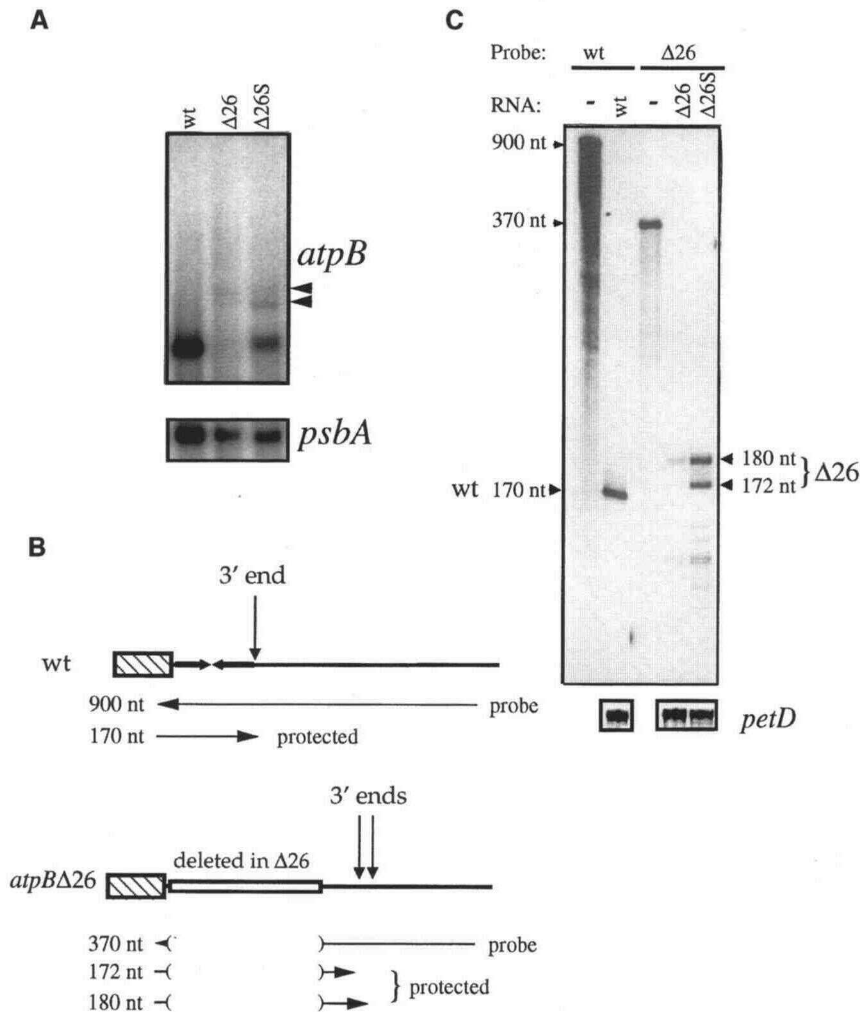

Figure 1. *atpB* Transcripts in Wild-Type and Suppressor Strains.

(A) RNA gel blot analysis. A filter containing 5 μg of total RNA from the indicated strains was probed with *atpB* or with the *psbA* coding region as a loading control (see Methods). The arrowheads mark the minor *atpB* transcripts common to Δ26 and Δ26S, as discussed in the text.

(B) The *atpB* 3′ region in wild-type and Δ26 cells. The dark arrows downstream of the gene in wild-type cells represent the inverted repeat sequence that is deleted in Δ26. The uniformly labeled antisense probes used for RNase protection are shown, as are the protected fragments (see **[C]**), for which sizes were calculated according to DNA size markers and the known size of the wild-type protected fragment (Stern and Kindle, 1993).

(C) RNase protection. Ten micrograms of RNA was hybridized with a uniformly labeled antisense RNA corresponding to the probes shown in **(B)**. The hybrids were digested with RNases for 15 min at 25°C and resolved in a 5% polyacrylamide–urea gel. A second RNase protection assay was performed using a *petD* RNA probe (bottom), which served as a loading control. Lanes marked with (−) are reactions containing 10 μg of yeast tRNA rather than Chlamydomonas RNA.

nt, nucleotide; wt, wild type.

2A). The 5′ end of the *petD* transcript, which is generated by a processing event (Sakamoto et al., 1994), was identical in the wild type and Δ26 and Δ26S, as determined by primer extension (data not shown). To detect possible differences at the 3′ ends of *petD* transcripts, we performed an RNase protection experiment. A tRNA^{Arg} gene (*trnR*) is located immediately downstream of the *petD* gene (Yu and Spreitzer, 1991). Although *trnR* probably has its own promoter, read-through transcription of *petD* is also expected because the 3′ UTR of *petD* is an inefficient transcription terminator (Rott et al., 1996). Therefore, we used as a probe a 535-nucleotide transcript complementary to the *petD* 3′ UTR and full-length

trnR, as shown at the bottom in Figure 2B. The sizes of the protected products were estimated using DNA size markers. The results shown in Figure 2B revealed two major protected bands in all strains: a smaller one of 73 nucleotides corresponding to the processed tRNA and a larger one of ~200 nucleotides representing the 3′ end of mature *petD* mRNA. In addition, two weaker high molecular weight bands were protected by the wild-type and Δ26 RNAs; these products migrated between the probe and the mature *petD* 3′ end. We presume that these represent processing intermediates between the 3′ end of *petD* and the 5′ end of *trnR*. In Δ26S, three additional protected fragments were detected (Figure 2B, arrowheads). Quantification showed that these additional transcript ends accumulated to <0.5% of the level of the mature 3′ end and therefore are unlikely to affect subunit IV expression. Nevertheless, their presence suggests that the gene mutated in Δ26S might be involved in *petD* mRNA 3′ end maturation.

The Suppressor Mutation Modifies the Abundance of Polycistronic Transcripts That Contain the *atpA* Coding Region

As shown above, Δ26S exhibited increased accumulation of several monocistronic *atpB* transcripts and accumulated

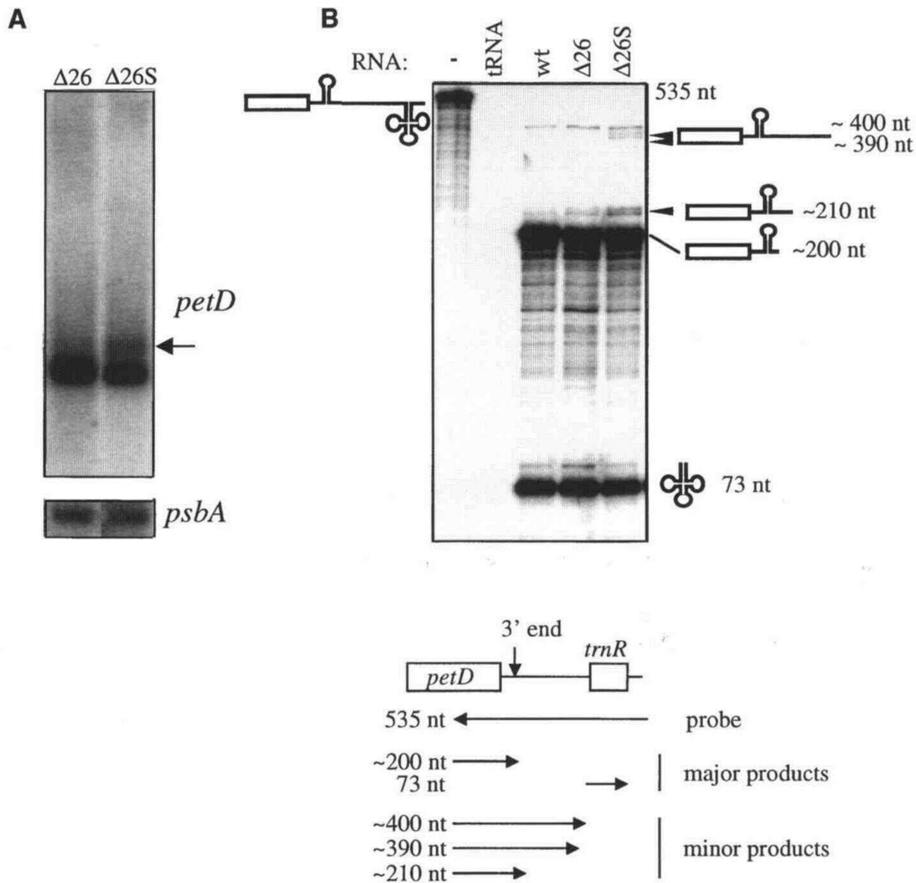

Figure 2. *petD* Transcripts in Wild-Type and Suppressor Strains.

(A) RNA gel blot analysis. A filter containing 5 μg of total RNA from the indicated strains was probed with *petD* or with the *psbA* coding region as a loading control. The arrow marks the novel *petD* transcript in Δ26S.

(B) The *petD* 3′ end in wild-type and suppressor strains. The probe and protected products are diagrammed at bottom. For the RNase protection reactions, 10 μg of Chlamydomonas total RNA or yeast tRNA was hybridized with the uniformly labeled antisense probe and treated with RNase, as described in Stern and Kindle (1993), for 10 min at 25°C. (−) indicates the lane containing the probe. The products were resolved in a 5% polyacrylamide–urea gel. The diagrams to the right of the gel represent putative intermediates in *petD* mRNA processing, as discussed in the text. nt, nucleotide; wt, wild type.

novel putative processing intermediates with 3′ ends between *petD* and *trnR*. To determine whether RNA accumulation in Δ26S was altered in the relatively complex context of a polycistronic gene cluster, we examined transcripts derived from the *atpA* region. Figure 3A shows that downstream of *atpA*, which encodes the α subunit of the ATP synthase (Dron et al., 1982), lies *psbI*, which encodes a nonessential photosystem II subunit (Kunstner et al., 1995), *ycf10* (GenBank accession number X90559), a *cemA* homolog that may encode a heme binding protein in the chloroplast inner membrane (Willey and Gray, 1990), and *atpH*, which encodes subunit III of the ATP synthase CF_0 complex (GenBank accession number X90559). Figure 3B shows that when an RNA blot was probed with the *atpA* coding region, four transcripts were identified in wild-type and Δ26 cells. We have mapped the 3′ ends of these transcripts (H. Suzuki, D.B. Stern, and K.L. Kindle, unpublished results). As shown in Figure 3A, these transcripts correspond to monocistronic, dicistronic, tricistronic, and tetracistronic messages containing the *atpA* coding region. *psbI* and *atpH* also accumulate in a monocistronic form (Kunstner et al., 1995; H. Suzuki, D.B. Stern, and K.L. Kindle, unpublished results), and *ycf10* also accumulates in non-*atpA*-containing transcripts (data not shown).

The RNA filter hybridization shown in Figure 3B revealed two quantitative changes in the *atpA*-containing transcripts of Δ26S relative to those of Δ26 and wild-type cells. First, the abundance of the dicistronic *atpA* message (transcript 2) was reduced to ~30 to 50% of the wild-type level, when normalized to *psbA*. Second, the tricistronic message (transcript 3) could not be detected even after long exposures. These results are consistent with instability of these two transcripts or with a failure to efficiently process precursor transcripts downstream of *psbI* and *ycf10*.

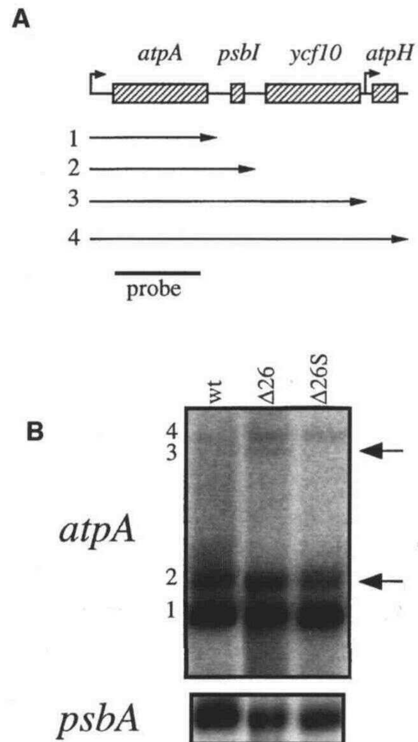

Figure 3. *atpA* mRNA in Wild-Type and Suppressor Strains.

(A) Map of the Chlamydomonas chloroplast *atpA* gene cluster. *atpA*-containing transcripts are numbered 1 to 4. Promoters, indicated by arrows, are known to exist upstream of the *atpA* and *atpH* genes (Dron et al., 1982; H. Suzuki, D.B. Stern, and K.L. Kindle, unpublished data).

(B) *atpA*-containing transcripts. RNA was size fractionated in an agarose–formaldehyde gel, transferred to a nylon filter, and hybridized with an *atpA* coding region probe, with *psbA* being used as a loading control. Arrows at right indicate the transcripts that differ between Δ26 and Δ26S. wt, wild type.

The Suppressor Mutation Is Encoded by the Nuclear Genome

The pleiotropic effects of the suppressor mutation suggested that it might affect a *trans*-acting protein that regulates RNA processing or stability. To determine whether the suppressor mutation was located in the nuclear or chloroplast genome, Δ26S (mt^+) was crossed with two mt^- strains that are wild type with respect to the suppressor mutation: Δ27^ (Kindle et al., 1994) and wt12 (see Table 1). Because chloroplast DNA is inherited uniparentally from the mt^+ parent, the tetrad progeny from these crosses should carry the Δ26 deletion in *atpB* as well as any chloroplast-encoded suppressor mutation. Eight complete tetrads from the two crosses were analyzed by RNA filter hybridization using an *atpB* probe. The results from a representative tetrad are presented in Figure 4A. It is clear that the accumulation of the discrete, wild-type–sized *atpB* transcript segregates 2:2, indicating that the suppressor mutation is encoded in the nucleus.

To determine whether the observed differences in the accumulation of *petD*, *atpA*, and *atpB* mRNAs were all caused by the same mutation, we examined the cosegregation of these phenotypes in tetrad progeny. For *atpA* and *atpB*, we analyzed six complete tetrads, two incomplete tetrads (three of four progeny cells), and ~40 random progeny. The *petD* phenotype was determined for two complete tetrads and two incomplete tetrads (three of four). The same progeny carrying the suppressor mutation, as revealed by the accumulation of a discrete *atpB* transcript, accumulated less of the dicistronic *atpA* message (Figure 4A) and also accumulated putative processing intermediates of the *petD* transcript (Figure 4B). In addition, the tricistronic *atpA*-containing transcript was absent in progeny containing the *atpB* suppressor, whereas the unsuppressed progeny accumulated both the tricistronic and

Table 1. Chlamydomonas Strains Used in This Study

Strain	Relevant Genotype[a]	Mating Type	Source
CC-373	*nit1 nit2* (Δ*atpB*)	+	Shepherd et al. (1979)
P17	*nit1 nit2*	+	Stern et al. (1991)
Δ26	*nit1 nit2* (*atpB*Δ26)	+	Stern et al. (1991)
Δ26S	*nit1 nit2 crp3* (*atpB*Δ26)	+	This study
Δ27^	*nit1 nit2* (*atpB*Δ27)	−	Kindle et al. (1994)
Δ4	*nit1 nit2* (*atpA*Δ4)	+	This study
Δ12	*nit1 nit2* (*atpB*Δ12)	+	This study
A10	*nit4*	−	R. Schnell, University of Minnesota, St. Paul, MN
wt12	Wild type, derived from culture collection strain CC-621		J. Girard-Bascou, IBPC, Paris[b]
CC-3396	*nit1 arg7*	−	U. Goodenough, Washington University, St. Louis, MO
Δ26S::A10	Diploid between Δ26S and A10	−	This study
Δ26S::UG126	Diploid between Δ26S and CC-3396	−	This study

[a] Chloroplast genotypes are enclosed in parentheses.
[b] IBPC, Institut de Biologie Physico-Chimique.

tetracistronic *atpA*-containing transcripts (Figure 4 and data not shown). These results suggest that a single nuclear locus affects transcript accumulation from these three genes.

Δ26S Carries a Recessive Suppressor Mutation

Diploid strains of Chlamydomonas can be selected after genetic crosses if the parents carry complementing auxotrophic mutations (Harris, 1989). To create heterozygous diploid Δ26 cells with one wild-type and one mutant allele of the suppressor mutation, we mated strains carrying the complementary auxotrophic markers *nit4* (A10) and *nit1 nit2* (Δ26S), which confer a requirement for a reduced nitrogen source. Diploids were selected by their ability to use nitrate as the sole source of nitrogen (see Methods). Initially, diploid chloroplasts were heteroplasmic; however, after several cycles of single-colony isolation, diploids homoplasmic for the Δ26 chloroplast genome were obtained.

The phenotypes of the diploid cells were analyzed by RNA filter hybridization using an *atpB* probe (Figure 5A) or by RNase protection using a *petD* probe (Figure 5B). In the case of *atpB*, the Δ26S::A10 diploid did not accumulate an abundant, discrete transcript akin to that of Δ26S. Thus, the suppression of *atpB* mRNA instability is a recessive trait. The RNase protection assay (Figure 5B) clearly indicates that the additional putative processing intermediates of the *petD* transcript that accumulated in the suppressed parent did not accumulate in the diploid. Further indication that the suppressor was recessive came from RNA filter hybridization analysis of *atpA* mRNAs in diploids made between Δ26S (*nit1 nit2*) and CC-3396 (*nit1 arg7*). In these diploids, the transcript pattern resembled that of wild-type cells (data not shown). Together, these results indicate that the multiple alterations in chloroplast transcript patterns are most likely a consequence of a single recessive mutation.

The Suppressor Mutation Decreases the Accumulation but Not the Stability of the Dicistronic *atpA–psbI* Message

To begin to examine the mechanism by which the suppressor mutation affects the accumulation of several chloroplast transcripts, we compared the half-life of the *atpA–psbI* transcript in Δ26 and Δ26S. We chose this transcript because its accumulation decreased substantially in Δ26S, but it was still abundant enough to be quantified. Its half-life was determined by measuring its abundance at varying times after the addition of actinomycin D to logarithmically growing cells. RNA isolated from equal numbers of cells was analyzed by filter hybridization with *atpA* followed by a *psbA* probe being used as a loading control. Blots such as the one shown in Figure 6 were quantified by using a PhosphorImager. The values for *atpA*-containing transcripts were normalized to the *psbA* values, because *psbA* abundance appeared to be unaffected by the suppressor mutation. This method does not allow absolute half-lives for the *atpA–psbI* transcript to be determined, because *psbA* mRNA also decays during the time course. However, the decay of *atpA–psbI* transcripts relative to *psbA* provides a useful measure of the effect of the suppressor mutation on *atpA–psbI* mRNA stability.

In Δ26, approximately half of the monocistronic *atpA* message remained after 12 hr in the presence of actinomycin D, whereas half of the dicistronic *atpA–psbI* transcript had decayed after only 30 min (see Figure 6). The tetracistronic

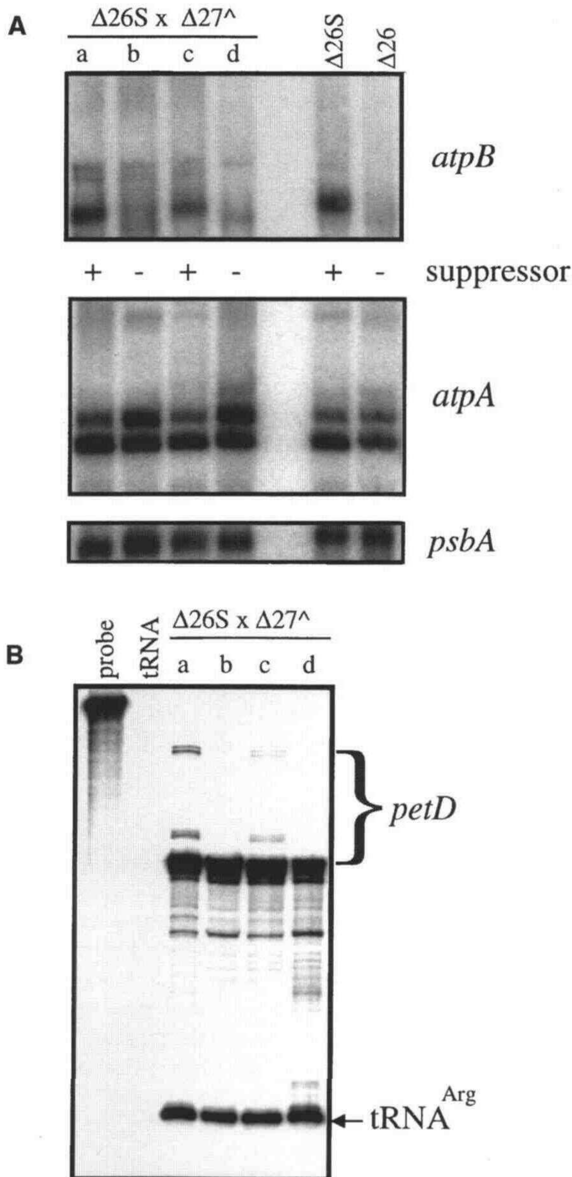

Figure 4. RNAs in Tetrad Progeny from Crosses between Δ26S and a Wild-Type Strain.

RNA filter hybridizations were performed using 5 μg of total RNA extracted from the indicated strains.

(A) The filters were probed with the *atpB* or the *atpA* coding region or with *psbA* as a loading control. a to d represent four progeny from a representative tetrad from the cross. The presence (+) or absence (−) of the suppressor mutation is indicated between the gels.

(B) RNase protection of the *petD–trnR* region was performed as described for Figure 2, using total RNA extracted from tetrad progeny.

transcript in Δ26 and Δ26S also declined during the course of the experiment, but the low signal intensity precluded quantification. The lower abundance of the *atpA–psbI* transcript in Δ26S was not due to a shorter half-life, because the rate of decay in Δ26S was the same or slightly slower than in Δ26. Thus, although this message accumulates to different steady state levels in Δ26 and Δ26S, this appears to be due to different rates of formation, perhaps at the level of RNA processing, rather than to differences in RNA stability. Because actinomycin D is a nonspecific inhibitor of both nuclear and organellar transcription, secondary effects, such as the depletion of a regulatory protein with a short half-life, cannot be ruled out. However, because the half-life of the dicistronic transcript is <30 min, such secondary effects should be minimized.

The Suppressor Mutation Acts on a Specific *cis* Element in the *psbI–ycf10* Intergenic Region

As shown in Figure 3, the suppressor mutation decreased the abundance of the *atpA–psbI* cotranscript. To address the question of whether the suppressor mutation acts

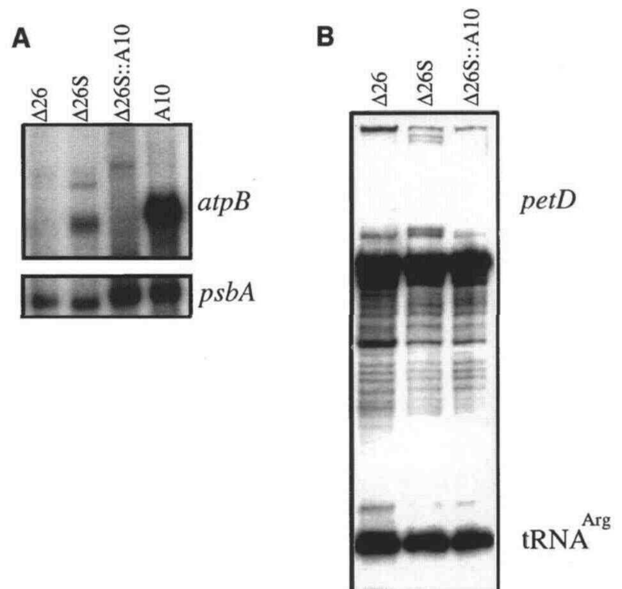

Figure 5. RNA in a Heterozygous Diploid Strain.

(A) RNA filter hybridization using 15 μg of total RNA extracted from three haploid strains (Δ26, Δ26S, and A10) and the diploid strain (Δ26S::A10) probed with *atpB* or *psbA*, which was used as a loading control.

(B) RNase protection of the *petD–trnR* region. The RNase protection assay was performed, as described for Figure 2, using total RNA extracted from the haploid strains Δ26 and Δ26S and the diploid strain Δ26S::A10.

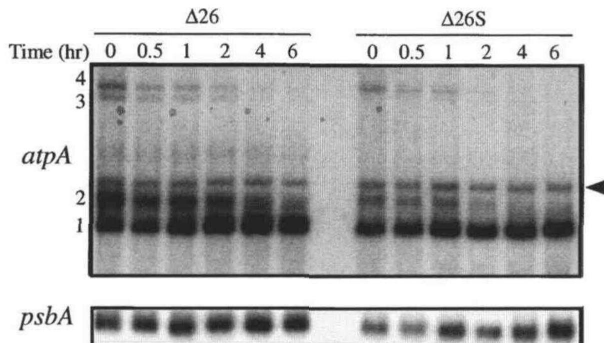

Figure 6. Decay of *atpA* Gene Cluster Transcripts after Actinomycin D Treatment.

Δ26 and Δ26S cells were treated for periods up to 6 hr with 100 mg/mL actinomycin D, as described in Methods. RNA was prepared and analyzed by filter hybridization with *atpA* and *psbA* probes. The *atpA* probe in these experiments was the 3.5-kb chloroplast EcoRI fragment 22, which also contains *psbI* and part of *ycf10*. This experiment was one of four independent experiments demonstrating the same general trend. The different *atpA*-containing transcripts are marked 1 to 4, according to the legend to Figure 3A. The additional transcript just above the *atpA–psbI* cotranscript (arrowhead) corresponds to one of the non-*atpA*-containing *ycf10* transcripts.

specifically on the 3′ end of the *atpA–psbI* cotranscript, we examined its effect on the accumulation of *atpA*-containing transcripts from strains containing derivatives of the *atpA* gene cluster in which the *atpA–psbI* or *psbI–ycf10* intergenic region was deleted (H. Suzuki, D.B. Stern, and K.L. Kindle, unpublished results). Figure 7A shows that strain Δ12 lacks the *atpA–psbI* intergenic region and failed to accumulate the monocistronic *atpA* transcript, whereas strain Δ4 lacks the *psbI–ycf10* intergenic region and did not accumulate the *atpA–psbI* cotranscript. Because of the deletion, however, the Δ12 "dicistronic" transcript migrates at a position similar to that of the monocistronic *atpA* transcript. Furthermore, the tricistronic and tetracistronic transcripts in Δ12 and Δ4 are slightly shorter than those in wild-type cells. These transcript profiles are shown in Figures 7B and 7C (lanes Δ12, Δ4, and wt). To determine the effect of the suppressor mutation on these altered *atpA* gene cluster transcripts, Δ12 and Δ4 (mt^+) were crossed to Δ26S (mt^-) and analyzed by RNA gel blot analysis (Figure 7B). We predicted that Δ12 transcript 2 would still be affected by the suppressor because the putative "target," the *psbI–ycf10* intergenic region, was still present. In contrast, this target was absent in Δ4; therefore, no effect of the suppressor was anticipated.

Figure 7B shows that the *atpA–psbI* cotranscript (transcript 2) in Δ12 × Δ26S progeny containing the suppressor mutation (+) accumulated to ~50% that of the level of the progeny lacking the suppressor mutation (−). The magnitude of this difference is similar to the relative levels of transcript 2 in Δ26 and Δ26S. In contrast, Figure 7C shows that all progeny of the cross Δ4 × Δ26S accumulated monocistronic *atpA* transcript to similar levels (in this cross, strains containing the suppressor can be identified by the lack of transcript 3). Together, these results support the hypothesis that the suppressor mutation acts on the 3′ end of the *atpA–psbI* cotranscript, a region that was deleted in Δ4.

DISCUSSION

In this article, we have described the isolation and characterization of strain Δ26S, which carries a recessive, pleiotropic nuclear mutation. This mutation increases the abundance of an *atpB* transcript lacking the wild-type inverted repeat at its 3′ end. Interestingly, the suppressor mutation did not appear to affect the processing or accumulation of a wild-type *atpB* transcript when it was introduced into the suppressed line by transformation or a genetic cross (data not shown). However, the mutation did affect transcripts from at least two other transcribed regions, the *atpA* gene cluster and the *petD–trnR* region. This distinguishes Δ26S from other Chlamydomonas nuclear mutations that act at the level of RNA processing or stability and that appear to act on single chloroplast genes. The most striking effect was on transcripts from the *atpA* gene cluster. Here, accumulation of two of the four *atpA*-containing transcripts was reduced. Because we failed to detect a significant difference in the half-life of the dicistronic *atpA–psbI* transcript, we presume that the suppressor mutation acts at the level of mRNA processing, that is, that the affected transcripts are not less stable but rather are generated at a slower rate. Although this predicts that the presumptive precursor of these transcripts, the tetracistronic transcript 4, would increase in abundance, this was not the case. This suggests either that the dicistronic and tricistronic transcripts do not require the tetracistronic message as a precursor or that in the absence of processing, the production or stability of the tetracistronic transcript is strictly limited. We cannot currently distinguish between these possibilities.

In the *petD–trnR* region, the suppressor mutation did not appear to affect tRNA processing. Instead, it allowed the accumulation of small amounts of putative processing intermediates of *petD* 3′ end maturation. In wild-type cells, two such intermediates accumulated, whereas in Δ26S at least five were detected (Figure 2). This suggests that the formation of the *petD* 3′ end could be a multistep process. Several (four or five) intermediates were also seen during an in vitro analysis of processing of the spinach chloroplast *psbA–trnH* region. This region also includes an mRNA 3′ end and downstream tRNA (Stern and Gruissem, 1987). It is not clear whether the processing intermediates seen in the *petD–trnR* region arise from the mRNA and/or tRNA processing pathway. In chloroplasts, cotranscription of protein-coding and tRNA genes is quite common (e.g., Christopher and Hallick, 1990; Delp et al., 1991; Stevenson and Hallick, 1994).

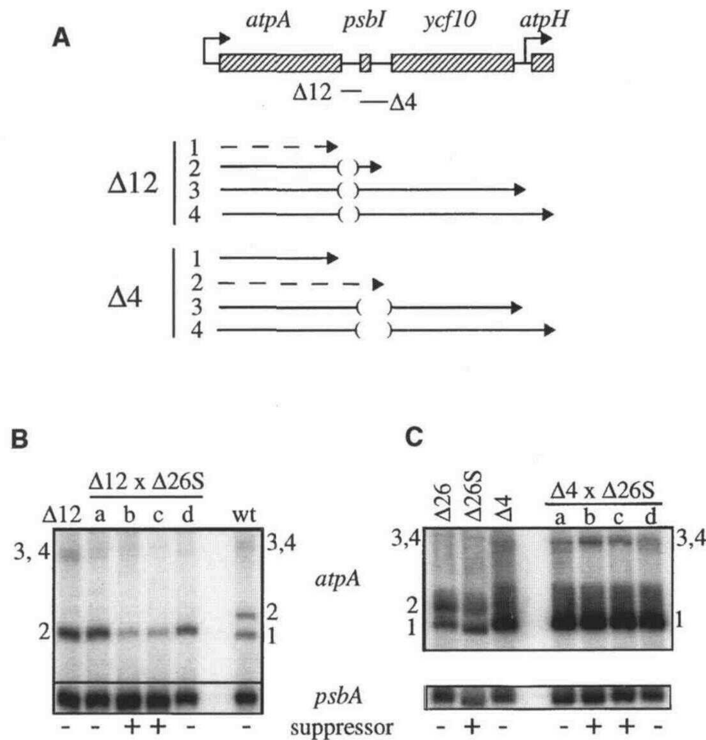

Figure 7. *atpA* mRNA in Tetrad Progeny from Crosses between Δ26S and Δ4 or Δ12.

(A) Map of the Chlamydomonas chloroplast *atpA* gene cluster. The extents of the deletions are marked by bars under the map (see Methods). The different *atpA*-containing transcripts from Δ4 and Δ12 are indicated by arrows and numbers. The dashed lines indicate transcripts that no longer accumulate as a result of the deletion.

(B) and **(C)** RNA filter hybridizations. Five micrograms of total RNA was extracted from the indicated strains, separated in gels, transferred to filters, and probed with the *atpA* coding region or with *psbA*, which was used as a loading control. a to d represent four progeny from a representative tetrad from each cross. The presence (+) or absence (−) of the suppressor is indicated below the gels; this was determined by the presence of the tricistronic transcript (Δ4) or the accumulation of the high molecular weight *petD* transcript (Δ12). wt, wild type.

Because we could detect no differences in the stability of the dicistronic *atpA–psbI* transcript and because the suppressor results in both increased and decreased accumulation of different chloroplast transcripts, we propose that the suppressor mutation affects a gene that encodes a protein involved in chloroplast RNA processing. We propose to call it *crp3* (for chloroplast RNA processing). Nuclear mutations *crp1* and *crp2* affect the processing of polycistronic transcripts in maize chloroplasts (Barkan et al., 1994). The *crp1* mutation blocks the formation of the monocistronic forms of chloroplast *petD* and *petB* mRNAs, although processing of other chloroplast transcripts appears to be normal. As discussed above, other Chlamydomonas nuclear mutations affecting chloroplast mRNA accumulation appear to be specific for a single gene and are thought to act at the level of mRNA stability. Although their mechanisms of action are unclear, the targets have been defined in at least two cases. The *nac2-26* mutation, for example, acts on the 5′ end of *psbD* mRNA (Nickelsen et al., 1994), whereas *ncc1* seems to act on the 3′ end of *atpA* mRNA (Drapier et al., 1992). *crp3* is distinct as an example of a nuclear mutation in Chlamydomonas that has pleiotropic effects.

The processing of mRNAs in chloroplasts may involve enzyme activities similar to those in bacteria. If Δ26S had an altered-function or loss-of-function mutation in a gene encoding such an enzyme, multiple effects on RNAs might be seen. Furthermore, if that enzyme were partially redundant, mutant phenotypes might be limited to a subset of transcripts. In *Escherichia coli*, the endoribonucleases RNase E and RNase III and the exoribonucleases polynucleotide phosphorylase (PNP) and RNase II play major roles in RNA processing and degradation (Deutscher, 1993). Possible homologs of RNase E have been identified in animal cells (Wennborg et al., 1995) and in spinach chloroplasts (Hayes et al., 1996); a PNP-like protein was also found in spinach (Hayes et al., 1996). Recently, it was demonstrated in *E. coli* that RNase E and PNP copurify and are likely to be part of the same protein complex (Carpousis et al., 1994). A multiprotein

complex associated with mRNA processing was also reported in spinach (Hayes et al., 1996). The *crp3* mutation could affect the activity of such a complex directly or indirectly either by possessing a processing activity itself or by altering the expression or activity of a processing enzyme. In any case, a defect in processing could result in decreased accumulation of some transcripts (e.g., the dicistronic and tricistronic *atpA* transcripts) if the mutated gene were involved in their maturation. In this case, the lack of processing would be reflected as accumulation to a lower level without any decrease in half-life. On the other hand, if the processing activity acted downstream of a processing intermediate or as part of a degradation pathway, the abundance of these molecules would increase as a result of slower degradation (e.g., the high molecular weight *petD* and *atpB* transcripts).

Because redundancy in ribonuclease activities is a hallmark of the *E. coli* proteins (Deutscher, 1993), it would not be surprising if the *crp3* background still allowed normal processing of most chloroplast messages. This is the case, for example, in *E. coli*. In this bacterium, mutations in the RNase E gene affect only a small number of transcripts. Ribonuclease mutations, however, can have complex effects on RNA accumulation from operons, for example, in the *rpsO–pnp* region, in which multiple factors determine RNA patterns and abundance (Haugel-Nielsen et al., 1996). Nonetheless, our findings are consistent with the involvement of a single protein in the maturation of multiple mRNAs in Chlamydomonas chloroplasts.

METHODS

Strains, Culture Conditions, and Genetic Analysis

The strains used in this study are shown in Table 1. In strain Δ4, a 267-bp HpaI-HindIII fragment was deleted; this fragment contains the *psbI* coding region and 158 bp of 3′ flanking sequences. In strain Δ12, a 314-bp HpaI fragment was deleted; this fragment contains most of the *atpA–psbI* intergenic region. The properties of these strains will be described in detail elsewhere (H. Suzuki, D.B. Stern, and K.L. Kindle, unpublished results). Cells were grown in HS medium (Harris, 1989) or HS medium supplemented with 1.2 g/L sodium acetate (HSA) under constant fluorescent lighting. Genetic crosses were performed using standard techniques (Harris, 1989). Diploid cells were selected from rapidly appearing colonies exhibiting complementation of the two auxotrophic defects. Diploid cells appeared larger than haploid cells, as determined by using light microscopy, and their mating types were confirmed to be mt$^-$.

Plasmids and Probes

Plasmid pA09, which contains most of the *atpA* coding region, was generated by subcloning the 0.9-kb EcoRI-PstI fragment of the chloroplast EcoRI fragment 22 into pBluescript SK+ (Stratagene). This 0.9-kb fragment was used as a probe in the RNA filter hybridizations. The *atpB* 3′ end was polymerase chain reaction (PCR) amplified from Δ26 and Δ26S by using primers 8906 and 9001 (Stern et al., 1991). This served as a template for DNA sequencing and a probe for the RNase protection assays. The 3′ end of the *petD* gene, including the downstream tRNA^{Arg} gene (*trnR*), was amplified using primers DBS6 and DBS7 (Rott et al., 1996). All PCR-amplified fragments were cloned into pBluescript SK+. The *Chlamydomonas reinhardtii psbA* gene used as a probe was EcoRI fragment 15. We used *psbA* as a loading control, because the abundance of this transcript was not affected by the suppressor mutation. The *atpB* probe was generated by PCR amplification of a 1-kb fragment from the 5′ coding region with the primers DBS2 (GCGTTAGTGAATAATC) and NS1B (GACCGTATACAAGAGCTAC).

Isolation of Nucleic Acids, Filter Hybridizations, and RNase Protection

For whole-cell nucleic acid preparations, cells were grown in HSA. RNA was isolated using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH), according to the protocol provided but with a minor modification: 1 mL of Tri-reagent was used at 55°C to lyse 3 to 5 × 10^7 cells. RNA was size fractionated in 1.1% agarose–6% formaldehyde gels for 3 hr at 80 V in Mops (20 mM) running buffer and transferred to nylon membranes (Amersham Corp.) by using a pressure blotter (Stratagene). Nucleic acids were labeled using the random priming method (Feinberg and Vogelstein, 1983). DNA:RNA hybridizations were performed according to Church and Gilbert (1984). The 3′ ends of the *atpB* and *petD* transcripts were mapped using the above-mentioned plasmids, and RNase protection assays were conducted as previously described (Stern and Kindle, 1993). Gel imaging and quantification were performed by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Transcription Inhibition Using Actinomycin D

To determine the actinomycin D concentration that inhibited chloroplast RNA synthesis, Δ26 was grown to logarithmic phase (2 to 3 × 10^6 cells per mL), and actinomycin D was added to a final concentration of 50 or 100 mg/mL. Samples (5 mL) were collected after 12 and 24 hr, RNA was immediately extracted, and the levels of *atpA* transcripts were determined by RNA filter hybridization. With 100 mg/mL actinomycin D, no *atpA* transcripts could be detected at the 24-hr time point. At 50 mg/mL, however, relatively high levels of *atpA* mRNA could still be detected; therefore, 100 mg/mL was used in the following experiments. To compare the decay rates of the dicistronic *atpA–psbI* transcript between Δ26 and Δ26S, cultures were grown to logarithmic phase, and actinomycin D was added to a final concentration of 100 mg/mL. Each culture was then divided into six 5-mL aliquots, which were harvested at the different time points, and RNA was prepared immediately. Half of the RNA from each time point was analyzed by filter hybridization and quantified by using PhosphorImager analysis. In this experiment, we used the complete 3.5-kb EcoRI fragment 22 as a probe to allow the high molecular weight *atpA*-containing transcripts to be detected. Because this fragment contains coding regions for *psbI* and part of *ycf10*, an additional non-*atpA*-containing *ycf10* transcript appeared above the dicistronic *atpA–psbI* cotranscript.

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