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Nuclear Mutants of Maize with Defects in Chloroplast Polysome Assembly Have Altered Chloroplast RNA Metabolism

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The molecular basis for the photosynthetic defect in four nuclear mutants of maize was investigated. Mutants *hcf7*, *cps1-1*, *cps1-2*, and *cps2* contained reduced levels of many chloroplast-encoded proteins without corresponding deficiencies in chloroplast mRNAs. Many chloroplast mRNAs were associated with abnormally few ribosomes, indicating that the protein deficiencies were due to global defects in chloroplast translation. These mutants were used to study the effects of reduced ribosome association on the metabolism of chloroplast RNAs. The level of the *rbcL* mRNA was reduced four-fold in each mutant, but was unaltered in other nonphotosynthetic mutants with normal chloroplast translation. These results suggest that the *rbcL* mRNA is destabilized as a consequence of its decreased association with ribosomes. The fact that many other chloroplast mRNAs accumulated to normal levels demonstrated that a decreased association with ribosomes does not significantly alter their stabilities or processing. *hcf7* seedlings had a gross defect in the processing of the 16S rRNA: the primary lesion in this mutant may be a defect in 16S rRNA processing itself.

INTRODUCTION

A vital component of leaf cell differentiation is the differentiation of proplastids into chloroplasts. The proplastid is a spherical organelle with little internal membrane structure, few ribosomes, and little pigmentation. During the differentiation of leaf cells, the proplastid differentiates into the much larger, more elongated chloroplast containing the abundant and highly organized thylakoid membrane (reviewed by Mullet, 1988). Accompanying these morphological changes are changes in lipid content (Leech et al., 1973), increases in the abundance of ribosomes and photosynthetic enzymes (reviewed by Mullet, 1988), transient increases in DNA copy number and transcriptional activity (Baumgartner et al., 1989), and an increase in the level of spliced mRNAs relative to their unspliced precursors (Barkan, 1989).

While the events accompanying chloroplast differentiation have been described in considerable detail, little is known about ways in which products of nuclear genes integrate these events with changes in the rest of the cell. Few nuclear genes with defined roles in chloroplast gene expression have been identified. Clones corresponding to a number of nuclear genes encoding chloroplast ribosomal proteins (reviewed by Subramanian et al., 1991) and translation factors (Baldauf et al., 1990) have been isolated. Several nuclear genes have been identified that encode chloroplast RNA binding proteins (Li and Sugiura, 1991; Schuster and Gruissem, 1991; Cook and Walker, 1992; Mieszczak et al., 1992), but the roles of these proteins in the chloroplast remain unknown.

A potentially powerful way to identify nuclear genes that play critical roles in chloroplast gene expression is to isolate nuclear mutations that block chloroplast function. Numerous nuclear mutants of this type exist (reviewed by Miles, 1982; Somerville, 1986; Taylor et al., 1987; Taylor, 1989), but the primary defects in most of these are unknown. This approach has been exploited most thoroughly in studies of Chlamydomonas (reviewed by Rochaix, 1992). It is likely that novel mechanisms will be unmasked by genetic studies in higher plants, considering the substantial differences between plants and algae in chloroplast gene organization and expression.

To identify and isolate nuclear genes that modulate chloroplast gene expression in higher plants, we are isolating transposon-induced mutants of maize with defects in chloroplast translation and mRNA metabolism. This paper concerns four nuclear mutants (*hcf7*, *cps1-1*, *cps1-2*, and *cps2*) that fail to assemble normal chloroplast polysomes. Mutant seedlings are slightly pale green relative to their normal siblings and photosynthetically inactive. To gain insight into the basis for the photosynthetic defects, the mutant phenotypes were analyzed in detail. The level of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the mutant seedlings was 20- to 40-fold lower than normal. *cps1* and *hcf7* mutants were deficient in many thylakoid membrane proteins as well. Their pigmentation was nonetheless near normal due to the normal accumulation of the light-harvesting complexes. While most chloroplast mRNAs were of normal size and abundance, they were

associated with very few ribosomes. Therefore, these mutants are likely to be defective in the initiation step of translation.

Based upon their phenotypes, the *cps1* and *cps2* mutants could have lesions in genes encoding ribosomal proteins or initiation factors. *hcf7* was unique in that it had a severe block in the maturation of the 16S rRNA; thus, the primary defect in *hcf7* may be in rRNA processing itself. The defects in polysome assembly correlated with altered metabolism of at least one chloroplast mRNA (*rbcL*). Therefore, changes in the chloroplast translation machinery during chloroplast development might be one factor that modulates the stabilities of a subset of chloroplast mRNAs.

RESULTS

Mutant Isolation and Genetic Analysis

Three of the mutants used in this study were isolated from maize lines harboring active *Mutator* (*Mu*) transposons. Because they proved to be defective in *c*hloroplast *p*rotein *s*ynthesis, these were named *cps* mutants. The three mutants arose independently and defined two complementation groups. Thus, they were named *cps1-1*, *cps1-2*, and *cps2*. All three *cps* mutations are unstable (i.e., dark green sectors arise on pale green mutant leaves), indicating that transposon insertions are responsible for the mutant phenotypes. These sectors were typically very small and were of similar frequency in all of the mutant material used for this study. The fourth mutant discussed here, *hcf7*, was isolated from a population of ethylmethane sulfonate–mutagenized material by D. Miles (University of Missouri, Columbia, Missouri). *hcf7* complemented both *cps1* and *cps2*, indicating that it defines a separate gene. Crosses with B-A translocation stocks (Beckett, 1978) revealed that *cps2* maps to the long arm of chromosome 6 and *hcf7* and *cps1* map to the long arm of chromosome 1.

hcf7, *cps1*, and *cps2* Mutants Have Reduced Levels of Photosynthetic Complexes

Protein gel blots were used to quantify levels of representative protein components of each photosynthetic complex. Dilutions of wild-type protein extracts were analyzed on each gel to aid in quantifying specific proteins in mutant extracts. Examples of these results are shown in Figure 1.

The level of the *rbcL* gene product (the large subunit of Rubisco) was reduced more than 20-fold in all four mutants (Figure 1A). The Rubisco level was consistently lower in *cps1-2* than in *cps1-1*, suggesting that the *cps1-1* allele is a "leakier" allele. Levels of each thylakoid membrane complex containing plastid-encoded components were also reduced in *hcf7* and *cps1* mutants (Figure 1B): subunits of photosystem I (psaA/B), photosystem II (psbA), the cytochrome b_6f complex

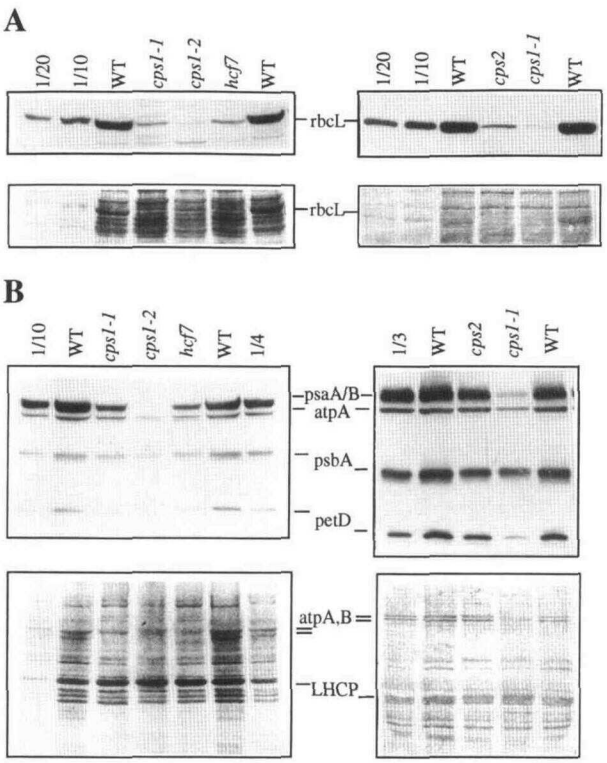

Figure 1. Immunoblot Analysis of Chloroplast Proteins.

(A) Level of Rubisco in mutant leaves. Equal amounts (or the indicated dilutions of the wild-type sample) of soluble leaf proteins were analyzed.
(B) Levels of specific thylakoid membrane proteins in mutant leaves. Equal amounts (or the indicated dilutions of the wild-type sample) of membrane-associated leaf proteins were analyzed.
The upper panel in each pair shows the results of probing blots with the indicated monospecific antisera. The lower panel in each pair shows the same blots stained with Ponceau S to visualize the total population of bound protein. The identities of several of the stained protein bands are labeled. The gels shown are examples of many independent analyses of this type. The signals obtained for one or more proteins sometimes did not decrease in proportion to the amount of protein applied to the wild-type (WT) dilution lanes. Such blots were not used for quantification of those particular proteins. In **(B)**, for example, differences in the level of the *atpA* gene product were more apparent on the stained filters than on the antibody detections. LHCP, light-harvesting chlorophyll *a*/*b* binding protein.

(petD), and the ATP synthase (atpA) accumulated to only 5 to 10% of wild-type levels. The *cps1-2* mutant had the most severe loss of thylakoid proteins, as it did of Rubisco. *cps2* exhibited only a minor reduction in thylakoid membrane proteins. Subunits of photosystems I and II, the cytochrome b_6f complex, and ATP synthase accumulated to 30 to 50% of normal levels (Figure 1B and data not shown).

The *psaD*, *petA*, *petC*, *psbC*, and *psbB* gene products were decreased to a similar extent in each mutant as the thylakoid membrane proteins shown in Figure 1 (Taylor et al., 1987; data

not shown). The fact that *hcf7* and *cps1* mutants were deficient in both membrane and soluble chloroplast-encoded proteins suggested that they might be defective in some general aspect of chloroplast gene expression. The loss of the nuclear-encoded PSAD and PETC proteins would be an expected outcome of such a global defect in plastid gene expression, because it has generally been found that the stabilities of the core subunits of each photosynthetic complex decrease dramatically when the synthesis of one core subunit is blocked (Schmidt and Mishkind, 1983; Spreitzer et al., 1985; Bennoun et al., 1986; Takahashi et al., 1991). Although *cps2* mutants had only minor losses of thylakoid proteins, it became apparent that they also had a global translation defect, for reasons that will be described below.

Most Plastid mRNAs Are of Normal Size and Abundance in *hcf7*, *cps1*, and *cps2* Mutants

To address the possibility that the global deficiencies in chloroplast-encoded proteins were due to defects in chloroplast

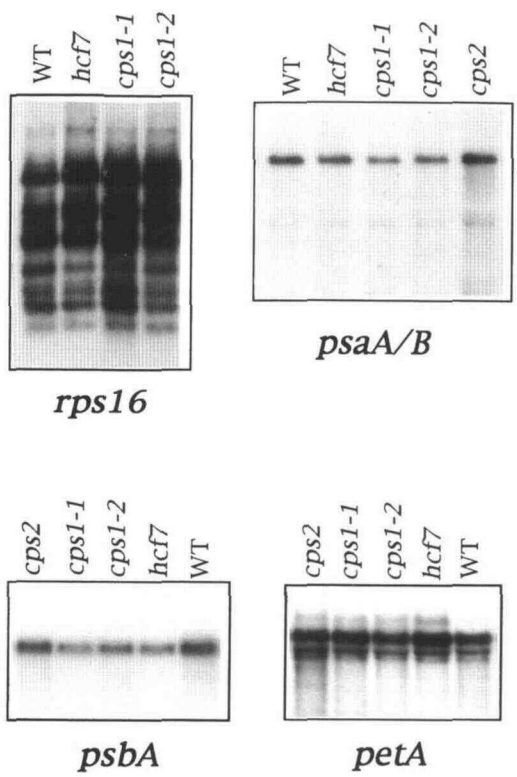

Figure 2. RNA Gel Blot Analysis of Chloroplast mRNAs.

Two micrograms of seedling leaf RNA was analyzed in each lane. The gene-specific probes are described in Methods. The filter labeled *psaA/B* was hybridized with a probe derived from the *psaB* gene; this probe detects the single polycistronic transcript encoding both *psaA* and *psaB*. WT, wild type.

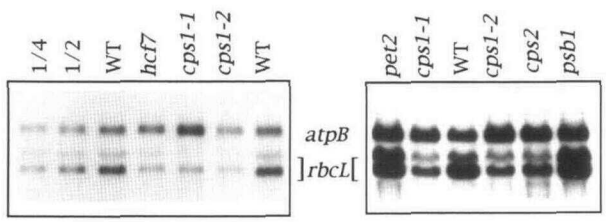

Figure 3. RNA Gel Blot Analysis of *rbcL* and *atpB* mRNAs.

An equal amount (or the indicated dilutions of the wild-type [WT] sample) of seedling leaf RNA was analyzed in each lane. *psb1* and *pet2* are nuclear mutants that lack, specifically, the photosystem II core complex and the cytochrome b_6f complex, respectively (A. Barkan, unpublished results). The precise ratio of the two *rbcL* transcripts varies slightly between different preparations of RNA, indicating that these minor differences are not due to differences in genotype. The filter shown on the left was reprobed with a 16S rRNA-specific probe (see Figure 5B).

mRNA metabolism, the size and abundance of various plastid mRNAs were analyzed on RNA gel blots. Transcripts of the *petA*, *psaA/B*, *psbA*, *rps16*, and *atpB* genes accumulated to normal levels in these mutants, as shown in Figures 2 and 3. The slight differences between samples in the levels of *psaA/B*, *psbA*, and *atpB* transcripts were less than twofold and were not reproducible. Transcripts of the *petB*, *petD*, *psaC*, *psbC*, *psbN*, *psbH*, *psbE*, *psbI*, *atpF*, *rps18*, *rpl33*, and *rps12* genes were of normal size and abundance as well (data not shown). This failure to detect defects in mRNA metabolism suggested that the protein deficiencies resulted from defects at a translational or post-translational step.

One mRNA (*rbcL*) whose level was reduced in all four of these mutants was detected (Figure 3). As is described below, this feature is a hallmark of mutants that do not assemble normal polysomes and is unlikely to be the sole cause of the reduced accumulation of Rubisco.

Chloroplast mRNAs Are Associated with Abnormally Few Ribosomes in *hcf7*, *cps1*, and *cps2*

To investigate the possibility that a defect in translation led to the loss of chloroplast proteins, the association of chloroplast mRNAs with ribosomes was examined. A decrease in the rate of translation initiation would result in a decrease in the average number of ribosomes associated with mRNAs. Seedling leaf lysates were prepared under conditions that maintain intact polysomes and size-fractionated in analytical sucrose gradients. Figure 4 shows the distribution of various chloroplast mRNAs in the gradient fractions, assayed by RNA gel blot hybridization.

Cytosolic translation appeared to be unaltered by these mutations because mutant seedlings were of normal size and

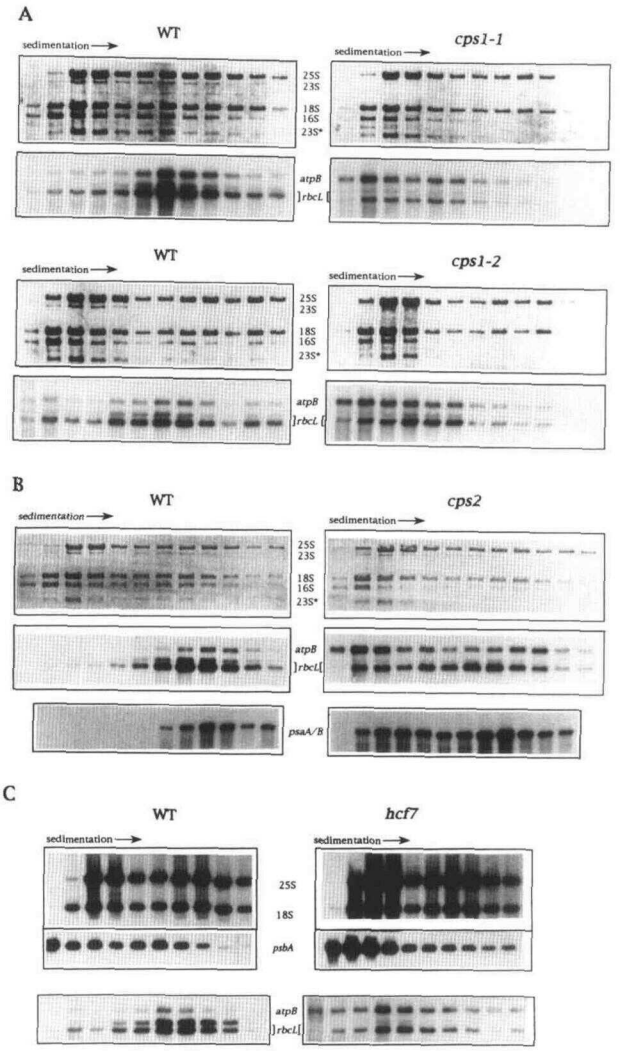

Figure 4. Association of Chloroplast mRNAs with Polysomes.

Total extracts of the apical half of the second and third leaves of single seedlings were fractionated on sucrose gradients. The gradients were fractionated into 12 **([A]** and **[B])** or 10 **([C])** fractions of equal volume. An equal proportion of the RNA purified from each fraction was analyzed by RNA gel blot hybridization. Each mutant was analyzed in parallel with a wild-type (WT) sibling. The same results were obtained with a second seedling of each genotype (data not shown). 23S* is a breakdown product of the plastid 23S rRNA. The decrease in the ratio of *rbcL* to other transcripts is characteristic of these mutants (see Figures 2 and 3). WT, wild type.

(A) Association of *rbcL* and *atpB* mRNAs with polysomes in *cps1* leaves. The distribution of rRNAs in each gradient, visualized by staining the nylon membrane with methylene blue, is shown in the upper panel in each pair. The lower panels show the results of hybridizing the same filters with a DNA fragment containing both *rbcL* and *atpB* sequences.

(B) Association of *rbcL*, *atpB*, and *psaA/B* mRNAs with polysomes in *cps2* leaves. The distribution of rRNAs in each gradient, visualized by staining the nylon membrane with methylene blue, is shown in the upper panels. The middle panels show the results of hybridizing the same filters with a radioactive DNA fragment containing both *rbcL* and *atpB* sequences. The bottom panels show the results of hybridizing a duplicate set of filters with a probe derived from the *psaB* gene.

(C) Association of *psbA*, *rbcL*, and *atpB* mRNAs with polysomes in *hcf7* leaves. The *psbA* mRNA and cytosolic rRNAs were visualized by consecutive hybridizations of the same filters. The *rbcL* and *atpB* mRNAs were visualized by hybridizing a duplicate set of filters with a radioactive DNA fragment containing both *rbcL* and *atpB* sequences.

morphology during their first few weeks of growth. As expected, the distribution of mutant and wild-type cytosolic rRNAs (25S and 18S) in the sucrose gradients was similar (Figure 4). The cytosolic rRNAs were distributed in two broad peaks, the upper peak corresponding to particles smaller than 80S (i.e., monosomes or smaller), the lower one corresponding to polysomes. The plastidic rRNAs (23S, 16S, and 23S*) in the wild-type samples were also distributed in two broad peaks. The majority of chloroplast RNA molecules sedimenting five or more fractions from the top of the gradients were associated with polysomes, because they were released to smaller particles (i.e., to the top three to four fractions) by treatment with EDTA or by treatment with puromycin in the presence of high salt (data not shown).

For each mutant, all chloroplast mRNAs examined showed a substantial shift toward the top of the gradient. For example, the majority of the *atpB*, *rbcL*, and *psaA/B* transcripts in wild-type leaves cosedimented with polysomes (Figure 4). In *cps1-1* and *cps1-2* mutants, however, the majority of the *rbcL* and *atpB* mRNA molecules were no longer associated with multiple ribosomes (Figure 4A). The defect in *cps2* was less severe: there was a dramatic increase in nonpolysomal *rbcL*, *atpB*, and *psaA/B* mRNAs in this mutant, but a substantial fraction of these mRNAs remained associated with multiple ribosomes (Figure 4B).

Whereas the *atpB*, *rbcL*, and *psaA/B* mRNA molecules were almost entirely polysome associated in wild-type chloroplasts, a significant proportion of the *psbA* mRNA molecules were not (Figure 4C). A similar observation was made during studies of barley chloroplasts (Klein et al., 1988); its significance is unknown. Nonetheless, the *hcf7* mutation caused a reduction in the proportion of *psbA* mRNA molecules that were polysome bound (Figure 4C). The number of ribosomes associated with the *rbcL* and *atpB* mRNAs was also decreased in *hcf7* seedlings (Figure 4C).

All chloroplast mRNAs assayed were associated with abnormally few ribosomes in these mutants. In addition to the data shown in Figure 4, the following mRNAs were assayed: *rpl16* mRNA in *hcf7*; the *rps4*, *psbA*, *psbB*, *psbC/D*, *psbE/F*, *atpA*, *atpF*, *psaA/B*, and *petA* mRNAs in *cps1* mutants; and the *petA* and *psbA* mRNAs in *cps2* (data not shown). It was striking that *cps2* mutants failed to assemble many different chloroplast mRNAs into polysomes of normal size, despite the fact that the proteins encoded by some of these mRNAs accumulated to near normal levels.

These results indicate that the *hcf7*, *cps1*, and *cps2* mutations each reduced the average number of ribosomes associated with most or all chloroplast mRNAs. This is most easily explained by a global decrease in the rate of an initiation step, rather than an elongation step, of translation. A reduction in the elongation rate would, by itself, result in an increase in the number of ribosomes associated with mRNAs, as is observed after treatment of chloroplasts with chloramphenicol (A. Barkan, unpublished results; Klaff and Gruissem, 1991), or eukaryotic cells with cycloheximide (Peltz et al., 1992).

It is of interest that the distributions of the two *rbcL* mRNAs in the gradients were similar (Figure 4). The larger of these is the primary transcipt; the smaller is a derivative resulting from processing at the 5′ end (Hanley-Bowdoin et al., 1985; Mullet et al., 1985). Their similar sedimentation in these gradients indicates that the 5′ processing does not dramatically alter the rate at which translation is initiated on this mRNA.

A Defect in Polysome Assembly Is Accompanied by a Fourfold Decrease in the Level of the *rbcL* mRNA

Because the *hcf7*, *cps1*, and *cps2* mutations cause a decrease in the number of ribosomes associated with chloroplast mRNAs, these mutants may be useful for revealing effects of ribosome association on the metabolism of plastid mRNAs. The fact that most plastid mRNAs were processed normally and accumulated to normal levels (Figures 2 and 3; data not shown) suggested that the spacing of ribosomes along the mRNA does not affect the metabolism of most chloroplast mRNAs. Figure 3 shows, however, that the metabolism of *rbcL* transcripts was altered, in that the level of the two *rbcL* mRNAs was reduced fourfold in each mutant.

The reduction in the level of *rbcL* transcripts is not a consequence of the photosynthetic deficiency because most nonphotosynthetic mutants have normal levels of this mRNA (e.g., *psb1* and *pet2* in Figure 3; Barkan et al., 1986). Among the 17 mutants for which we have analyzed the association of plastid mRNAs with polysomes, there was a perfect correlation between decreased polysome size and decreased *rbcL* mRNA accumulation: all 13 mutants in which plastid mRNAs were incorporated normally into polysomes had normal levels of *rbcL* mRNA (Barkan et al., 1986; data not shown); the four mutants (defining three genes) that were defective in polysome assembly had a fourfold reduction in the level of *rbcL* mRNAs (Figure 3). Whereas this reduction could be due either to a decrease in their rate of synthesis or to an increase in their rate of decay, the simplest explanation is that the *rbcL* mRNA is destabilized as a consequence of decreased association with ribosomes.

Chloroplast rRNAs in *hcf7*, *cps1*, and *cps2* Mutants: Pleiotropic or Primary Defects in rRNA Processing?

To assess whether the translation defects in these mutants were associated with defects in chloroplast rRNA synthesis or processing, the structure and abundance of chloroplast rRNAs were examined. Figure 5 shows the results of RNA gel blots in which clones derived from the maize chloroplast rRNA genes were used as probes. Although a small fraction of the maize chloroplast 23S rRNA is recovered intact, most of it is recovered as two smaller molecules: a 1.8-kb fragment is derived from the 5′ portion of the 23S rRNA, and a 1.2-kb fragment is derived from the 3′ portion (Figure 5A; Kössel et al., 1985). Both of the "23S" fragments were unaltered in size and accumulated to normal levels in each mutant. Therefore, the translation defects are unlikely to be due to altered metabolism of the 23S rRNA.

It is interesting that the mature, intact 23S rRNA was undetectable in *cps1* and *hcf7* mutants, while larger transcripts, presumably corresponding to pre-23S rRNA, were detected at normal or increased levels (Figure 5A). Nonphotosynthetic mutants with normal chloroplast translation were not affected in this way, although their ratio of pre-23S rRNA to the mature form was often somewhat higher than normal (see *crp2* and

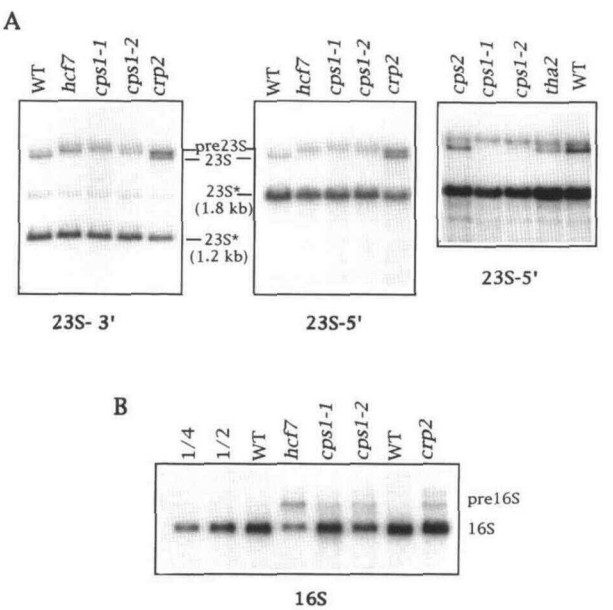

Figure 5. RNA Gel Blot Analysis of Chloroplast rRNAs.

An equal amount (or the indicated dilutions of the wild-type [WT] sample) of leaf RNA was analyzed in each lane. *tha2* and *crp2* are nuclear mutants with defects in thylakoid assembly and chloroplast mRNA processing, respectively (A. Barkan, unpublished results).

(A) Transcripts of the 23S ribosomal DNA. The 23S 3′ probe was a 4.3-kb PstI-EcoRI fragment encoding the 3′ half of the maize 23S rRNA and some downstream sequences. The 23S 5′ probe was a 3-kb PstI fragment that encodes the 5′ half of the maize 23S rRNA and some upstream sequences.

(B) Transcripts of the 16S ribosomal DNA. These results were obtained by reprobing the filter shown in the left panel of Figure 3. The maize chloroplast Bam13 fragment was used as the hybridization probe.

tha2 in Figure 5A). The 23S rRNAs detected in *cps2* were indistinguishable from those in nonphotosynthetic mutants with normal chloroplast translation.

These results indicate that the *hcf7* and *cps1* mutations cause a decrease in the rate of 23S rRNA maturation and/or a decrease in the stability of the mature form. Despite the fact that pre-23S rRNAs were detected in the mutants, the "broken" molecules of 1.8 and 1.2 kb appeared to have normal termini, because they comigrated with their counterparts in normal chloroplasts. Therefore, the formation of the "hidden break" near residue 1800 of the 23S rRNA seems to be coupled to the processing of the termini.

Analysis of 16S rRNA revealed a clear distinction between *hcf7* and the other mutants. The mature 16S rRNA in *cps1* and *cps2* mutants accumulated to near normal levels (Figure 5B and data not shown). However, the *hcf7* mutation caused a fourfold reduction in the level of this molecule (Figure 5B). The loss of the mature 16S rRNA was accompanied by an increase in the abundance of precursor forms that were undetectable in wild-type plants. Whereas a very small amount of pre-16S rRNA was also detected in the *cps* mutants, this amount was no greater than that observed in nonphotosynthetic mutants with normal chloroplast translation (e.g., *crp2*). Therefore, a slight decrease in the rate of maturation of the 16S rRNA may be a consequence of many photosynthetic defects.

To determine whether the pre-16S rRNA in *hcf7* chloroplasts functions in translation, its association with polysomes was assessed. Figure 6 shows that most of the pre-16S rRNA was excluded from the polysomal fractions, whereas the mature form was not. Therefore, this pre-16S rRNA does not function efficiently in translation.

The termini of the pre-16S rRNA molecules found in *hcf7* plants were mapped by S1 nuclease protection, as shown in Figure 7. Wild-type RNA protected a 410-nucleotide fragment of the "5′ probe," consistent with the previous mapping of the 5′ end of mature 16S rRNA (Strittmatter et al., 1985). RNA from *hcf7* leaves protected an additional fragment of the 5′ probe of ~510 nucleotides. The 5′ end of the most prominent pre-16S rRNA is therefore ~100 nucleotides upstream of that of the mature 16S rRNA. This end maps near to or at the transcription start site for the rRNA gene cluster (Strittmatter et al., 1985), which is 117 nucleotides upstream of the mature 5′ end.

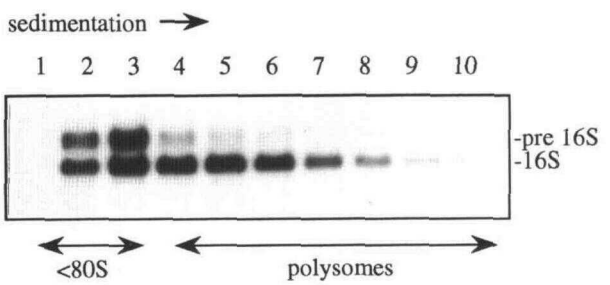

Figure 6. Sedimentation of Pre-16S rRNA in Polysome Gradients.

A lysate of an *hcf7* leaf was fractionated on a sucrose gradient, as described in Methods, and analyzed by RNA gel blot hybridization. The maize chloroplast Bam13 fragment was used as a probe.

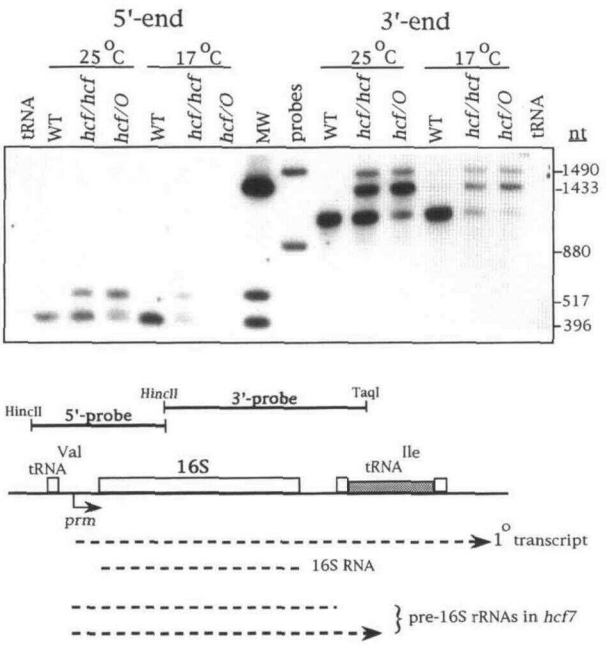

Figure 7. S1 Nuclease Mapping of the Pre-16S rRNA in *hcf7* Leaves.

Reactions contained total leaf RNA from phenotypically normal siblings (WT), diploid mutant plants (*hcf/hcf*), mutant plants hemizygous for chromosome 1L (*hcf/0*; see text), or no maize RNA (tRNA). RNA was purified from plants grown at either 25 or 17°C, as indicated. Molecular size (MW) markers of 1433, 517, and 396 bp were HinfI fragments of pUC18. One nanogram of each double-stranded probe was run in the lane marked "probes." The 3′ probe was 1490 bp; the 5′ probe was 880 bp. nt, nucleotides.

hcf7 RNA protected one additional fragment of the 5′ probe, which was slightly larger than the fragment protected by mature 16S rRNA. In analyses of the most severely affected plants (*hcf/0* grown at 17°C; see below), this band was barely visible while the band corresponding to the fully processed 5′ end was undetectable. This fragment may correspond to a minor processed end in wild-type plants that maps 30 nucleotides upstream of the mature end (Strittmatter et al., 1985).

The maturation of the 3′ end of the 16S rRNA was also aberrant in *hcf7*. Wild-type RNA protected a 1090-nucleotide fragment of a probe spanning the 3′ end of the 16S rRNA (Figure 7). This is consistent with the deduced 3′ end of the mature 16S rRNA (Schwarz and Kössel, 1980). RNA from *hcf7* plants protected an additional fragment of ~1400 nucleotides. The 3′ end that this corresponds to is very near to the start of the $tRNA^{Ile}$ coding sequence (see map in Figure 7). A substantial amount of the 3′ probe was fully protected by RNA from mutant seedlings, indicating that many transcripts continue

an unknown distance past the end of the probe used in the experiment.

Taken together, these results indicate that the pre-16S rRNA that accumulates in *hcf7* is incompletely processed at both its 5′ and 3′ ends. Most of the aberrant molecules begin very near to the transcription start site and end near the RNase P cleavage site that defines the beginning of $tRNA^{Ile}$ downstream. These termini are consistent with the mobility of the pre-rRNA in agarose gels. The pre-16S rRNA migrated slightly more slowly than the 18S rRNA and is, therefore, ~400 nucleotides longer than the mature 16S rRNA. The S1-mapping experiments indicate that this is due to an extension at the 3′ end of ~300 nucleotides and an extension at the 5′ end of ~100 nucleotides.

The severity of the defect in 16S rRNA processing in *hcf7* relative to the other mutants cannot be explained simply as a consequence of a tighter block in translation: *cps1-2* mutants had a more severe translation defect (Figure 1), but contained much less pre-16S rRNA and much more mature 16S rRNA than *hcf7* (Figure 5B). Therefore, the primary defect in *hcf7* may be in the processing of the 16S rRNA itself. A defect of this nature would result in a decrease in functional 30S ribosomal subunits (Figure 6). The primary defects in *cps1* and *cps2* mutants probably lie elsewhere, because these mutants accumulated no more pre-16S rRNA than other nonphotosynthetic mutants with normal chloroplast translation.

The *hcf7* Phenotype Is Sensitive to Gene Dosage and Temperature

While mapping the *hcf7* mutation to a chromosome arm, it became apparent that the mutant phenotype was more severe in plants carrying one copy of the mutant allele rather than two. Plants heterozygous for the mutation were crossed with a series of plants carrying a reciprocal translocation between the B chromosome and various standard chromosome arms. Because the B chromosome frequently undergoes nondisjunction at the second pollen mitosis, progeny plants hemizygous for the translocated chromosome arm were obtained at high frequency (Beckett, 1978).

When plants heterozygous for the *hcf7* mutation were pollinated by plants carrying TB-1La (a reciprocal translocation between the B chromosome and the long arm of chromosome 1), 15 of 100 progeny seedlings were small and pale yellow. An example is shown in Figure 8 (*hcf*/0). Approximately the same number were equally small, but fully green (+/0 in Figure 8). The morphology of the small seedlings was characteristic of plants hemizygous for the 1L chromosome arm (Beckett, 1978).

It seemed likely that the pale yellow seedlings were plants in which the *hcf7* mutation had been "unmasked" (i.e., that had the genotype *hcf7*/0) due to the nondisjunction of the B^{1L} chromosome. In support of this, no pigment-deficient plants were obtained when homozygous wild-type plants were crossed by the same translocation-bearing plants, and crosses of *hcf7*/+ plants by stocks carrying B-A translocations involving eight other chromosome arms never gave rise to pigment-deficient progeny.

To test the hypothesis that the pale yellow progeny represented a more severe expression of the *hcf7* mutation, the accumulation of pre-16S rRNAs was examined in S1 protection experiments and by RNA gel blot hybridization, as shown in Figures 7 and 9. The pale yellow plants (designated *hcf*/0) had an even more dramatic increase than *hcf7/hcf7* plants in the ratio of the precursor to the mature form of the 16S rRNA. Several albino mutants were analyzed in parallel to evaluate the possibility that this defect in rRNA processing was simply a consequence of arrested plastid development. Although the albino mutants contained reduced levels of 16S rRNA, pre-16S rRNA molecules were only barely detectable (Figure 9). Therefore, the severe defect in 16S rRNA processing is a hallmark of the *hcf7* mutation, and the pale yellow seedlings almost certainly are hemizygous for *hcf7*. Taken together, these results strongly suggest that the *hcf7* mutation maps to the long arm of chromosome 1. These results also imply that the

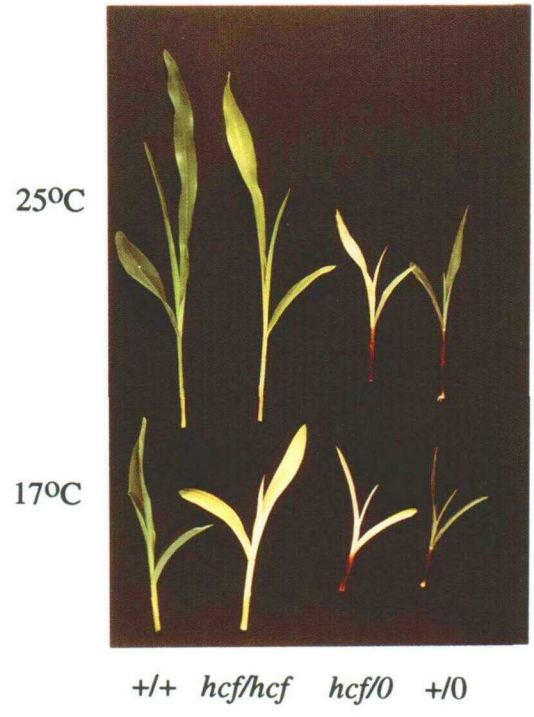

Figure 8. Gene Dosage and Temperature Dependence of Pigmentation in *hcf7* Seedlings.

Plants were germinated and grown in a growth chamber at the indicated temperature under a 16-hr day (light intensity of 400 $\mu E\ m^{-2}\ sec^{-1}$), 8-hr night cycle. Plants hemizygous for chromosome 1L have decreased stature (compare +/+ to +/0), as has been previously observed (Beckett, 1978). This decrease in stature is independent of the *hcf7* genotype. Plants were harvested after 10 days of growth at 25°C or 20 days of growth at 17°C.

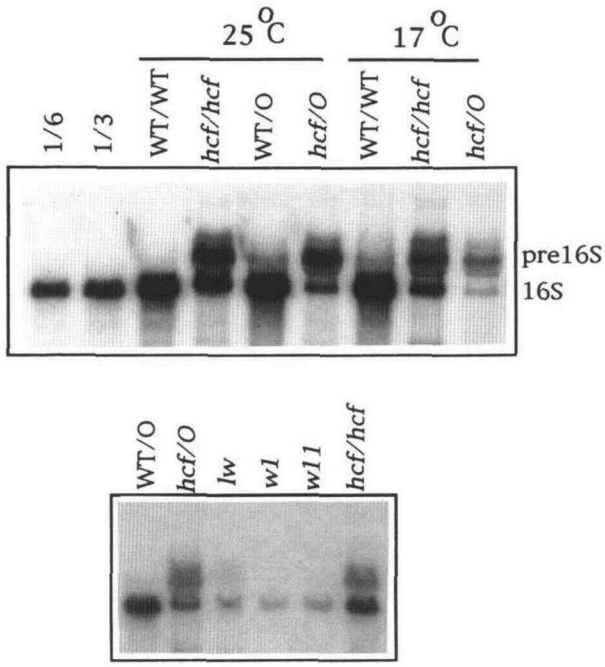

Figure 9. Gene Dosage and Temperature Dependence of 16S rRNA Processing in *hcf7* Seedlings.

Leaf RNA was purified from plant material grown as described in the legend to Figure 8. An equal amount (or the indicated dilutions of the wild-type [WT] sample) of total RNA was analyzed by RNA gel blot hybridization, using the maize chloroplast Bam13 fragment as a probe. Lanes labeled *lw*, *w1*, and *w11* contained RNA purified from albino seedlings homozygous for *lw1*, *w1*, or *w11*. The primary defect in *lw1* mutants appears to lie in the carotenoid biosynthetic pathway, because mutant kernels are white, and chlorophyll accumulates in seedlings grown under low-intensity light (Bachmann et al., 1973).

mutation is leaky: deficiencies in pigmentation and in 16S rRNA processing are both more severe in plants hemizygous for the mutant allele (*hcf7/0*) than in homozygous *hcf7/hcf7* plants.

The severity of the *hcf7* phenotype was also increased by growth at low temperatures. Mutant seedlings grown at 17°C had substantially less pigment (Figure 8) and had a lower proportion of mature 16S rRNA (Figures 7 and 9) than plants grown at 25°C. These properties are similar to those of several "virescent" mutants of maize for which growth at low temperatures induces chlorosis and loss of plastid ribosomes (Hopkins and Elfman, 1984). Based upon the variety of phenotypes seen with *hcf7*, it seems quite likely that some ivory, pale yellow, and virescent mutants are allelic to mutants like those described here.

Expression of Nuclear Genes Encoding Chloroplast Proteins in Chloroplast Translation Mutants

It has been postulated that a "signal" from the chloroplast is necessary for the normal transcription of a subset of nuclear genes encoding chloroplast proteins (reviewed by Taylor, 1989). Experiments using inhibitors of chloroplast transcription and translation have led to the model that the transmission of this signal is dependent upon chloroplast transcription (Rapp and Mullet, 1991) and translation (Oelmuller et al., 1986) at critical stages of development. Because the *hcf7* defect was apparent even at the very earliest stages of the proplastid-to-chloroplast transition (data not shown), this model predicts that the *hcf7* mutation would have pleiotropic effects on the transcription of those nuclear genes that are sensitive to the putative chloroplast signal.

Because the level of the mRNA encoding the major light-harvesting chlorophyll *a/b* binding protein has been shown previously to be sensitive to the putative chloroplast signal (Mayfield and Taylor, 1984), its level was compared in leaves of *hcf7/hcf7*, *hcf7/0*, and wild-type seedlings, as shown in Figure 10. The level of this mRNA was only slightly decreased in *hcf7/0* plants grown at 25°C. However, in plants grown at 17°C its level was reduced three- and sixfold in *hcf7/hcf7* and *hcf7/0* leaves, respectively. Therefore, the translation defect in its milder expression does not appear to block the transmission of the putative chloroplast signal. The more severe translation block expressed at low temperatures, however, does appear to block the signal to some extent.

DISCUSSION

The results of these studies indicate that the *cps1*, *cps2*, and *hcf7* gene products facilitate the translation of most or all chloroplast mRNAs. Mutants defective in these genes had reduced levels of many chloroplast-encoded proteins without corresponding deficiencies in chloroplast mRNAs. Chloroplast

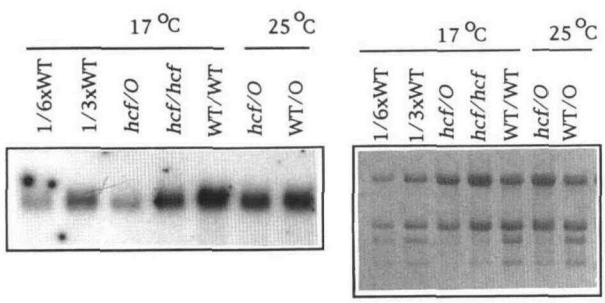

Figure 10. RNA Gel Blot Analysis of Light-Harvesting Chlorophyll *a/b* Binding Protein mRNA.

Equal amounts of total seedling leaf RNA (or the indicated dilutions of the wild-type [WT] sample) were applied to each lane. In the panel at right, the rRNAs that bound to the nylon filter were visualized by staining with methylene blue. At left is shown the results of hybridizing the filter with a probe for the major light-harvesting chlorophyll *a/b* binding protein (Mayfield and Taylor, 1984). Plants used for these experiments were all harvested between 11 AM and 1 PM to avoid fluctuations in transcript levels due to circadian rhythms.

mRNAs in mutant seedlings were associated with abnormally few ribosomes, suggesting a global reduction in the rate of plastidic translation initiation. The genes defined by these mutations may therefore encode components of the chloroplast translation machinery, enzymes that modify the translation machinery, or factors involved in the expression of that machinery.

Analysis of the mutant phenotypes has provided insights into the primary defect in each mutant and into general features of chloroplast rRNA metabolism, mRNA metabolism, and translation.

Chloroplast rRNA Metabolism

The *hcf7* mutant is unique with regard to its severe defect in the maturation of the 16S rRNA. Because the precursors accumulate at the expense of the mature form, the rate of maturation of 16S rRNA in *hcf7* chloroplasts appears to be reduced. Without a clone of the *hcf7* gene, it is not possible to be certain whether this processing defect is a direct consequence of the *hcf7* mutation or a pleiotropic consequence of a defect in ribosome assembly/function. However, the fact that *cps1-2* mutants have a more severe translation block than *hcf7* but accumulate very little pre-16S rRNA argues that the *hcf7* gene product functions directly in the processing of the 16S rRNA. Because the pre-16S rRNA does not function efficiently in translation (Figure 6), a defect in 16S rRNA processing would result in a decrease in the number of functional 30S ribosomal subunits and, therefore, a decrease in the translation rate.

The *hcf7* defect is not a global defect in rRNA maturation because the "broken" 23S rRNA fragments were of normal size and abundance. An RNase III–like activity may be involved in the maturation of chloroplast rRNAs (Delp and Kössel, 1991), but the *hcf7* phenotype is unlikely to result from a defect in a chloroplast homolog of RNase III for two reasons: in bacteria, RNase III is involved in generating both 23S and 16S rRNAs, and RNase III–deficient strains of *Escherichia coli* synthesize 16S rRNA with the mature 5′ end at the normal rate (reviewed by Srivastava and Schlessinger, 1990). Mutants like *hcf7* should be helpful for defining the enzymes that process chloroplast rRNAs.

Rye leaf cells that differentiate at suitably high temperatures accumulate few chloroplast ribosomes and little chlorophyll (reviewed by Feierabend, 1982). A 16S rRNA precursor of similar size to that found in *hcf7* has been observed in heat-bleached rye leaves (Subramanian et al., 1991). Perhaps the rye nuclear genome enodes a temperature-sensitive component of the same enzyme that is defective in *hcf7*.

Alterations in rRNA metabolism shared by several of these mutants are likely to be pleiotropic consequences of a reduction in translation rate or defective ribosome assembly. For example, *hcf7* and *cps1* mutants failed to accumulate mature intact 23S rRNA. Analysis of other nonphotosynthetic mutants revealed that this property is not simply a consequence of a photosynthetic defect. It is likely to be a hallmark of many types of lesions in the chloroplast translation machinery and will be useful for quickly identifying such mutants in the future. The final stages of bacterial rRNA processing are coupled with ribosome formation and/or translation (Srivastava and Schlessinger, 1990). The properties of these maize mutants suggest that normal maturation of chloroplast rRNAs also depends upon a functional translation apparatus.

The bulk of the chloroplast 23S rRNA in higher plants is recovered as several smaller molecules (reviewed by Delp and Kössel, 1991). It is interesting that the broken "23S" molecules derived from mutant and normal leaves were the same size, even though the unbroken molecules in the mutants accumulated only as pre-23S rRNAs. These results suggest that the break does not occur unless the ends are matured, perhaps because the break occurs only in translating ribosomes.

Chloroplast Translation

Analysis of two Chlamydomonas mutants with defects in chloroplast translation led to the hypothesis that ribosomal protein mRNAs may be preferentially translated under conditions of reduced chloroplast protein synthesis (Liu et al., 1989). One of the mutants used in those studies, *cr-1*, resembled *hcf7* in that it was deficient, specifically, in the small ribosomal subunit (Harris et al., 1974). *hcf7* and *cps1* might be useful for determining whether this model holds true for higher plant chloroplasts. If mRNAs encoding ribosomal proteins were preferentially translated in these maize mutants, this might be reflected by a relatively unaltered association of ribosomal protein mRNAs with ribosomes. However, this was not found to be the case. Sucrose gradient fractionations revealed that two mRNAs encoding ribosomal proteins (*rpl16* and *rps4*) were also associated with abnormally few ribosomes in *hcf7* and *cps1* mutants, suggesting that these mRNAs are not preferentially translated. It remains possible that other ribosomal protein mRNAs are preferentially translated in these mutants, or that the differences in translation are not reflected by obvious differences in ribosome association.

It should be noted that many ribosomal proteins do appear to accumulate to normal levels in these mutants, despite the global losses of proteins involved in photosynthesis. Protein gel blots probed with antibodies raised against the spinach 50S or 30S ribosomal subunits revealed similar populations of proteins in extracts of *hcf7*, *cps1*, and wild-type leaves (data not shown). Unfortunately, the identities of individual bands were not known, so it is unclear which, if any, of the proteins detected were encoded by chloroplast genes.

Chloroplast mRNA Metabolism

The stabilities of certain chloroplast mRNAs appear to change during the maturation of spinach leaves (Klaff and Gruissem, 1991) and after illumination of dark-grown barley seedlings (Klein and Mullet, 1990). It is not clear what mediates these changes, although a role for specific mRNA binding proteins has been proposed (Stern and Gruissem, 1987). In both

prokaryotic and eukaryotic cells, certain mRNAs are stabilized and others are destabilized by their association with translating ribosomes (reviewed by Brawerman, 1989). To address the possibility that developmentally induced changes in chloroplast mRNA stabilities might be mediated, in some cases, by changes in their translation, various chloroplast mRNAs were quantified in these mutants. The level of the *rbcL* mRNA was reduced fourfold in *cps1*, *cps2*, and *hcf7* mutants; in contrast, the *rbcL* mRNA level was normal in 13 other nonphotosynthetic mutants with unaltered polysome recruitment. Among this latter group were three mutants with 10-fold reductions in the Rubisco protein (A. Barkan, unpublished results), indicating that the absence of Rubisco protein per se does not alter the accumulation of its mRNA.

This correlation between polysome incorporation and *rbcL* mRNA level seems most likely to be due to a change in the stability of the *rbcL* mRNA rather than a change in its rate of synthesis. There is ample precedent for this, in that numerous prokaryotic mRNAs are destabilized as a consequence of a reduction in their association with ribosomes (Nilsson et al., 1987; Brawerman, 1989; Yarchuk et al., 1991). On the other hand, it is difficult to envision a mechanism linking the transcription of, specifically, the *rbcL* gene with changes in the recruitment of chloroplast mRNAs into polysomes. If there were a plastid-encoded transcription factor specific for *rbcL*, a reduction in chloroplast translation might lead to a specific decrease in the transcription of the *rbcL* gene. This is unlikely for two reasons. First, treatment of dark-grown barley seedlings with chloramphenicol actually stimulated light-activated *rbcL* transcription (Klein, 1991). Second, three maize mutants that appear to have global blocks in chloroplast translation elongation exhibit the global protein losses seen in *hcf7* and *cps1* mutants, but recruit mRNAs into polysomes and have normal levels of *rbcL* mRNA (A. Barkan, unpublished results). Therefore, a decrease in the association of chloroplast mRNAs with polysomes rather than a reduction in the rate of translation appears to cause the decrease in the level of the *rbcL* mRNA.

The conclusion that a decrease in polysome recruitment destabilizes the *rbcL* mRNA may appear to be at odds with the results of Klaff and Gruissem (1991) who found that lincomycin, an antibiotic that decreases the association of chloroplast mRNAs with polysomes, stabilized the *rbcL* mRNA in spinach leaves. The mutations described here also decrease polysome association of mRNAs, but result in the destabilization of the maize *rbcL* mRNA. This difference may simply reflect a difference in chloroplast mRNA metabolism in maize and spinach. However, both sets of results are consistent with the following common mechanism. Lincomycin inhibits the formation of only the first peptide bond in newly initiated polypeptides. After sufficient time to allow run-off of previously initiated ribosomes, this results in mRNAs that are not devoid of bound ribosomes, but rather have one or two that remain near the start codon (Vazquez, 1979). If there were a particularly labile site on the *rbcL* mRNA very close to the start codon (as is true of some bacterial mRNAs; reviewed by Brawerman, 1989), lincomycin might increase the stability of this mRNA by stalling a ribosome over that site. The maize mutations described here would lead to increased exposure of this labile site, because the few ribosomes that do initiate translation probably do not remain at the start codon. Thus, the two sets of results together may indicate that an important determinant for the stability of the *rbcL* mRNA is a labile site near its start codon.

Despite an extensive analysis of chloroplast transcripts in *hcf7*, *cps1*, and *cps2* mutants, no other significant difference in mRNA size or abundance was observed. Minor differences in the levels of certain transcripts or slight changes in the ratios of transcripts from certain transcription units were observed. However, these differences were also observed in nonphotosynthetic mutants with normal chloroplast translation, indicating that they result from the altered physiology in nonphotosynthetic chloroplasts. Therefore, decreased recruitment of chloroplast mRNAs into polysomes has little effect on the stability or processing of most chloroplast mRNAs.

cps2 Causes a General Decrease in Polysome Assembly but a Specific Loss of Rubisco

The specificity of the protein loss in *cps2* is intriguing. All chloroplast mRNAs examined were associated with abnormally few ribosomes, suggesting a global decrease in the translation initiation rate. However, the level of Rubisco was decreased 20-fold while the accumulation of many chloroplast-encoded thylakoid membrane proteins was reduced only twofold. How can one explain this discrepancy? We considered the possibilities that the translation defect was specific to bundle sheath chloroplasts (the primary site of Rubisco synthesis) or that bundle sheath chloroplast differentiation was blocked. However, analysis of polysomes in separated bundle sheath and mesophyll cells revealed that polysome assembly was defective in both bundle sheath and mesophyll chloroplasts (J. Mendel-Hartvig and A. Barkan, unpublished data). Furthermore, malic enzyme, another enzyme specific to bundle sheath chloroplasts, accumulated to normal levels in *cps2* (data not shown). A defect in the synthesis of the small subunit of Rubisco is unlikely to be responsible for the loss of the large subunit because *rbcS* mRNAs accumulate normally (data not shown). In addition, the chloroplast chaperonin-60 was found at normal levels (data not shown), eliminating the possibility that its absence leads to the specific loss of Rubisco. Although the reason for the specific loss of Rubisco in *cps2* remains a mystery, the following scenarios are consistent with our observations.

Because *cps2* mutants accumulated somewhat more Rubisco than *hcf7* and *cps1* mutants (Figure 1), it appears that, of these, *cps2* has the "leakiest" translation defect. Thus, the accumulation of Rubisco seems to be a much more sensitive indicator of decreased polysome recruitment than other prominent chloroplast-encoded proteins. The fact that the *rbcL* mRNA level was decreased as a consequence of decreased polysome association certainly contributes to this effect by amplifying what might otherwise be small decreases in the translation

rate. But the fourfold reduction in the *rbcL* mRNA level is not sufficient to account for the 10-fold differential between the accumulation of Rubisco and the accumulation of some thylakoid complexes in *cps2* mutants. It may be that the *rbcL* mRNA has a particularly low affinity for ribosomes, such that a small decrease in the ability of ribosomes to initiate translation results in a disproportionate decrease in the rate of *rbcL* translation. Alternatively, the disproportionate decrease in the level of Rubisco could be explained if many chloroplast-encoded thylakoid proteins are ordinarily synthesized in excess of the amount that is stabilized by incorporation into photosynthetic complexes, whereas the *rbcL* gene product is not synthesized in as great an excess.

What Are the Functions of the *hcf7*, *cps1*, and *cps2* Genes?

To define the biochemical roles of the genes defined by these mutations, it will be essential to clone them. It is unfortunate in this regard that *hcf7* is a chemically induced mutation. On the other hand, the *cps1* and *cps2* mutants are transposon induced: they arose in maize lines harboring active *Mu* transposons, and they are unstable, exhibiting small revertant sectors of dark-green tissue on pale green mutant leaves. It should therefore be possible to clone these genes by virtue of their association with a *Mu* transposon. The phenotypes of the *cps1* and *cps2* mutants are consistent with the possibility that their mutations lie in nuclear genes encoding previously defined components of the chloroplast translation machinery. Even this apparently trivial possibility would, if confirmed, enhance our understanding of the chloroplast translation machinery. Only approximately one-third of the nuclear genes encoding chloroplast ribosomal proteins have been cloned (reviewed by Subramanian et al., 1991). More importantly, there are a number of plastid-specific ribosomal proteins with no bacterial homologs, whose roles in the chloroplast are not understood (Gantt, 1988; Zhou and Mache, 1989; Johnson et al., 1990). A mutant phenotype associated with mutation of any of these structural genes would be invaluable for assigning function.

It is useful to consider the types of mutants that might arise in future screens for transposon-induced mutants defective in chloroplast translation. Among the mutants with global reductions in chloroplast-encoded proteins and decreased chloroplast polysome size will be mutants with defects in chloroplast rRNA processing (like *hcf7*), and possibly mutants with specific defects in the expression of one or more chloroplast genes encoding ribosomal proteins. This latter class could include mutants with defects in the *trans*-splicing of the *rps12* mRNA and mutants with defects in the transcription or mRNA maturation of any of the other chloroplast-localized ribosomal protein genes. Mutants may also arise that are defective in light-activated (Gamble et al., 1989) or developmentally induced translation, rather than simply in constitutive translation. Because of these exciting possibilities, we are looking forward to recovering more transposon-induced mutants of this type in the future.

METHODS

Mutant Isolation, Propagation, and Genetic Analysis

hcf7 was recovered as a nonphotosynthetic mutant by D. Miles following an ethylmethane-sulfonate mutagenesis performed by G. Neuffer (University of Missouri, Columbia). A brief account of this mutant was presented previously (Taylor et al., 1987). *cps1-1*, *cps1-2*, and *cps2* were recovered from lines harboring active *Mu* transposons that were propagated by S. Hake, M. Freeling, and coworkers (University of California at Berkeley). These mutants were first identified by their pale green, seedling lethal phenotype. All four mutations are recessive. They were propagated by outcrossing heterozygotes to inbred lines, followed by self-pollination of heterozygotes to recover homozygous mutant seedlings. The mutations were inherited through the pollen and segregated as single Mendelian traits, indicating that they are localized to single nuclear genes. Except where noted otherwise, plants used in these studies were grown in a greenhouse for 10 days, at which time the first two leaves were fully expanded and the third leaf was only partially expanded. At this stage, the size and morphology of mutant and normal plants were indistinguishable. All plants were harvested between 11 AM and 2 PM. Within seconds after harvesting, plants were frozen in liquid nitrogen. Frozen tissue was stored at −80°C until use.

Complementation tests were performed by intercrossing plants heterozygous for each mutation. Both crosses made between pairs of plants heterozygous for *cps1-1* and *cps1-2* yielded one-quarter pale green, lethal progeny; these mutant progeny had the global losses of chloroplast proteins seen in the two parents. Therefore, *cps1-1* and *cps1-2* do not complement, and are most likely allelic. Numerous crosses involving one wild-type and one heterozygous plant did not yield any mutant progeny.

All four crosses made between pairs of plants heterozygous for *hcf7* and *cps1* failed to yield any mutant progeny. Therefore, these mutations complement one another and are almost certainly not allelic. A cross between plants heterozygous for *cps2* and *cps1* failed to yield mutant progeny, indicating that these mutations also complement one another.

Mutations were mapped to chromosome arms by crosses with B-A translocation stocks. The *cps2* mutation was mapped to chromosome 6L: crosses of three *cps2*/+ plants by TB-6Lc pollen yielded pale green lethal seedlings lacking Rubisco. The *cps1* mutations were mapped to chromosome 1L: crosses of five *cps1*/+ plants by TB-1La pollen yielded pale green seedlings with hypoploid morphology and lacking Rubisco. The *hcf7* mutation was mapped to chromosome 1L, as described in this paper.

Electrophoretic Fractionation and Immunological Detection of Protein

Approximately 0.2 g of leaf tissue in 0.5 mL of ice cold homogenization buffer (40 mM β-mercaptoethanol, 10% sucrose, 100 mM Tris-HCl, pH 7.2, 5 mM EDTA, 5 mM EGTA, 2 μg/mL aprotinin, 2 mM phenylmethylsulfonyl fluoride) was ground to a fine slurry with a mortar and pestle. Fibrous material was removed by filtration through a glass wool plug

in a small syringe. Membranes were pelleted by centrifugation at 4°C for 3 min at 12,000 rpm in a microcentrifuge. The supernatant was removed and used as the "soluble" fraction. Membranes were then rinsed once in homogenization buffer and resuspended to equal chlorophyll concentrations in homogenization buffer.

Protein samples were solubilized by the addition of an equal volume of 7 M urea, 5.8% SDS, 12.5% β-mercaptoethanol, 25% glycerol, 0.17 M Tris-HCl, pH 6.8, with incubation at 65°C for 15 min. Samples were then electrophoresed on 13% polyacrylamide gels containing 4 M urea, prepared using the buffer system of Laemmli (Laemmli, 1970). Protein was electrophoretically transferred to nitrocellulose (Sambrook et al., 1989) and visualized on the membrane by staining with Ponceau S (Sigma). Antibody/antigen complexes were detected with the ECL system (Amersham International).

In some experiments, the signal obtained with a particular antibody did not decrease in proportion to the decreasing amounts of protein applied to the wild-type dilution lanes. Such blots were not used to quantify that particular protein. At least five seedlings with each mutant genotype were analyzed, and several sets of gels were run for each mutant. Single examples are shown, due to space limitations.

RNA Gel Blot Analysis

Total leaf RNA was purified from 10-day-old seedlings as described by Martienssen et al. (1989). RNA was fractionated in agarose-formaldehyde gels as described previously (Barkan, 1988), transferred to nylon membrane by capillary blotting, and fixed to the membrane by UV cross-linking. The rRNA bound to membranes was stained with methylene blue by immersing the membrane briefly in a solution of 0.03% methylene blue, 0.3 M NaAc, pH 5.2, and destained by rinsing in water. Hybridizations took place at 65°C in 7% SDS, 0.5 M sodium phosphate, pH 7, and 1 mM EDTA. Membranes were washed for 1.5 hr at 65°C in five changes of 0.2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS (for DNA probes), or 0.1 × SSC, 0.5% SDS (for RNA probes).

Transcripts of the *psaA/B*, *petA*, *petB*, *petD*, *psbC*, *psbH*, *psbE*, and *atpF* genes were detected by hybridization with radiolabeled, gel-purified DNA fragments that mapped internal to the coding region of the indicated maize chloroplast gene. The specific DNA fragments used have been described previously (Barkan et al., 1986; Barkan, 1988, 1989). The *rbcL* and *atpB/E* transcripts were detected by hybridization with the maize chloroplast Bam9 fragment. DNA probes were radiolabeled by random hexamer priming.

RNA probes were used to detect transcripts of the *psbA*, *rps16*, *psaC*, *psbN*, *psbI*, *rps18*, *rpl33*, and *rps12* genes. The following DNA fragments were used as templates for the production of RNA probes: *psbA*, a 1.2-kb BglII-XbaI fragment of spinach chloroplast DNA that includes most of the *psbA* coding sequence; *rps16*, a 1.4-kb BamHI-EcoRI fragment of the maize chloroplast Bam11 fragment; *psaC*, a 700-bp EcoRI fragment of maize chloroplast DNA that also encodes the N terminus of the *ndhD* gene; *rps12*(3′), a 1-kb BamHI- PstI fragment of the maize chloroplast Bam23 fragment; *rps12*(5′), a 400-bp HindIII-KpnI fragment of the maize chloroplast Bam10 fragment; *psbN*, an 800-bp fragment of the maize chloroplast Bam9′ fragment extending between the 3′ end of *psbB* and the 5′ end of *psbH*; *psbI*, a 200-bp BamHI-EcoRI fragment of the maize chloroplast Bam2 fragment; *rps18* and *rpl33*, a 2-kb KpnI fragment of the maize chloroplast Bam10 fragment. The maize chloroplast BamHI fragments are named as described by Larrinua et al. (1983). RNA probes were transcribed with the appropriate RNA polymerase in the presence of ^{32}P-UTP, as described by the manufacturer.

Polysome Analysis

Polysomes were isolated by grinding ~0.3 g of frozen leaf tissue in liquid nitrogen to a fine powder with a mortar and pestle. One milliliter of polysome extraction buffer (0.2 M Tris-HCl, pH 9, 0.2 M KCl, 35 mM $MgCl_2$, 25 mM EGTA, 0.2 M sucrose, 1% Triton X-100, 2% polyoxyethylene-10-tridecyl ether, 0.5 mg/mL heparin, 100 mM β-mercaptoethanol, 100 μg/mL chloramphenicol, 25 μg/mL cycloheximide) was added, and the leaf material was ground further until thawed. After 10 min on ice, nuclei and insoluble material were pelleted by centrifugation for 5 min at 12,000 rpm in a microcentrifuge. Sodium deoxycholate was added to the supernatant to a concentration of 0.5%, and the sample was placed on ice for 5 min. Remaining insoluble material was then pelleted by centrifugation at 12,000 rpm for 15 min. The supernatant (0.4 mL) was layered onto 4.4-mL sucrose gradients that were prepared, centrifuged, and fractionated as described previously (Barkan, 1988). RNA was purified from each fraction by detergent treatment, phenol extraction, and ethanol precipitation (Barkan, 1988). Each mutant was analyzed in parallel with a normal sibling. Samples were kept at 4°C throughout preparation.

S1 Nuclease Mapping

The probe for the 5′ end of the pre-16S rRNA was an 880-bp HincII fragment including the rRNA promoter and extending ~500 bp into sequences encoding the mature 16S rRNA (Schwarz and Kössel, 1980; Strittmatter et al., 1985). The 3′ probe was a 1490-bp fragment with one end at the HincII site in the middle of the 16S coding region and the other end at a *TaqI* site in the *trnI* intron (Koch et al., 1981). This DNA fragment extends ~500 bp past the 3′ end of the mature 16S rRNA.

Twenty nanograms of gel-purified probe fragment, 100 ng (*hcf7/hcf7* and *hcf7/0* at 25°) or 50 ng (remaining samples) of total leaf RNA, and 6 μg of yeast tRNA were suspended in 15 μL of S1 hybridization buffer (80% formamide, 400 mM NaCl, 40 mM Pipes, pH 6.4, 1 mM EDTA). The mixture was heated to 80°C for 5 min to denature the DNA, cooled to 58°C, and incubated overnight. These conditions prevented reannealing of the DNA probe, but allowed the formation of RNA/DNA hybrids. S1 reaction buffer (150 μL; 250 mM NaCl, 30 mM NaAc, pH 5.5, 1 mM $ZnSO_4$, 10 μg/mL denatured calf thymus DNA) containing 150 units of S1 nuclease (Bethesda Research Laboratories) was added to each hybridization and incubated at 37°C for 1 hr. Reactions were terminated by the addition of 40 μL of 2.5 M NH_4Ac, 50 mM EDTA, 400 μg/mL tRNA. Nucleic acids were precipitated by the addition of 2 volumes of ethanol and resuspended in 15 μL of 10 mM Tris, pH 8, 1 mM EDTA. Samples were electrophoresed on 1.5% agarose gels, denatured in alkali, and transferred to nylon membranes. Blots were hybridized with the Bam13 fragment of maize chloroplast DNA, which encodes all of the mature 16S rRNA.

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