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A Nuclear Gene in Maize Required for the Translation of the Chloroplast *atpB*/*E* mRNA

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To elucidate mechanisms that regulate chloroplast translation in land plants, we sought nuclear mutations in maize that disrupt the translation of subsets of chloroplast mRNAs. Evidence is presented for a nuclear gene whose function is required for the translation of the chloroplast *atpB*/*E* mRNA. A mutation in *atp1* results in a failure to accumulate the chloroplast ATP synthase complex due to reduced synthesis of the AtpB subunit. This decrease in AtpB synthesis does not result from a change in *atpB* mRNA structure or abundance. Instead, the *atpB* mRNA is associated with abnormally few ribosomes in *atp1-1* mutants, indicating that *atp1* function is required during translation initiation or early in elongation. Previously, only one nuclear gene that is required for the translation of specific chloroplast mRNAs had been identified in a land plant. Thus, *atp1* will be a useful tool for dissecting mechanisms of translational control in chloroplasts.

INTRODUCTION

The chloroplast translation machinery resembles that in eubacteria in many respects (reviewed in Sugiura et al., 1998). Chloroplast ribosomes are similar to bacterial ribosomes in size and antibiotic sensitivities, and the sequences of many chloroplast ribosomal proteins are closely related to those of their prokaryotic ancestors. Despite these similarities, there is increasing evidence that translation mechanisms in chloroplasts differ in several important ways from those in *Escherichia coli.* For example, chloroplast ribosomes include several proteins that do not have bacterial counterparts (Subramanian, 1993), Shine–Dalgarno sequences play a less prevalent role in chloroplast translation (Sakamoto et al., 1994; Hirose and Sugiura, 1996; Fargo et al., 1998), and the sequence of chloroplast start codons affects translational efficiency but plays only a minor role in positioning the start site (Chen et al., 1995). In addition, there is strong genetic evidence that chloroplast translation is commonly regulated by positively acting regulatory proteins (Goldschmidt-Clermont, 1998), which is a phenomenon that is rare in *E. coli* (McCarthy and Gualerzi, 1990).

The translation of chloroplast mRNAs is regulated by a variety of factors. For example, light stimulates the translation of both the *rbcL* and *psbA* mRNAs (Malnoe et al., 1988; Berry et al., 1990; Staub and Maliga, 1993; Kim et al., 1994) and modulates the rates of both the initiation and elongation steps (Berry et al., 1988, 1990; Kim et al., 1991; Edhofer et al., 1998). For *psbA*, this regulation is mediated in part by the 5′ untranslated region (UTR) of the mRNA (Staub and Maliga, 1993). Failure to synthesize one component of a photosynthetic complex can influence the translation of other subunits of the same complex: the chloroplast-encoded large subunit of ribulose bisphosphate carboxylase (Rubisco) is translated at reduced rates when synthesis of the nucleus-encoded small subunit is disrupted (Khrebtukova and Spreitzer, 1996; Rodermel et al., 1996), and the rate of translation of the *petA* mRNA is reduced when the *petD* gene is disrupted in Chlamydomonas (Choquet et al., 1998). Impairment of the chloroplast translation machinery in Chlamydomonas results in the preferential translation of mRNAs encoding ribosomal proteins (Liu et al., 1989), and an analogous phenomenon may occur in nonphotosynthetic plastids in spinach roots (Deng and Gruissem, 1988).

One approach that has been used to identify factors that regulate chloroplast translation is to identify proteins that are capable of binding in vitro to the 5′ UTRs of regulated mRNAs. The focus on the 5′ UTR was prompted by observations that mutations in several 5′ UTRs in Chlamydomonas chloroplasts altered translation of the downstream open reading frame (Rochaix et al., 1989; Sakamoto et al., 1994; Stampacchia et al., 1997; Zerges et al., 1997) and that mutations that suppress the requirement for nucleus-encoded translational activators map to 5′ UTR sequences (Rochaix et al., 1989; Stampacchia et al., 1997). A variety of proteins that bind chloroplast 5′ UTRs have been identified (Danon and Mayfield, 1991; Hauser et al., 1996; Hirose and Sugiura, 1996; Yohn et al., 1998a). Although proteins that modulate translation are likely to be among these, definitive experiments establishing a role in translation have not been reported.

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Nuclear genes in Chlamydomonas that are required for the translation of the chloroplast *psbA* (Girard-Bascou et al., 1992; Yohn et al., 1998b), *psbC* (Rochaix et al., 1989; Zerges and Rochaix, 1994), *psaB* (Stampacchia et al., 1997), and *atpA* (Drapier et al., 1992) mRNAs have been identified by genetic analysis. A mutation in each of these genes disrupts the translation of a specific chloroplast mRNA, suggesting that each mRNA requires specific translational activators. In several cases, it has been established that these nuclear gene products act via *cis*-acting sequences in the 5′ UTR of the target mRNAs (reviewed in Goldschmidt-Clermont, 1998). However, the cloning of these genes has not been reported, and their mechanism of action is not understood.

In land plants, genetic screens have yielded numerous nuclear mutants with global translation defects in the chloroplast (Barkan, 1993; Goldschmidt-Clermont, 1998). However, only one nuclear gene in plants that functions in the translation of a subset of chloroplast mRNAs has been reported previously: *crp1* in maize. *crp1* mutants are defective in the translation of the chloroplast *petA* and *petD* mRNAs, encoding subunits of the cytochrome b_6f complex, and also fail to process a monocistronic *petD* mRNA from its polycistronic precursor (Barkan et al., 1994).

To understand the molecular mechanisms responsible for regulated chloroplast translation in land plants, we sought additional nuclear mutations in maize that disrupt the translation of subsets of chloroplast mRNAs. Here, we describe one such mutant, *atp1-1*, which specifically lacks the chloroplast ATP synthase complex. The failure to accumulate the ATP synthase results from a defect in the translation of the *atpB*/*E* mRNA. This mRNA is associated with abnormally few ribosomes, and the rate of synthesis of the *atpB* gene product is markedly reduced. This is only the second genetically identified translational regulator in land plant chloroplasts and as such will be a useful tool for understanding mechanisms that regulate chloroplast translation.

RESULTS

atp1-1 Is a Nuclear Mutation That Leads to the Loss of the Chloroplast ATP Synthase Complex

The *atp1-1* mutation arose in a maize line harboring active *Mutator* (*Mu*) transposons. Mutant seedlings were initially identified by their pale green, nonphotosynthetic phenotype. The protein gel blots in Figure 1 show that these properties reflect a loss of the chloroplast ATP synthase: representative subunits of the photosystem I core complex (PsaD), the cytochrome b_6f complex (PetD), and the photosystem II core complex (PsbA) accumulate to normal levels in *atp1-1* seedlings, but subunits of the chloroplast ATP synthase are reduced in abundance >10-fold (Figure 1A). Subunits of both the membrane extrinsic portion of the complex (AtpA, AtpB, and AtpE) and the membrane intrinsic portion (AtpF and AtpG) are reduced to a similar extent (Figure 1B). Therefore, the *atp1* gene is essential for the accumulation of the chloroplast ATP synthase complex.

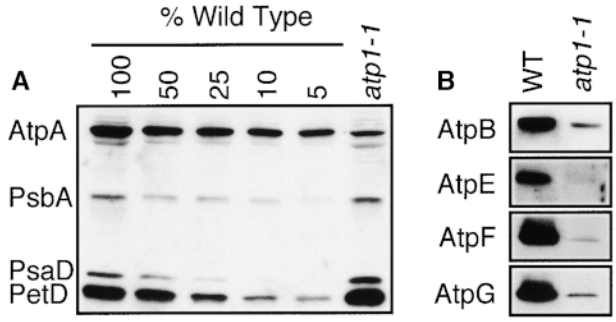

Figure 1. Immunoblots Demonstrating the Loss of the Chloroplast ATP Synthase in *atp1-1* Mutants.

(A) Accumulation of the major thylakoid membrane complexes. Total leaf proteins (5 μg or the indicated dilutions of the wild-type sample) were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with an antisera cocktail that detects one subunit of each of the major thylakoid membrane complexes (AtpA, PsbA, PsaD, and PetD). Because core subunits of the photosynthetic complexes are rapidly degraded if any single subunit is unavailable for assembly (reviewed in Barkan et al., 1995), the abundance of each complex was assessed by monitoring a single subunit. Staining of leaf proteins fractionated by SDS-PAGE revealed that Rubisco accumulates to normal levels in *atp1-1* leaves (data not shown).

(B) Accumulation of other ATP synthase subunits. Total leaf proteins were analyzed as given for **(A)**. Immunoblots were probed with monospecific antisera for AtpB, AtpE, AtpF, and AtpG. WT, wild type.

atp1 Is Not Required for the Accumulation of the mRNAs Encoding ATP Synthase Subunits

To address the possibility that the loss of the ATP synthase in *atp1-1* mutants was due to a defect in the metabolism of mRNAs encoding one or more of its subunits, these mRNAs were assayed by RNA gel blot hybridization. The ATP synthase is composed of six chloroplast-encoded subunits: AtpA, AtpB, AtpE, AtpF, AtpH, and AtpI. AtpB and AtpE are translated from the dicistronic *atpB*/*E* mRNA, the accumulation of which is unaltered in *atp1-1* mutants (Figure 2). AtpA, AtpF, AtpH, and AtpI are all encoded by a second transcription unit, whose polycistronic primary transcript is processed to yield a variety of smaller mRNAs (Stahl et al., 1993). Transcripts corresponding to four genes were similar in the wild-type and mutant samples (Figure 2). One *atpI* transcript, however, accumulated to reduced levels in *atp1-1* mutants (Figure 2, see asterisk). The affected transcript is polycistronic, with *atpI* sequences at its 5′ end, followed by *atpH*

and unspliced *atpF* sequences (data not shown). We suspect that this change in mRNA metabolism is a consequence rather than the cause of the ATP synthase deficiency because the total amount of *atpI* mRNA is reduced to only a small extent and because a nonallelic ATPase mutant exhibits a similar *atpI* transcript pattern (data not shown).

The ATP synthase also includes three nucleus-encoded subunits: AtpC, AtpG, and AtpD. *atpC* and *atpG* mRNAs accumulated to normal levels in *atp1-1* mutants (Figure 2). *atpD* mRNA was not assayed due to lack of a suitable probe. These data indicate that the absence of the ATP synthase in *atp1-1* mutants is not due to a defect in the metabolism of the chloroplast-encoded ATP synthase mRNAs and is unlikely to be due to a defect in the nucleus-encoded mRNAs.

Translation of the *atpB/E* mRNA Is Disrupted in *atp1-1* Mutants

To determine whether the *atp1-1* mutation disrupts translation of the chloroplast-encoded ATPase mRNAs, the association of these transcripts with polysomes was analyzed. Total leaf lysates were fractionated in sucrose gradients under conditions that maintain polysome integrity (Barkan, 1993). Specific mRNAs were localized in the gradients by performing RNA gel blot hybridizations with RNA purified from gradient fractions. The distribution of the chloroplast and cytosolic rRNAs was similar in the gradients containing mutant and wild-type samples (Figure 3A). The *rbcL* mRNAs were distributed similarly as well (Figure 3C), correlating with the normal accumulation of Rubisco in mutant seedlings. The distribution of the *atpA*, *atpF*, *atpH*, and *atpI* mRNAs also

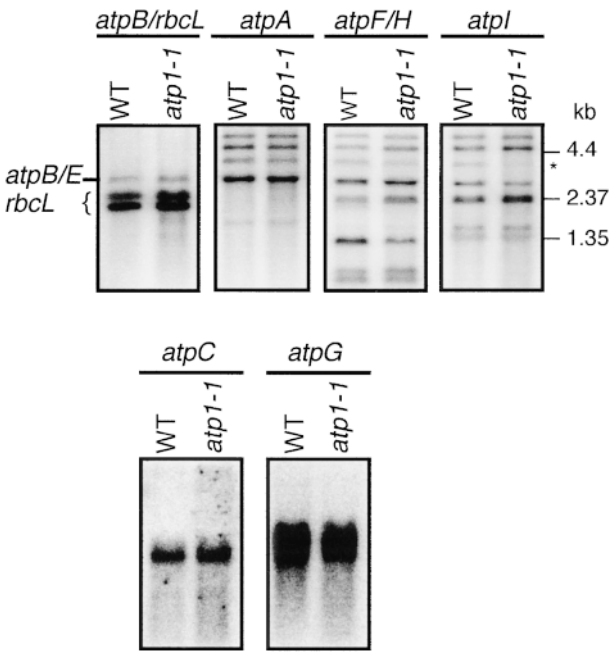

Figure 2. RNA Gel Blot Hybridizations Showing mRNAs Encoding ATP Synthase Subunits.

Total leaf RNA (6 μg) was analyzed. The *atpB*/*rbcL* probe contained portions of both genes. The brackets indicate the two *rbcL* mRNAs, which differ at their 5′ ends (Crossland et al., 1984). *atpI*, *atpH*, *atpF*, and *atpA* are cotranscribed; the primary transcript is processed to give multiple mRNAs (Stahl et al., 1993). RNA molecular weight markers are given at left in kilobases. The asterisk indicates an *atp1* transcript that accumulates to reduced levels in *atp1-1* mutants. WT, wild type.

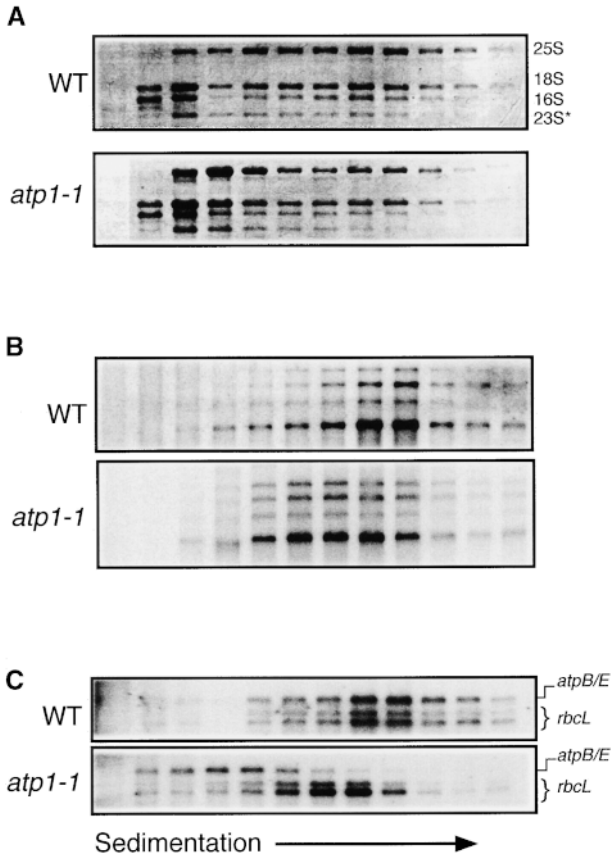

Figure 3. Association of the Chloroplast ATP Synthase mRNAs with Polysomes.

Sucrose gradients were divided into 12 fractions. An equal proportion of the RNA purified from each fraction was analyzed by RNA gel blot hybridization. The top four fractions contained particles <70S (i.e., monosomes and smaller). WT, wild type.

(A) RNA gel blots of polysome gradient fractions were stained with methylene blue to visualize rRNAs. 23S* is a breakdown product of the chloroplast 23S rRNA.

(B) The filter shown in **(A)** was hybridized with a probe specific for *atpA*.

(C) A duplicate filter was hybridized with a probe that detects *atpB*/*E* and *rbcL* mRNAs.

was similar in the wild-type and mutant samples, although these mRNAs (like the *rbcL* mRNA) were associated with slightly smaller particles in the mutant sample (Figure 3B; data not shown). However, a clear defect was observed in the sedimentation of the *atpB*/*E* mRNA. Whereas the bulk of *atpB*/*E* mRNA in the wild-type sample was found in the polysomal region of the gradient, most of this mRNA in the *atp1-1* sample sedimented with material <70S (Figure 3C). The reduced sedimentation rate of the *atpB*/*E* mRNA indicates a reduction in the number of associated ribosomes, implying that translation initiation and/or a step very early in elongation is blocked in the *atp1-1* mutant.

To test whether the reduced polysome association of the *atpB*/*E* mRNA is reflected by a reduced rate of AtpB synthesis, rates of protein synthesis were examined by performing pulse-labeling experiments. Leaf proteins were pulse-labeled in vivo for 25 min by applying ^{35}S-methionine to seedling leaves that had been lightly perforated (Barkan, 1998). Proteins were then solubilized and immunoprecipitated with a mixture of antisera specific for AtpB and PsbA (a photosystem II subunit used as an internal standard) (Figure 4A, top). Accumulation of radiolabeled AtpB was markedly reduced in the *atp1-1* seedlings, whereas radiolabeled PsbA accumulated to a similar extent in the wild-type and mutant seedlings. This finding supports the conclusion that *atpB* mRNA translation is disrupted in *atp1-1* mutants. To assess the rate of synthesis of another ATP synthase subunit, AtpA was immunoprecipitated from the same extracts (Figure 4A, bottom). The accumulation of radiolabeled AtpA was only slightly reduced in *atp1-1* plants. This decrease may result from a small decrease in the rate of AtpA synthesis or an increase in its rate of degradation. The latter is an expected consequence of the defect in ATP synthase assembly that would result from the loss of AtpB synthesis.

Failure to detect radiolabeled AtpB protein suggested that its rate of synthesis was reduced in *atp1-1* mutants. However, it was also possible that AtpB was degraded with a half-life of several minutes. To enhance our ability to detect the synthesis of proteins with a very short half-life, a 5-min pulse was achieved by applying radiolabeled amino acids to isolated chloroplasts. Proteins were solubilized and immunoprecipitated with a mixture of AtpB and PsbA antisera (Figure 4B). The accumulation of radiolabeled AtpB was again reduced significantly in the mutant, whereas radiolabeled PsbA accumulated to increased levels in the same sample. These results, in conjunction with the polysome defect, demonstrate that the *atpB* mRNA is translated inefficiently in *atp1-1* mutants.

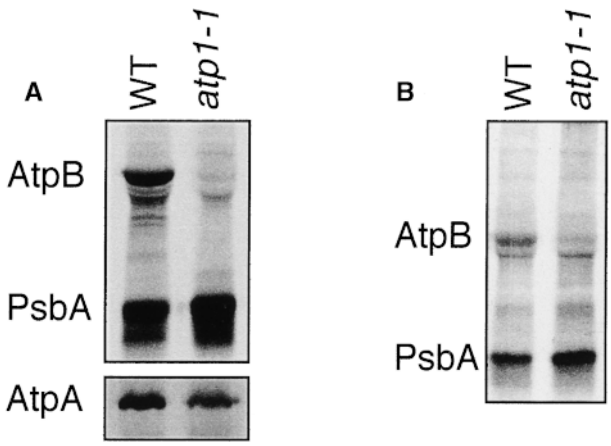

Figure 4. Pulse-Labeling Analysis of AtpB Synthesis.

(A) In vivo labeling. Leaf proteins were radiolabeled for 25 min with ^{35}S-methionine/cysteine. Total proteins were solubilized and subjected to immunoprecipitation with monospecific antisera. Aliquots of solubilized protein containing 200,000 counts per min were used for each immunoprecipitation. The upper panel shows the results of immunoprecipitating with a mixture of anti-AtpB and anti-PsbA antisera. The lower panel shows the results of immunoprecipitating with an anti-AtpA antiserum.

(B) In organello labeling. Intact chloroplasts were incubated with ^{35}S-methionine/cysteine for 5 min. Aliquots of solubilized protein containing 100,000 counts per min were immunoprecipitated with a mixture of anti-AtpB and anti-PsbA antisera.

WT, wild type.

Structure of the *atpB*/*E* mRNA Is Not Altered in *atp1-1* Mutants

It was plausible that failure to translate the *atpB*/*E* mRNA was a consequence of a subtle change in its structure. Although this mRNA appeared normal by RNA gel blot hybridization (Figure 2), higher resolution methods are required to map precisely the positions of the 5′ and 3′ ends.

The 5′ end of the *atpB*/*E* mRNA was determined by primer extension analysis (Figure 5A). The 5′ end detected in both the wild-type and the *atp1-1* samples mapped to ~296 nucleotides upstream of the *atpB* start codon, similar to the previously published site (Krebbers et al., 1982). The 3′ termini of the *atpB*/*E* mRNA were mapped by RNase protection analysis (Figure 5B). The same three probe fragments were protected by wild-type and *atp1-1* RNA. Two of these bands correspond to 3′ ends terminating at ~136 nucleotides and ~206 nucleotides downstream of the *atpE* stop codon; the third corresponded to unspliced *trnV*, encoded downstream. These results confirm that failure to translate the *atpB* mRNA in *atp1-1* mutants is not due to even subtle defects in the processing of its termini.

DISCUSSION

Plastid gene expression is controlled by the activities of a large number of nuclear genes, the identification and cloning

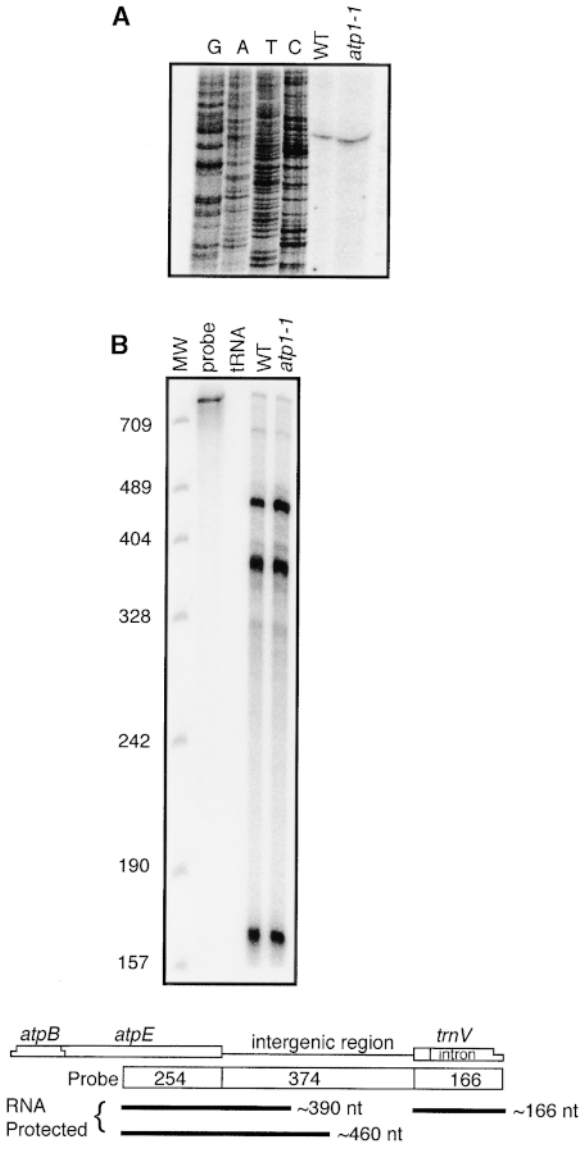

Figure 5. Termini of the *atpB*/*E* mRNA Are Unaltered in *atp1-1* Seedlings.

(A) Primer extension analysis of the 5′ terminus. The primer was complementary to a sequence mapping 90 nucleotides downstream of the *atpB* start codon. A sequencing ladder primed with the same primer was fractionated in adjacent lanes to position the 5′ terminus precisely. WT, wild-type.

(B) RNase protection mapping of the 3′ termini of the *atpE* mRNA. Total leaf RNA (2 μg) was hybridized with a radiolabeled probe complementary to the 3′ end of the *atpE* mRNA. A radioactive antisense transcript containing 254 nucleotides of the *atpE* coding region and 540 nucleotides of the downstream sequence was annealed with wild-type (WT) or *atp1-1* leaf RNA. Hybrids were treated with RNase A and T1 and fractionated on a denaturing polyacrylamide gel. The size in nucleotides (nt) of each molecular weight (MW) marker is indicated to the left.

of which will be essential for understanding the regulatory mechanisms that govern these processes. We have presented evidence for a nuclear gene in maize, *atp1*, which functions in the translation of the chloroplast *atpB*/*E* mRNA. A mutation in *atp1* reduced the synthesis of AtpB dramatically, with no corresponding change in mRNA structure or abundance. Furthermore, *atpB* mRNA was associated with few ribosomes in *atp1-1* mutants, indicating that the mutation disrupts a step in translation initiation or early in elongation. This is the only nuclear gene identified to date that is required specifically for the translation of the *atpB* mRNA.

That the *atp1-1* mutation disrupts the translation of only the *atpB*/*E* mRNA was suggested initially by the results of the polysome analyses. In support of this notion, pulse-labeling experiments indicated that the AtpA subunit was synthesized at near normal rates in the mutant. However, it is likely that AtpE, also encoded on the *atpB*/*E* mRNA, is translated at a reduced rate in the mutant. The *atpE* start codon overlaps the *atpB* stop codon, and these genes are translationally coupled when expressed in *E. coli* (Gatenby et al., 1989); the same is likely to be true in chloroplasts. Unfortunately, we were unable to measure AtpE synthesis directly because the available anti-AtpE antibody was not suitable for immunoprecipitation experiments.

The fact that AtpA synthesis is not significantly reduced in *atp1-1* mutants contrasts with results obtained with a Chlamydomonas mutant lacking the ATP synthase. The nuclear mutation *thm24* in Chlamydomonas causes the loss of the *atpB* mRNA; the AtpA subunit was also synthesized at reduced rates in this mutant, although its mRNA accumulated normally (Drapier et al., 1992). These and other data have been used to formulate a model called “control by epistasy of synthesis,” which posits that the availability of one subunit of each photosynthetic enzyme complex controls the synthesis of the other subunits (Choquet et al., 1998). Accordingly, it was postulated that the failure to synthesize AtpB in the *thm24* mutants caused the reduction in AtpA synthesis (Drapier et al., 1992). The results presented here suggest that this relationship may not hold true in land plants. However, it is also possible that even the small amount of AtpB that is synthesized in *atp1-1* mutants may be sufficient to prevent a significant reduction in AtpA synthesis.

The *atp1* gene is unique among known genes in land plants in that it is required for the synthesis of a single chloroplast gene product; other nuclear mutations recovered in plants disrupt the expression of several or many chloroplast genes (reviewed in Goldschmidt-Clermont, 1998; Leon et al., 1998). In contrast, numerous nuclear mutations affecting chloroplast gene expression in a gene-specific fashion have been discovered in Chlamydomonas (reviewed in Goldschmidt-Clermont, 1998). This may reflect fundamental differences in regulatory mechanisms that have evolved in concert with the divergence of chloroplast gene organization in plants and algae. For example, in land plants, the chloroplast genes encoding the ATP synthase subunits are organized in two polycistronic transcription units (Stahl et al., 1993),

whereas these genes are distributed throughout the chloroplast genome in Chlamydomonas (Woessner et al., 1987). A continued comparison between plants and algae will indicate whether differences in the types of mutations recovered reflect differences in the regulatory circuits or simply the different strategies employed to identify mutants.

The cloning of the nuclear genes that modulate chloroplast translation will be crucial for understanding translational regulatory mechanisms. *crp1*, a maize gene required for the translation of the chloroplast *petA* and *petD* mRNAs, was recently cloned (Fisk et al., 1999). The *crp1* gene product is related to nuclear genes in fungi that play an analogous role in mitochondrial gene expression, suggesting an underlying mechanistic similarity. As more nuclear genes involved in chloroplast translation are cloned, it will be interesting to discover whether it is typical that regulators of chloroplast and mitochondrial gene expression are related.

METHODS

Plant Material

atp1-1 arose in a maize line with active *Mutator* (*Mu*) transposons. The mutation is inherited as a single, recessive Mendelian trait. It results in seedling lethality at ~3 weeks after germination, as is typical of nonphotosynthetic maize mutants, due to depletion of endosperm stores. Except where otherwise noted, plants were grown for 10 to 12 days in a growth chamber at 26°C in 16 hr of light (400 μE m^{-2} sec^{-1}) and 8 hr of dark. At that time, normal and mutant seedlings had three leaves and were indistinguishable on the basis of their size or morphology.

Protein Extraction and Analysis

For immunoblot analysis, proteins were extracted from seedling leaf tissue and analyzed as described previously (Barkan, 1998).

For pulse-labeling experiments, progeny of self-pollinated *atp1-1*/+ plants were germinated and grown in light/dark cycles for 3 to 4 days until the coleoptiles began to emerge from the soil; then they were transferred to complete darkness until the third leaf began to emerge (~8 days after planting). They were then shifted into continuous light (400 μE m^{-2} sec^{-1}) for 24 hr before the pulse-labeling experiment. Mutant plants were distinguished from their normal siblings by immunoblot analysis of leaf tip extracts to score ATP synthase accumulation.

In vivo labeling experiments were conducted as described in Barkan (1998). In brief, 50 μCi of ^{35}S-methionine/cysteine EXPRESS protein labeling mix (>1000 Ci $mmol^{-1}$; New England Nuclear, Beverly, MA) was applied to small perforations on the midsection of the second leaf. Seedlings were incubated in an illuminated hood for 25 min, after which a 2-cm leaf segment surrounding the perforations was excised. Protein was extracted, solubilized, and immunoprecipitated, as described previously (Barkan, 1998). The concentration of ATPase antigen in the mutant extracts is less than that in wild-type extracts because of their ATPase deficiency. If the antibody is not in sufficient excess, this results in the immunoprecipitation of a greater proportion of the antigen from the mutant samples, leading to an overestimate of synthesis rates. In analogous studies performed previously, we compensated for this deficiency by adding nonradioactive wild-type leaf proteins to immunoprecipitations of mutant samples (Barkan et al., 1994; Voelker and Barkan, 1995). However, the immunoprecipitations shown here were not adjusted in this way; thus, the amount of radiolabeled AtpA and AtpB precipitated from the mutant samples may be an overestimate of their true rates of synthesis.

In organello pulse-labeling experiments were as described in Barkan (1998), except that intact chloroplasts were purified on Percoll gradients before the 5-min incubation with ^{35}S-methionine/cysteine.

Antibodies generated against PsaD, PsbA, and PetD were described previously (Barkan et al., 1994). Antibodies generated against spinach chloroplast AtpB and AtpE were raised in rabbits by injecting purified recombinant AtpB and AtpE generously provided by Dr. P. Gegenheimer (University of Kansas, Manhattan). The rabbit anti–AtpG antibody was raised against a recombinant fusion protein generated with a maize *atpG* cDNA clone provided by Pioneer Hi-Bred (Johnston, IA). Antibodies used for detecting AtpA and AtpF were generated from recombinant fusion proteins made with the maize chloroplast protein coding sequences.

RNA and Polysome Extraction and Analysis

Total leaf RNA and polysome fractions were prepared as described previously (Barkan, 1998). RNA gel blot analysis was performed as described in Barkan (1998). The gene-specific probe for *atpA* mRNA was described in Barkan (1989). *atpB* and *rbcL* mRNAs were detected with the maize chloroplast Bam9 fragment (Larrinua et al., 1983). *atpH* and *atpF* mRNAs were detected with a cloned 1200-bp fragment of maize chloroplast DNA that included most of *atpH* and 620-bp of *atpF.* The *atpI* mRNA was detected with a cloned DNA fragment containing the entire maize *atpI* coding region. The *atpC* mRNA was detected with a full-length maize *atpC* cDNA (GenBank accession number AA030720). The *atpG* mRNA was detected with a 500-bp fragment of a maize *atpG* cDNA generously provided by Pioneer Hi-Bred.

RNase protection experiments were performed as described previously (Barkan et al., 1994), except that both RNase A and T1 were used to degrade unannealed probe.

For primer extension analysis, an oligonucleotide that binds 90 nucleotides downstream of the *atpB* start codon (5′-CACGGGTCCAATAATTTGATC-3′) was radiolabeled at its 5′ end with polynucleotide kinase and γ-^{32}P-ATP. Total leaf RNA was denatured and annealed to 1 pmol of primer by heating to 65°C for 3 min, cooling in liquid N_2 for 1 min, and heating to 48°C for 10 min. Avian myeloblastis virus reverse transcriptase (Boehringer Mannheim) (1.25 units) and deoxynucleoside triphosphates (1 mM final concentration) were added to the reactions, which were incubated at 48°C for 45 min. Reactions were stopped by the addition of 16 μL of a solution containing 80% formamide, 10 mM NaOH, and 1 mM EDTA. Extension products were separated on denaturing polyacrylamide gels.

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