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The Nucleus-Encoded *HCF107* Gene of Arabidopsis Provides a Link between Intercistronic RNA Processing and the Accumulation of Translation-Competent *psbH* Transcripts in Chloroplasts

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To understand the functional significance of RNA processing for the expression of plastome-encoded photosynthesis genes, we investigated the nuclear mutation *hcf107* of Arabidopsis. The mutation is represented by two alleles, both of which lead to a defective photosystem II (PSII). In vivo protein labeling, in vitro phosphorylation, and immunoblot experiments revealed that the *psbB* gene product (CP47) and an 8-kD phosphoprotein, the *psbH* gene product (PsbH), are absent in mutant plants. PsbH and PsbB are essential requirements for PSII assembly in photosynthetic eukaryotes, and their absence in *hcf107* is consistent with the PSII-less mutant phenotype. RNA gel blot hybridizations showed that the *hcf107* mutation specifically impairs the accumulation of some but not all oligocistronic *psbH* transcripts that are released from the pentacistronic *psbB-psbT-psbH-petB-petD* precursor RNA by intergenic endonucleolytic cleavage. In contrast, neither the levels nor the sizes of *psbB*-containing RNAs are affected. S1 nuclease protection analyses revealed that *psbH* RNAs are lacking only where *psbH* is the leading cistron and that they are processed at position −45 in the 5′ leader segment of *psbH*. These data and additional experiments with the cytochrome b_6f complex mutant *hcf152*, which is defective in 3′ *psbH* processing, suggest that only those *psbH*-containing transcripts that are processed at their −45 5′ ends can be translated. Secondary structure analysis of the 5′ *psbH* leader predicted the formation of stable stem loops in the nonprocessed transcripts, which are unfolded by processing at the −45 site. We propose that this unfolding of the *psbH* leader segment as a result of RNA processing is essential for the translation of the *psbH* reading frame. We suggest further that HCF107 has dual functions: it is involved in intercistronic processing of the *psbH* 5′ untranslated region or the stabilization of 5′ processed *psbH* RNAs, and concomitantly, it is required for the synthesis of CP47.

INTRODUCTION

Plastidial genes of land plants typically are organized in polycistronic transcription units (Herrmann et al., 1992; Sugiura, 1992). These transcription units often are composed heterogeneously and reflect their postendosymbiotic assembly from different cyanobacterial progenitor genes or operons (Douglas, 1994). The evolutionary origin also may explain the highly complex transcript patterns of plastid transcription units and the many auxiliary and regulatory factors that are required for the expression of these operons (reviewed by Barkan and Goldschmidt-Clermont, 2000).

The complex transcript patterns are caused in part by multiple transcriptional initiation. However, the great majority of the transcripts are generated by RNA processing. Processing involves the splicing of introns, endonucleolytic cleavage in intercistronic regions, exonucleolytic and/or endonucleolytic trimming of RNA 5′ and 3′ ends, polyadenylation, and RNA editing (reviewed by Monde et al., 2000). The functional significance of these processing events is only beginning to be understood, but a detailed explanation

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of the in vivo function of plastidial RNA processing is still lacking.

Both the 5′ and 3′ ends of plastid transcripts carry determinants for regulating RNA turnover. The 3′ ends often are folded into stem and loop structures that bind specific proteins and that appear to act as barriers against 3′ exonucleolytic degradation (reviewed by Monde et al., 2000). There is evidence that ribonucleoprotein complexes also are assembled at the 5′ RNA segments and that these structures serve to determine the stability of the corresponding RNA (Salvador et al., 1993; Nickelsen et al., 1994). Besides their function in RNA stability, the 5′ untranslated regions play an important role in translation (reviewed by Bruick and Mayfield, 1999; Zerges, 2000). Transregulatory factors have been identified that bind to *cis*-active elements within these segments and regulate the translational activity of the RNA (Danon and Mayfield, 1991; Zerges et al., 1997). Differences in the size of the 5′ untranslated region attributable to multiple processing sites may influence the translational activities of the RNAs. This notion is supported by the analysis of the high-chlorophyll fluorescence mutant *crp1* (for *chloroplast RNA processing*) of maize, which revealed that the fully processed *petB* and *petD* RNAs are better templates for the translational machinery than are their polycistronic precursors (Barkan et al., 1994). Findings in bacteria suggest that depending on their length, the 5′ untranslated regions may fold into different secondary structures, which affects their translational activity (reviewed by Lindahl and Hinnebusch, 1992).

We are interested in understanding the functional significance of RNA processing for the expression of plastid genes and are pursuing a genetic approach with Arabidopsis as the model plant. Our focus is on the *psbB-psbT-psbH-petB-petD* operon, which serves as a paradigm for a typical plastidial, polycistronic transcription unit (Barkan, 1988; Westhoff and Herrmann, 1988). The genes of this unit encode five thylakoid membrane polypeptides that belong to two different protein complexes, photosystem II (PSII) (*psbB*, *psbT*, and *psbH*) and the cytochrome b_6f complex (*petB* and *petD*). *psbB*, *psbT*, and *psbH* encode the CP47 chlorophyll apoprotein and the T subunit and H subunit of PSII, the latter of which has been called the 10-kD phosphoprotein. *petB* and *petD* encode cytochrome b_6 and subunit IV of the cytochrome b_6/f complex. The peculiar feature of this transcription unit is the presence of another PSII gene, *psbN*, that is located in the intercistronic segment of *psbT* and *psbH* but is transcribed in the opposite direction and thus is not a component gene of the *psbB-psbT-psbH-petB-petD* operon (Kohchi et al., 1988).

Here we report a recessive nuclear mutation in Arabidopsis, named *hcf107* (Meurer et al., 1996b), that is represented by two alleles (*hcf107-1* and *hcf107-2*). The mutants lack PSII as a result of a deficiency in the synthesis of subunits CP47 (PsbB) and PsbH. At the RNA level, *hcf107* mutant plants are affected in the accumulation of processed *psbH*-containing RNAs. We show that only *psbH* transcripts with a −45 5′ end are absent, whereas processing at the *psbH* 3′ site proceeds normally. The deficiency in *psbH* transcripts with the −45 5′ end is correlated with the inability to synthesize the PsbH protein. We discuss the presence of secondary structures in the unprocessed 5′ *psbH* leader region and propose that unfolding of the *psbH* leader segment attributable to RNA processing is essential for the translation of the *psbH* reading frame.

RESULTS

Two Allelic, Recessive High-Chlorophyll Fluorescence (*hcf*) Mutants with a Defective PSII

By standardized screening of ethyl methanesulfonate– and T-DNA–mutagenized lines of Arabidopsis for *hcf* phenotypes (Meurer et al., 1996b), two pale green mutants, *hcf107* (Meurer et al., 1996b) and DEI117 (this study), were identified that were defective in the RNA pattern of the *psbB-psbT-psbH-petB-petD* transcription unit (see below). Homozygous mutant plants were found to be seedling lethal when grown on soil but could be maintained on sucrose-supplemented agar medium, on which they grew like wild-type plants. Segregational analysis of the mutant phenotype in backcrosses of heterozygous mutant plants to wild type revealed that both mutations were recessive. Crosses of heterozygous *hcf107* to heterozygous DEI117 mutant plants resulted in a 3:1 segregation of wild-type to mutant phenotypes (data not shown). This finding demonstrated that the two mutations were allelic; consequently, the mutants were named *hcf107-1* and *hcf107-2*.

Chlorophyll fluorescence induction experiments showed that both mutants lacked variable fluorescence, which is diagnostic for a defective PSII (Meurer et al., 1998). The P700 redox kinetics revealed that the electron flow to PSI is inhibited but that the photosystem itself is functional (*hcf107-1*, Meurer et al., 1996b; *hcf107-2*, data not shown). The deficiency in PSII function was reflected at the protein level. Immunoblot analyses (Figure 1) with a representative set of antisera specific for the major subunits of PSII indicated that in both mutants, the relative amounts of the PSII reaction center core subunits CP47 and D1 were below the level of detection and that CP43 and D2 were reduced drastically. In contrast, the relative levels of the 33- and 23-kD proteins of the water-splitting complex and of cytochrome b_{559} were not altered significantly.

When equal amounts of thylakoid membrane proteins are analyzed and PSII levels are reduced drastically, a relative increase in the level of the other thylakoid membrane complexes is to be expected. Figure 1 shows that such relative increases in the levels of PSI (subunits PSI-A and PSI-D), the cytochrome b_6f complex (Cyt *f* and Cyt b_6), and the ATP synthase (α and β subunits) were observed in both allelic mutants. The joint results of the spectroscopic and immunoblot analyses indicate that the mutations in *hcf107-1* and

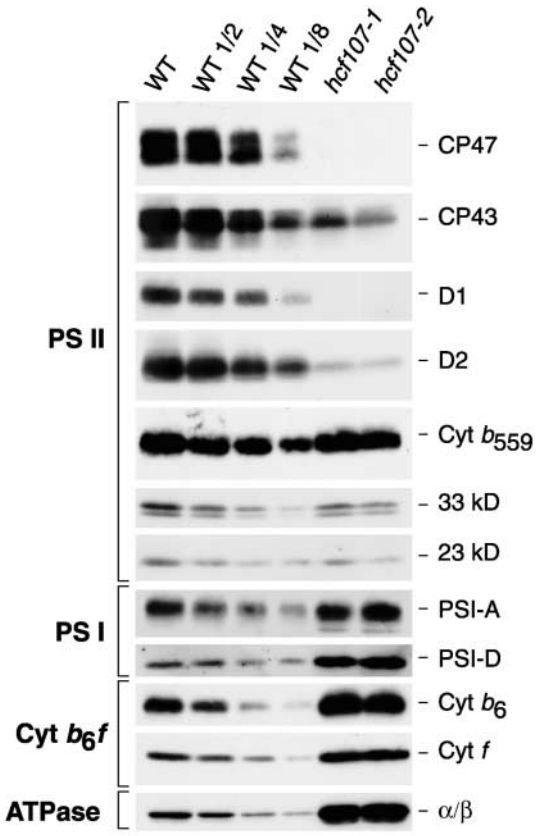

Figure 1. Immunoblot Analysis of Thylakoid Membrane Proteins from *hcf107* and Wild Type.

The lanes were loaded with 10 μg (wild type [WT], *hcf107-1*, and *hcf107-2*), 5 μg (WT 1/2), 2.5 μg (WT 1/4), or 1.25 μg (WT 1/8) of total membrane proteins from ~3-week-old plants grown on a sucrose-supplemented Gelrite medium (see Methods). Proteins were detected with the indicated antiserum to subunits of PSII, PSI, the cytochrome (Cyt) b_6f complex, and the chloroplast ATP synthase (ATPase) as described (Meurer et al., 1996b).

hcf107-2 primarily affect PSII and that these mutants are to be classified as PSII mutants.

Hcf107 Mutant Seedlings Are Deficient in the Synthesis and/or Stability of CP47 and an 8-kD Plastome-Encoded Protein

Diminished amounts of proteins may be attributable to either impaired translation or accelerated degradation of proteins. To distinguish between these two possibilities, the synthesis of thylakoid membrane proteins was investigated in intact mutant seedlings by pulse-labeling experiments with ^{35}S-methionine. To obtain an easily interpretable labeling pattern, the synthesis of the nucleus-encoded chloroplast proteins was blocked with cycloheximide, and only the plastome-encoded proteins were investigated.

Figure 2A shows that the protein-labeling patterns of mutant *hcf107-1* and the wild type were qualitatively similar after a 15-min labeling period. Subunits CP43 and D2 of PSII, the A and B subunits of PSI (PSI-A/B), and the α and β subunits of the chloroplast ATP synthase (CF_1-α/β) were synthesized at approximately wild-type levels. In contrast, the incorporation of ^{35}S label into the D1 polypeptide was reduced significantly. Moreover, the labeling of CP47 and a small polypeptide of ~8 kD was not detectable in *hcf107* (Figure 2A), regardless of whether short (5 min) or longer (20 min) labeling periods were used (Figure 2B). We conclude from these findings that *hcf107* is defective in the synthesis of both CP47 and the 8-kD protein or that the lack of labeling of these proteins is attributable to a very rapid turnover.

The 8-kD Protein Exhibits the Typical Features of the PsbH Protein

The apparent molecular mass of the 8-kD protein is almost identical to the deduced size of the PsbH subunit of PSII (8.1 kD), whose gene is part of the *psbB-psbT-psbH-petB-petD* transcription unit. The PsbH protein is a constituent subunit of the PSII reaction center and, in green plants, an essential requirement for the assembly of this photosystem (Summer et al., 1997; O'Connor et al., 1998). To confirm the identity of the 8-kD protein as the *psbH* gene product, two experimental lines were followed. First, an antiserum was raised against a glutathione *S*-transferase fusion of the PsbH protein and used to quantitate PsbH levels in the two allelic mutants (Figure 3A). Second, because the PsbH subunit of PSII is a phosphoprotein (Farchaus and Dilley, 1986; Michel and Bennett, 1987), in vitro phosphorylation experiments were performed (Figure 3B).

Figure 3A shows that the levels of immunologically detectable PsbH protein were depleted drastically in the two allelic *hcf107* mutants. Although traces of PsbH protein were found in *hcf107-1*, the protein appeared to be completely absent in *hcf107-2*.

In vitro phosphorylation assays provided additional proof that the PsbH protein is depleted in *hcf107* plants (Figure 3B). Wild-type thylakoids showed the expected phosphorylation pattern, with CP43, D2, D1, the light-harvesting chlorophyll *a/b* binding proteins of PSII (LHCII), and the PsbH protein being the major phosphoproteins of the thylakoid membrane (Rintamäki et al., 1997). The phosphorylation pattern of *hcf107-1* thylakoids was strikingly different. Almost no ^{32}P label was detected in the PsbH protein, and the label in the other PSII proteins (i.e., CP43, D2, D1, and LHCII) was reduced drastically. The lack of label in D1 and PsbH was to be expected, because *hcf107* was depleted in these proteins. However, CP43, LHCII, and, to a lesser extent, D2 were present in *hcf107* (see Figure 1 for CP43 and

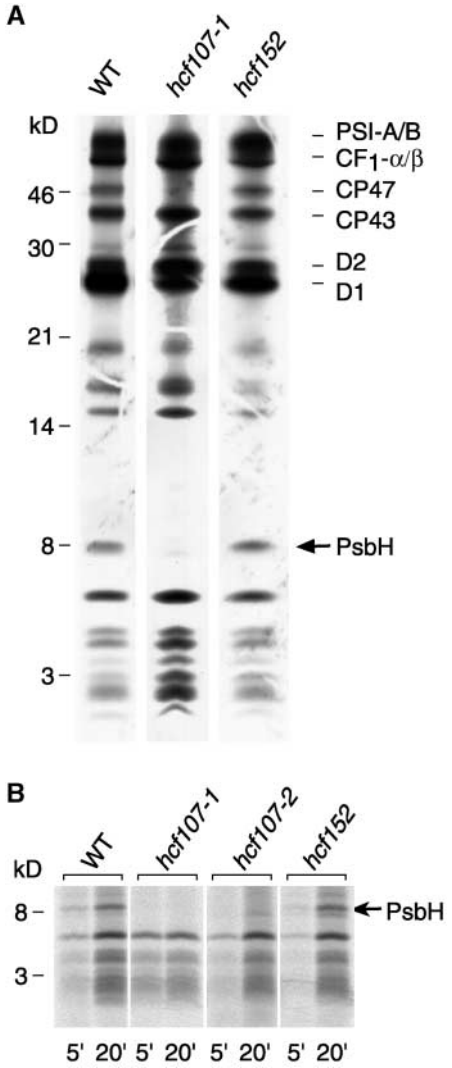

Figure 2. In Vivo Protein Synthesis of Plastome-Encoded Membrane Proteins of *hcf107-1*, *hcf107-2*, *hcf152*, and Wild-Type (WT) Plants.

(A) Pulse labeling for 15 min. Wild-type and mutant proteins with equivalent amounts of radioactivity (100,000 cpm) were separated electrophoretically on a 12.5% polyacrylamide-SDS/urea gel (Schägger and von Jagow, 1987), blotted onto a nitrocellulose membrane, and analyzed by fluorography. The position of the PsbH subunit of PSII is indicated. CF_1-α/β, α and β subunits of the chloroplast ATP synthase; WT, wild type.

(B) Kinetics of ^{35}S incorporation into the 8-kD protein. The labeling periods were 5 min (5′) and 20 min (20′). Only the lower part of the polyacrylamide-SDS/urea gel is shown. The gel was loaded with the following amounts of protein and radioactivity: WT, 5 μg of protein (5′, 20,000 cpm; 20′, 80,000 cpm); *hcf107-1*, 10 μg of protein (5′, 25,000 cpm; 20′, 40,000 cpm); *hcf107-2*, 15 μg of protein (5′, 20,000 cpm; 20′, 60,000 cpm); and *hcf152*, 15 μg of protein (5′, 20,000 cpm; 20′, 90,000 cpm). Radiolabeled proteins were detected by phosphorimaging.

D2; data not shown for LHCII), and the lack of phosphorylation of these proteins may be a secondary effect of the mutation. It is known that the phosphorylation of the PSII proteins CP43, D2, D1, and LHCII is redox controlled via reduction of plastoquinone and the cytochrome b_6f complex (Allen et al., 1981; Bennett et al., 1988), and it is plausible that a PSII-less mutant is defective in this type of control.

Two major conclusions can be drawn from these in vivo protein synthesis, immunoblotting, and in vitro phosphorylation experiments: (1) the PsbH protein is not detectable (*hcf107-2*) or is only barely detectable (*hcf107-1*) in the mutant background, and (2) this depletion is caused by a drastic decrease in its rate of synthesis or by a very rapid turnover.

The *hcf107* Mutation Affects the Accumulation of *psbH*-Containing RNAs

Preliminary RNA gel blot analyses indicated that the *hcf107* mutation affects only the RNA pattern of the *psbB-psbT-psbH-petB-petD* transcription unit, because no significant changes in the RNA levels or patterns of other plastid- and nucleus-encoded genes for photosynthetic proteins were detected (Meurer et al., 1996b; data not shown). To precisely identify the defect in the RNA pattern of the *psbB-psbT-psbH-petB-petD* transcription unit, all genic and extragenic regions of this transcription unit of Arabidopsis were cloned (Felder, 1999) and used as probes in RNA gel blot hybridization experiments. The *psbN* gene, which is located between *psbT* and *psbH* but is transcribed from the opposite strand, was included in this analysis.

Figure 4 shows that the defects in both *hcf107-1* and *hcf107-2* affected only the *psbH* part of the *psbB-psbT-psbH-petB-petD* transcription unit. Oligocistronic *psbB*, *psbT*, *petB*, and *petD* transcripts accumulated in *hcf107* mutant plants as in the wild type. Specifically, neither the amounts nor the apparent sizes of the two dicistronic *psbB-psbT* transcripts of 1900 and 2000 nucleotides and of the two tricistronic *psbB-psbT-psbH* transcripts of 2600 and 2700 nucleotides differed between the wild type and mutant. These doublets of *psbB-psbT* and *psbB-psbT-psbH* transcripts are conserved in monocotyledonous and dicotyledonous plants (Barkan, 1988; Westhoff and Herrmann, 1988; Hird et al., 1991). It reflects two different *psbB* RNA 5′ ends that arise by transcriptional initiation and an additional processing step (Westhoff, 1985; Westhoff and Herrmann, 1988). The accumulation of the *psbN* transcript also was not disturbed by the mutation. In both mutants, the 350-nucleotide single *psbN* RNA was detected, although the abundance of this RNA was somewhat lower in *hcf107-1* and *hcf107-2* than in wild-type plants.

The primary (5600 nucleotides), partially spliced (4900 nucleotides), and fully spliced (4100 nucleotides) pentacistronic *psbB-psbT-psbH-petB-petD* transcripts were present as in the wild type. However, the amounts of these RNAs

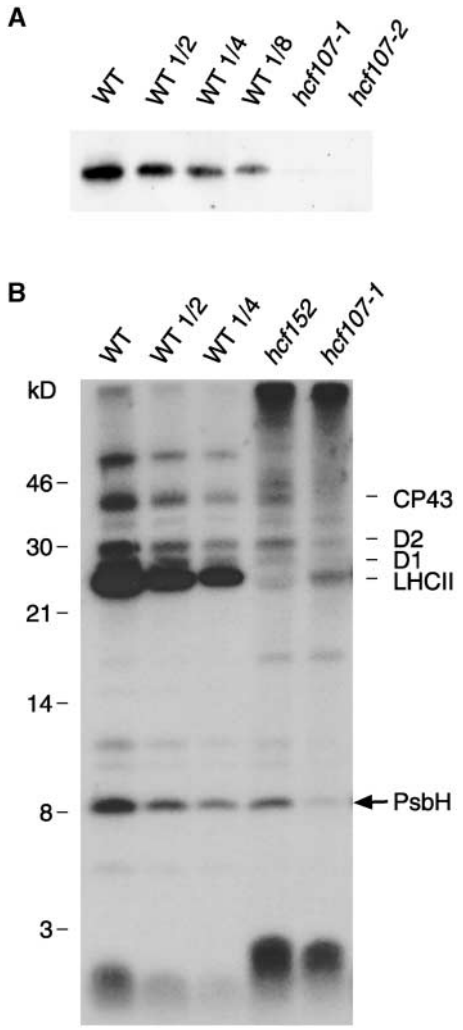

Figure 3. Identification of the 8-kD Protein by Immunoblotting and in Vitro Phosphorylation Experiments.

(A) Immunoblot analysis with an antiserum to the PsbH subunit of PSII. The analysis was performed and labels are as described in the legend to Figure 1.

(B) In vitro phosphorylation with thylakoids of *hcf107* (*hcf107-1*), *hcf152*, and wild-type (WT) seedlings. For experimental details, see Methods. Wild-type and mutant proteins with equivalent amounts of radioactivity (80,000 cpm) were separated electrophoretically on a 12.5% polyacrylamide-SDS/urea gel (Schägger and von Jagow, 1987) and analyzed by fluorography.

were higher in both allelic mutants than in the wild type, suggesting that the processing of the polycistronic transcripts into oligocistronic *psbH* RNAs was slowed down. This conclusion was confirmed by further inspection of the RNA pattern of the *psbH* region. Although the tricistronic *psbB-psbT-psbH* RNAs (2600 and 2700 nucleotides) accumulated to wild-type levels, other processed *psbH*-containing transcripts (i.e., those of 2600, 1800, 1200, and 400 nucleotides) were reduced drastically or below the level of detection (Figure 4). The 2600- and 1800-nucleotide RNAs are tricistronic *psbH-petB-petD* transcripts, with *petB* containing its intron [*psbH-petB*(I_B)*-petD*; 2600 nucleotides] or not [*psbH-petB-petD*; 1800 nucleotides]. Note that the *psbH-petB*(I_B)*-petD* RNA is of the same size as the tricistronic *psbB-psbT-psbH* transcript. The 1200-nucleotide transcript is a dicistronic *psbH-petB* RNA, and the 400-nucleotide transcript represents a monocistronic *psbH* RNA. All of these depleted RNAs have in common that *psbH* is their leading cistron. This finding suggests that the *hcf107* mutation causes a defect in intercistronic processing between *psbT* and *psbH*.

hcf107 Mutant Plants Do Not Accumulate *psbH* RNAs with the −45 5′ End

To substantiate these findings and to take into account the fact that the intercistronic processing between *psbT* and *psbH* was reported to result in *psbH* RNAs with two different 5′ ends (Westhoff and Herrmann, 1988), S1 nuclease mapping experiments were performed with RNA from wild-type and *hcf107-1*/*hcf107-2* plants.

With wild-type RNA and a single-stranded *psbH* 5′ DNA probe, the S1 nuclease assays resulted in two protected fragments of 287 and 257 nucleotides (Figure 5). This would correspond to *psbH* RNA 5′ ends located at −45 and −75 with respect to the translation initiation codon of *psbH* (Figure 6). The 287-nucleotide protected fragment was significantly stronger than the 257-nucleotide fragment, suggesting that transcripts with the −75 5′ untranslated *psbH* leader segment accumulate to higher levels than do those with the −45 end. Only relatively small amounts of fully protected probe were obtained with wild-type RNA (Figure 5). This was not in accord with our expectation, because the amounts of *psbH* transcripts that are not processed between *psbT* and *psbH*, that is, the pentacistronic *psbB-psbT-psbH-petB-petD* RNAs of 5600, 4900, and 4100 nucleotides and the tricistronic 2600/2700-nucleotide *psbB-psbT-psbH* RNAs (Figure 4), were significantly larger than the combined levels of the 2600-, 1800-, 1200-, and 400-nucleotide processed *psbH* RNAs. Consequently, S1 nuclease treatment of the hybridization reaction should have resulted in correspondingly large amounts of fully protected 5′ *psbH* probe.

S1 protection analysis with the 3′ *psbH* probe resulted essentially in only one partially protected fragment (Figure 5), indicating that there is a single major processing site between *psbH* and the 5′ exon of *petB*. In addition, large amounts of fully protected probe were obtained (Figure 5), reflecting the corresponding level of the unprocessed *psbH-petB* RNA segment (Figure 4).

S1 nuclease protection mapping with *psbN* 5′ and 3′ probes yielded a single RNA 5′ and 3′ end, respectively

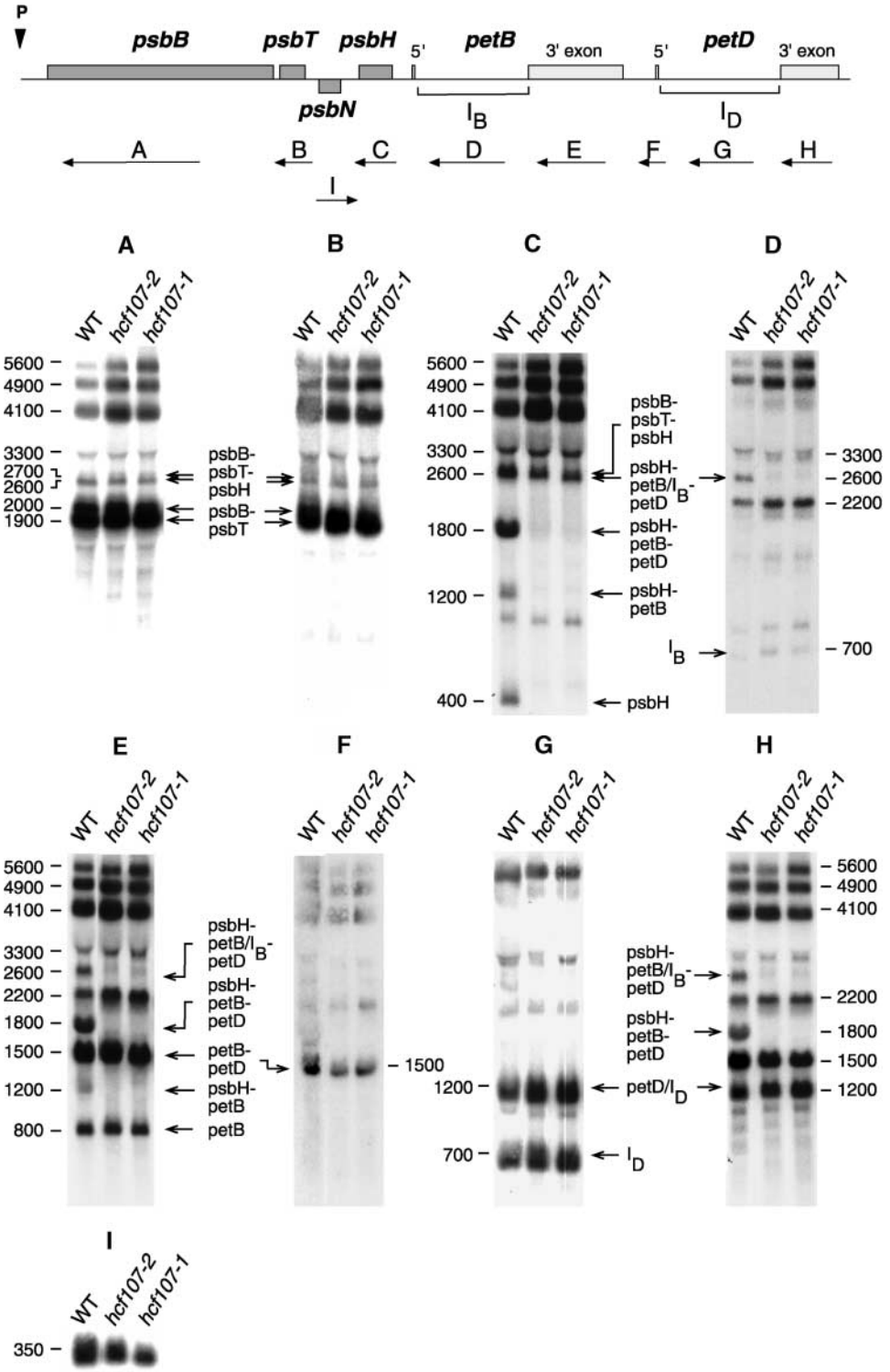

Figure 4. Transcript Pattern of the *psbB-psbT-psbH-petB-petD* Transcription Unit and *psbN* in the Mutants *hcf107-1* and *hcf107-2*.

Eight micrograms of total leaf RNA from 3-week-old mutant and wild-type (WT) seedlings was analyzed in each RNA gel blot hybridization (**[A]** to **[H]**). The hybridization probes and their locations are indicated with arrows and letters (A to H) at the top. The introns of *petB* and *petD* are marked as I_B and I_D. Sizes of the transcripts (in nucleotides) referred to in the text and their genic compositions are indicated.

(Figure 7). The 5′ end of the *psbN* RNA overlaps approximately six nucleotides with the putative −75 *psbH* 5′ end (Figure 6).

This close proximity of the *psbN* 5′ end and the −75 *psbH* 5′ end raises doubts about whether the −75 *psbH* 5′ end could be an artifact that is produced by the concurrent hybridization of complementary RNAs and a single-stranded DNA probe. During hybridization of the 5′ *psbH* DNA probe to unprocessed *psbT-psbH* RNA segments, the *psbN* RNA present also hybridizes to these transcripts. Because RNA-RNA hybrids are more stable than DNA-RNA hybrids under the chosen hybridization conditions (Sugimoto et al., 1995), this concurrent reaction results in *psbT-psbH* nonprocessed RNAs that have hybridized both the *psbN* RNA and the 5′ *psbH* single-stranded DNA probe. S1 nuclease digests the overlapping DNA probe; therefore, the 287-nucleotide protected fragment does not necessarily indicate the existence of the −75 RNA but may have to be considered as an artifact. The finding that full-size protection of the *psbH* 5′ probe is drastically lower than full-size protection of the *psbH* 3′ probe supports this conclusion (Figure 5). We conclude, therefore, that processing between *psbT* and *psbH* most likely yields only one major RNA 5′ end and that this 5′ end is located at position −45 in front of the *psbH* start codon.

Hybridization of the *psbH* 5′ probe with RNA from both *hcf107-1* and *hcf107-2* yielded only the putative −75 5′ end; that is, *psbH* RNAs with the −45 5′ end were not detectable (Figure 5). No differences between *hcf107* and the wild type were observed when S1 nuclease protection analysis was performed with a *psbH* 3′ probe (Figure 5) or with *psbN* 5′ and 3′ probes (Figure 7). It follows that the *hcf107* mutation prevents processing at the −45 processing site between *psbT* and *psbH* or, alternatively, the stabilization of *psbH* RNAs with the 5′ end. Because this defect is associated with a lack of PsbH accumulation, a free *psbH* 5′ end appears to be essential for PsbH synthesis, and *psbH* transcripts with a nonprocessed *psbT-psbH* intercistronic segment are translationally inactive.

Defects in *psbH* 3′ End Processing Do Not Affect *psbH* Translation

To confirm these conclusions, the Arabidopsis mutant *hcf152* was included in the analysis. *hcf152* is defective in the cytochrome b_6f complex but contains a functional PSII (K. Meierhoff and S. Felder, unpublished data). Accordingly, PsbH was synthesized (Figure 2) and phosphorylated (Figure 3) in *hcf152* as in the wild type. More specifically, *hcf152* was affected in the processing of RNAs from the *psbH-petB* part of the *psbB-psbT-psbH-petB-petD* transcription unit (K. Meierhoff and S. Felder, unpublished data). This is illustrated by the RNA gel blots shown in Figure 8. As in *hcf107*, the 1800-nucleotide tricistronic *psbH-petB-petD* transcript, the 1200-nucleotide dicistronic *psbH-petB* transcript, and

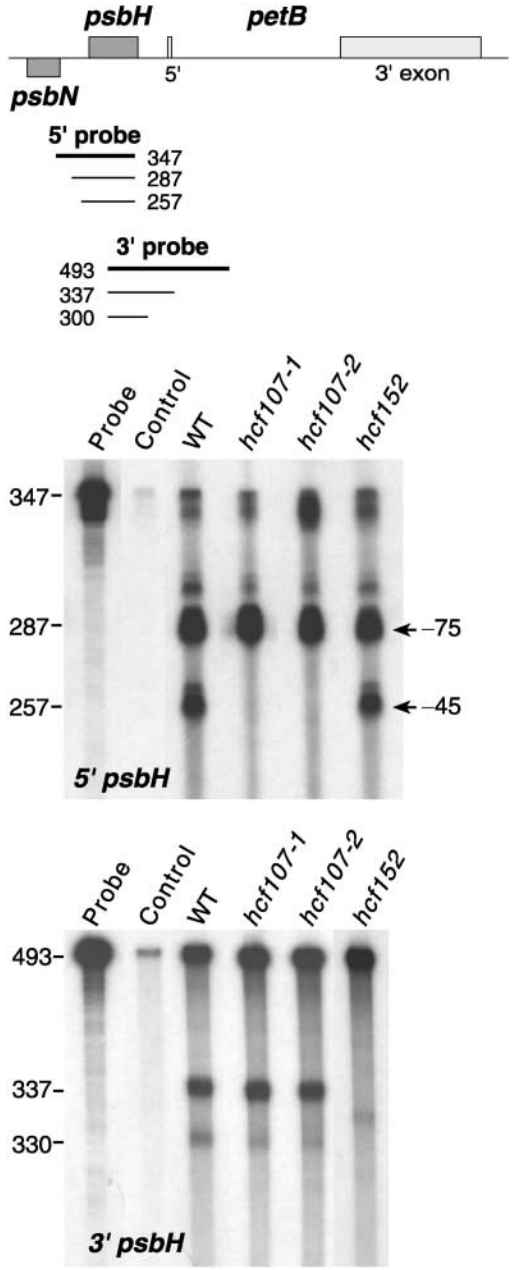

Figure 5. S1 Nuclease Protection Mapping of the 5′ and 3′ Termini of *psbH* Transcripts in Wild-Type, *hcf107-1*, *hcf107-2*, and *hcf152* Mutant Plants.

Total leaf RNA was annealed with probes spanning the 5′ and 3′ ends of *psbH*. The control reaction contained no RNA. Sizes of protected fragments (in bases) were determined by coelectrophoresis with a DNA sequence ladder. WT, wild type.

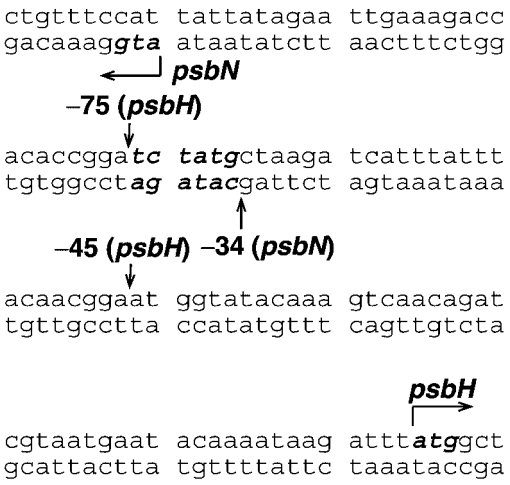

Figure 6. Nucleotide Sequence of the Intergenic Region between *psbN* and *psbH* in Arabidopsis with Mapped RNA 5′ Ends.

Start codons are printed in boldface italic letters. The directions of translation are indicated by horizontal arrows, and the directions of mapped 5′ sites are indicated by vertical arrows.

the 400-nucleotide monocistronic *psbH* RNA were lacking or at least depleted drastically. On the other hand, the 2600-nucleotide *psbH-petB*(I_B)*-petD* RNA and a *psbH-petB*(I_B) RNA of ~1900 nucleotides were present in *hcf152*. Thus, *hcf152* was depleted in some but not all processed *psbH*-containing transcripts with a free *psbH* 5′ end. S1 nuclease protection analysis (Figure 5) revealed that *hcf152* was impaired in 3′ *psbH* processing, whereas the 5′ *psbH* processing was normal and the −45 RNA 5′ end was generated. We conclude from these findings that defective 3′ *psbH* processing does not affect PsbH synthesis provided that *psbH* RNAs with the −45 5′ end are available.

DISCUSSION

The expression of plastid genes depends on the activities of a large number of auxiliary and regulatory factors, the majority of which are encoded by nuclear genes (Barkan and Goldschmidt-Clermont, 2000). The identification of these factors and the elucidation of their function within the network of plastidial gene regulation are a prerequisite if one wishes to understand how plant cells accumulate photosynthetically active chloroplasts. Here we discuss the nuclear mutation *hcf107* of Arabidopsis, which causes a defect in the synthesis and/or turnover of the PSII reaction center proteins CP47 (PsbB) and PsbH. We show that *hcf107* mutants specifically lack processed *psbH*-containing transcripts, whereas they are not affected in the accumulation of processed *psbB* RNAs. Our analysis of this mutation reveals novel information about the mechanisms that control chloroplast translation.

Chlorophyll fluorescence measurements (Meurer et al., 1996b) indicated a functional defect of PSII that was reflected at the level of PSII proteins. The PSII reaction center core proteins CP47, D1, and PsbH were not or were barely detectable in mutant plants. Subunits CP43 and D2 were present but reduced significantly, whereas the accumulation of cytochrome b_{559} was not impaired. Both spectroscopic and immunological data indicated that the other thylakoid membrane protein complexes (i.e., PSI, the cytochrome b_6f complex, and the ATP synthase) were not affected primarily by the *hcf107* mutation.

The lack of intact PSII complexes correlated with a deficiency in the accumulation of radioactively labeled CP47 (PsbB) and PsbH. Because the two proteins were not la-

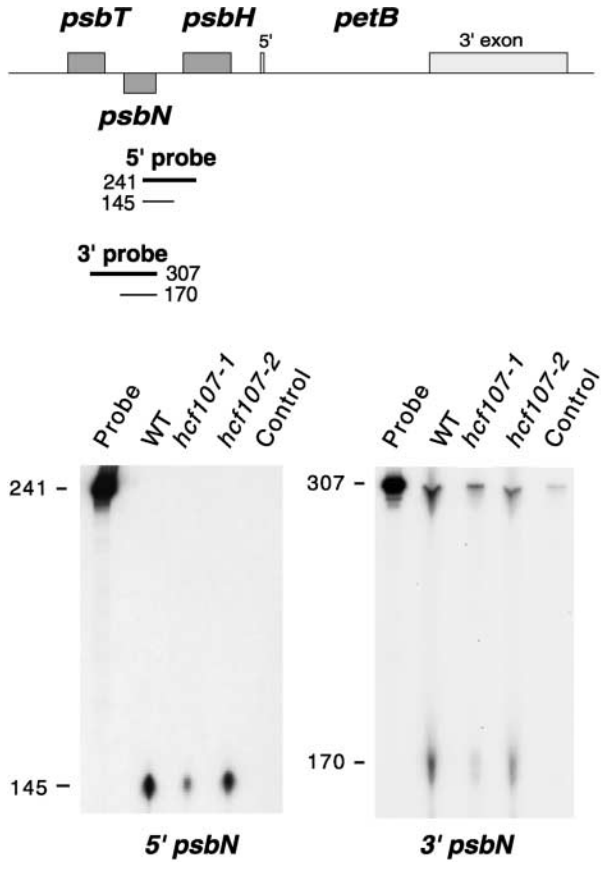

Figure 7. S1 Nuclease Protection Mapping of the 5′ and 3′ Termini of *psbN* Transcripts in Wild-Type, *hcf107-1*, and *hcf107-2* Mutant Plants.

The positions and sizes of the 5′ and 3′ probes (thick lines) and the S1 protected fragments (thin lines) are depicted in the map at the top of the figure. WT, wild type.

beled even when short pulses were applied, we conclude that most likely their synthesis and not their stability is impaired in *hcf107*. CP47 and PsbH are the only detectable plastome-encoded PSII proteins that are not synthesized in *hcf107* chloroplasts, suggesting that this defect is primarily responsible for the accumulation of truncated, nonfunctional PSII reaction centers.

Mutational analyses and/or directed gene inactivation experiments with the cyanobacterium *Synechocystis* PCC 6803, the unicellular green alga *Chlamydomonas reinhardtii*, and the higher plant Arabidopsis support this conclusion. In both the cyanobacterium (Vermaas et al., 1988) and the two photosynthetic eukaryotes (Monod et al., 1992; Meurer et al., 1996a), the presence of CP47 is absolutely required for the assembly and/or stability of PSII reaction center complexes. In contrast, the requirement of PsbH for PSII assembly appears to differ between photosynthetic prokaryotes and eukaryotes. The protein is necessary for the assembly and/or stability of PSII reaction centers in *C. reinhardtii* (Summer et al., 1997; O'Connor et al., 1998), although it is not essential for PSII biogenesis in the cyanobacterium at low light intensities. PsbH, however, is needed for PSII assembly/stability in the cyanobacterium under high light conditions (Mayers et al., 1993). The exact functions of these proteins in the assembly of PSII are not understood. Kinetic analysis of PSII assembly in *hcf107* by pulse labeling and subsequent separation of PSII assembly intermediates by two-dimensional gel electrophoresis revealed that the formation of the first detectable assembly intermediate (i.e., the D1–D2–cytochrome b_{559} complex) was not disturbed (H. Plücken, B. Müller, P. Westhoff, and L. Eichacker, unpublished data). This finding indicates that in Arabidopsis, CP47 and PsbH are required at later stages in the PSII assembly process.

At the RNA level, the deficiency in CP47 and PsbH synthesis was correlated only with defects in the *psbH* part of the *psbB-psbT-psbH-petB-petD* transcript profile but not with defects in the *psbB* segment. Neither the levels nor the sizes of the oligocistronic *psbB*-containing RNAs (i.e., the two dicistronic *psbB-psbT* transcripts and the tricistronic *psbB-psbT-psbH* RNAs) were altered in the *hcf107* mutant background (Figure 4). This finding suggests that the transcriptional initiation in front of *psbB* and the 5′ processing of the transcript produced is normal in *hcf107* mutants. In contrast, all processed *psbH* RNAs with *psbH* as the leading cistron did not accumulate in *hcf107*.

It may be surprising that PsbH synthesis and accumulation were impaired dramatically in *hcf107* although pentacistronic *psbH-psbT-psbH-petB-petD* RNAs and the tricistronic *psbB-psbT-psbH* RNA accumulated. However, these observations are similar to findings in the *crp1* mutant of maize (Barkan et al., 1994; Fisk et al., 1999). *crp1* cannot generate a monocistronic *petD* RNA and is disturbed concomitantly in the biogenesis of the cytochrome b_6f complex. In both mutants, the processing of the polycistronic precursor transcript to a monocistronic RNA appears to be

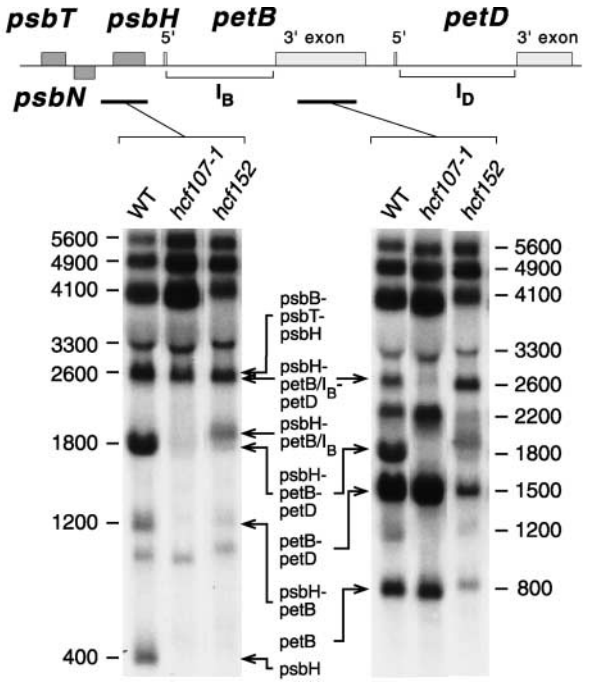

Figure 8. Comparative RNA Gel Blot Hybridization Analysis of the *psbH-petB* Segment of Mutants *hcf152* and *hcf107-1*.

The probes used are indicated in the map at the top of the figure. Experimental conditions were the same as those described in the legend to Figure 4. WT, wild type.

necessary to generate translatable RNAs. However, our comparison of *hcf107* with *hcf152* demonstrated that the translation of *psbH* does not depend on the presence of a monocistronic *psbH* transcript per se. *hcf152*, like *hcf107*, is depleted in the monocistronic *psbH* transcript because the mutant cannot cleave the *psbH-petB* intergenic RNA segment. Nevertheless, *hcf152* contains an active PSII. The fundamental difference is the formation of the *psbH* 5′ leader. Both mutants, like the wild type, accumulate *psbH* RNAs with the putative −75 end, provided that this RNA 5′ end actually exists (see Results for more details). However, only *hcf152* is capable of generating *psbH* RNAs with the −45 5′ end. It follows that translation of *psbH* is possible only when a *psbH* 5′ leader with the −45 site has been formed and that processing of the 3′ trailer is not necessary for translation to occur.

The requirement of the −45 *psbH* leader for translation raises the question of the inhibiting mechanism for the translation of those *psbH* transcripts that do not start at the −45 site. A likely answer to this question emerges when the 5′ upstream sequences of *psbH* are compared for their potential to form secondary structures (Figure 9). The unprocessed *psbH* leader, like the −75 leader, can fold into stable

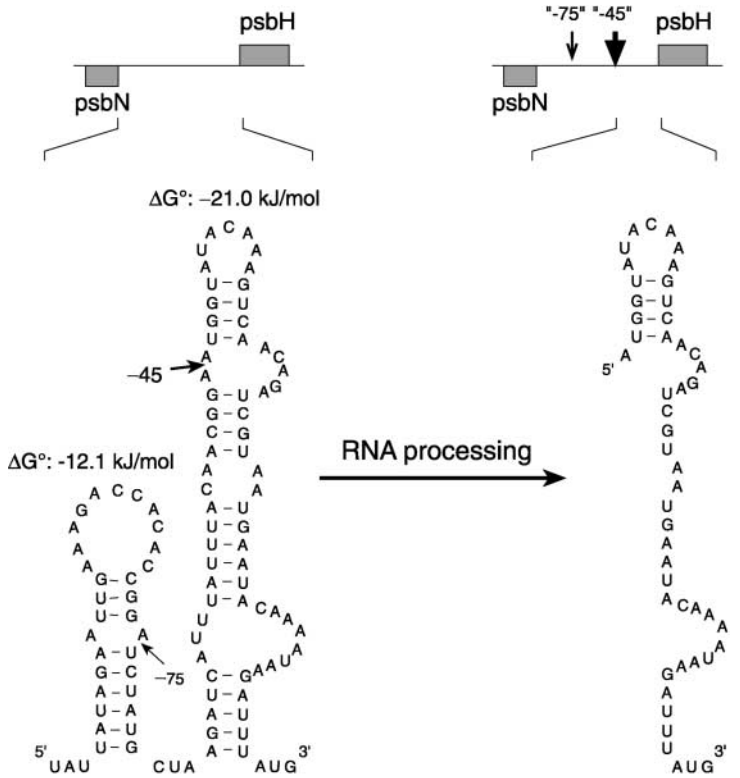

Figure 9. Secondary Structure Model of the Unprocessed and Processed *psbT-psbH* Intergenic RNA.

Structures were determined at 35°C and 1 M NaCl as an extrapolation to cellular conditions. The −45 processing site is labeled by a thick arrow, and the probably artifactual processing site at −75 is indicated by a thin arrow. The start codon of the psbH reading frame is printed in boldface. ΔG°, standard free energy change.

stem and loop structures in which the −75 and −45 processing sites are located in single-stranded bulges. However, no thermodynamically stable secondary structures can be predicted if the *psbH* leader is processed at −45. It is tempting to assume, therefore, that processing at −45 is required to unfold the *psbH* leader, which enables ribosome binding and subsequently the initiation of translation.

Masking of translational initiation sites by intramolecular RNA folding is well documented for prokaryotes (McCarthy and Gualerzi, 1990; Lindahl and Hinnebusch, 1992), and there is increasing evidence that the formation of intramolecular or intermolecular RNA/RNA duplexes functions as a translational controlling mechanism in plastids as well. In barley, methyl jasmonate treatment leads to a decline in in vitro–translatable *rbcL* RNA, although the total levels of *rbcL* transcripts do not change significantly. The decline in translational activity is correlated with a shift in the length of the 5′ leader of *rbcL* from −59 to −94. It has been suggested that intermolecular base pairing of a 35-base motif in the distal part of the 5′ leader with the extreme terminal part of 16S rRNA is the reason why the translation of the −94 *rbcL* transcripts is impaired (Reinbothe et al., 1993). The *crp1* mutant of maize mentioned above may represent one example of translational blockage by intramolecular base pairing. Cleavage of the *petD* message from the polycistronic precursor transcript could release the translation initiation region from inhibitory interactions with upstream RNA sequences (Barkan et al., 1994). Similarly, the intercistronic cleavage of the dicistronic *ndhD-psaC* transcript appears to abolish the intramolecular interaction between an eight-nucleotide sequence in the *psaC* coding region and its complementary eight-nucleotide sequence in the 5′ untranslated leader of *ndhD*. As a consequence, the blockage in *ndhD* translation is removed (Hirose and Sugiura, 1997). In summary, we propose that the *HCF107* gene product is necessary, directly or indirectly, for the unfolding of secondary structures within the *psbH* 5′ leader. This releases the inhibition of *psbH* translation in transcripts with the unprocessed or the −75 *psbH* leader.

The *hcf107* mutants exhibit deficiencies similar to those of the *Mbb1* mutant of *C. reinhardtii*. Like *hcf107*, *Mbb1* does not synthesize CP47—the synthesis of PsbH has not been analyzed yet—and is defective in the assembly of PSII complexes (Monod et al., 1992). *Mbb1* accumulates neither di-

A

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HCF107    MHFFFVPNSSSSSPSPANTSSFSLSFLTPQIPENLCKSPTKIHIGTHGISGQSFLSHPTF
Mbb1      MSLVPFSQLWRGVRTRGPVEQASSSSSSSSSSRRTWYAPARSQTG---VQVAAYEPTAVL
          *                    * *               *     *

HCF107    SSKNTYLYAVVDRSSSGVFSPQKESANGEGEESNTEEGVLVVRRPLLENSDKESSEEEGK
Mbb1      QLAPSAVSRRSTPVRSSIIADLSSSGSGDGE------GE--RGD--ATGSRDEASSAFAG
                       *         *  * **      *            *  * *

HCF107    KYPARIDAGLSNIAKKMPIFEPERSESSSSSSAAAAARAQERPLAVNLDLSLYKAKVLAR
Mbb1      --SSKVLK----INIDLLLWR-----CRTSRIRARQTLDLNE-----------------R
                    *                 *   *                          *

HCF107    NFRYKDAEKILEKCIAYWPEDGRPYVALGKILSKQSKLAEARILYEKGCQSTQGENSYIW
Mbb1      KSLYKAAEDGLRRCLALDPADPRAYVVLGKTLVQQKRYDEARQLYQDGCANTGNVNPYIW
             ** **  *  * *  * * * ** *** *  *    *** **  **  *   * ***

HCF107    QCWAVLENRLGNVRRARELFDAATVADKKHVAAWHGWANLEIKQGNISKARNLLAKGLKF
Mbb1      SAWGWLEARTGNVERARKLYDAAVVVDGTHACAWHKWGMLEKGQGNFTRARDLWMQGIQR
            *  ** * *** *** * *** * *  *  *** *  **  ***   ** *   *

HCF107    CGR-----NEYIYQTLALLEAKAGRYEQARYLFKQATICNS--RSCASWLAWAQLEIQQE
Mbb1      CRRKPQSQNAYLYNALGCMAAQLGRVGEARSWFEEGTRSAEGAASVALWQAWAVLEAKQG
          * *      * * *  *     *  **   **  *   *        * * * *** **  *

HCF107    RYPAARKLFEKAVQASPKNRFAWHVWGVFEAGVGNVERGRKLLKIGHALNPRDPVLLQSL
Mbb1      DPTVVRYLFRKALGANPRSRYVHLAWALWERRQGNPQHCLALLRRGCELNPTDPALYQAW
               * ** **  * *  *     *   *   **      **  *  *** ** * *

HCF107    GLLEYKHSSANLARALLRRASELDPRHQPVWIAWGWMEWKEGNTTTARELYQRALSIDAN
Mbb1      ALVEKQAGRIERARELFEQGLRADPSDLYMWQAYGVMEAEQGNMDRARQLFQEGVWADPR
           * *          ** *        **     * * * **   **   ** * *     *

HCF107    TESASRCLQAWGVLEQRAGNLSAARRLFRSSLNINSQSYVTWMTWAQLEEDQGDTERAEE
Mbb1      SPSTVYVFHAWGALEWQAGNVQTARELFKAAVRVDPKSETTWASWIAMESELGEIERVDE
            *       *** **  ***   ** **          *  **  *   *   *  **  *

HCF107    IRNLYFQQRTEVVDDASWVTGFLDIIDPALDTVKRLLNFGQNNDNNRLTTTLRNMNRTKD
Mbb1      LRIRQAERQWEFVVPAGFTTRPAPGLVDTLARFFSARGFGSDGNGSSSSNGGAGGQQAGS
           *        * *  *   *          *         **

HCF107    SQSNQQPESSAGREDIETGSGFNLDVFLRSKLSLDPLKLDVNLDSK--------------
Mbb1      EAAAGIRAADSVDLTVDGGGQLRFKDVERLVESNDLSALPDFLSSDDDVEASLRPPGAAG
                          *          *   * *   *   * *

HCF107    RLERFTRGR------INGA------------------------
Mbb1      RRQQQPAGSGTGGDNINGSAGYGKLQVPSLVPRPKATPLRSMG
          *      *       ***
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B

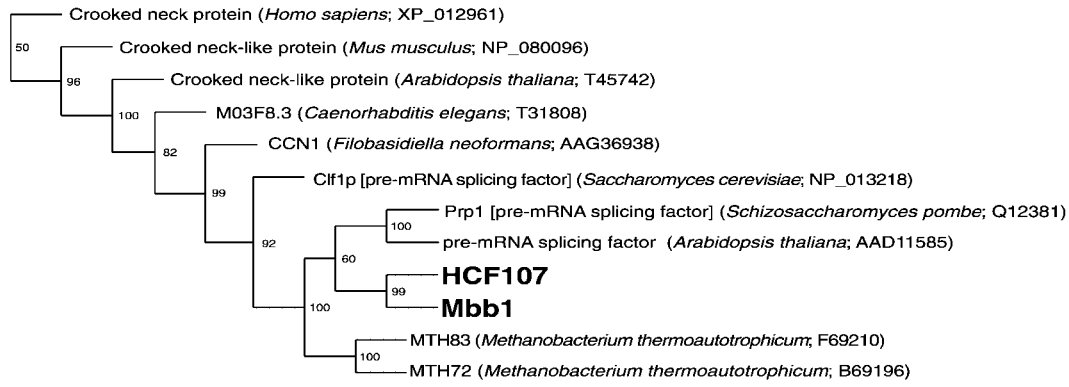

Figure 10. Sequence Comparison and Evolutionary Analysis of HCF107 and Mbb1.

(A) Sequence alignment of the predicted HCF107 and Mbb1 precursor proteins. The putative plastid transit peptides of the two proteins were determined by ChloroP search (http://www.cbs.dtu.dk/services/ChloroP/) and are indicated in boldface. The tetratricopeptide-like repeat of HCF107 is labeled by a thick line above the sequence.

(B) Phylogenetic analysis of HCF107 and Mbb1. HCF107 was used as the query sequence for a BLASTP search (http://www.ncbi.nlm.nih.gov/BLAST/). Protein sequences with alignment scores >50 were subjected to phylogenetic analysis using parsimony (PAUP 4.0b8; Sinauer Associates, Sunderland, MA). Only the relevant part of the tree is shown. Bootstrap values and protein identifiers are indicated.

cistronic *psbB-psbT* transcripts nor monocistronic *psbH* RNAs (Vaistij et al., 2000b). In contrast, the *hcf107* mutation of Arabidopsis affects only the accumulation of *psbH* RNAs with the −45 leader and leaves all other *psbH* RNAs as well as all *psbB* transcripts unaffected. Hence, with respect to their effects on RNA accumulation, the two mutations are clearly different.

What mechanistic functions can be imagined for HCF107 that would explain the effects on both CP47 and PsbH synthesis? HCF107 might act as a site-specific endonuclease that cleaves the *psbH* untranslated region at position −45 or as an accessory component of a nonspecific endonuclease, thereby conferring site specificity to that endonuclease. Alternatively, the *HCF107* gene product may be primarily a stabilizer of *psbH*-containing transcripts once they are processed at −45. However, these suggestions for HCF107 function do not take into account the fact that HCF107 also is required for the synthesis of CP47 without an apparent effect on *psbB* transcripts. The effects of the *hcf107* mutations on both targets can be reconciled more easily if one assumes that HCF107 is an essential part of a protein complex that is multifunctional. This complex would be necessary for the synthesis of CP47 and perhaps also PsbH by serving as a translational activator. The HCF107 complex also provides RNA processing and/or stabilization functions that are needed for *psbH*, but not for *psbB*, transcripts.

The recent cloning of the *Mbb1* gene of Chlamydomonas (Vaistij et al., 2000a) and of *HCF107* from Arabidopsis (Figure 10A; A. Sane, B. Stein, and P. Westhoff, unpublished data) is in line with this suggestion. HCF107 is identical to the tetratricopeptide repeat protein BAA9482 of Arabidopsis that Vaistij et al. (2000a) have identified as a homolog of Mbb1. BLAST searches followed by a phylogenetic analysis, moreover, indicate that the two proteins are true evolutionary orthologs (Figure 10B). The tetratricopeptide repeat motif is well known for mediating protein–protein interactions (Blatch and Lässle, 1999), and for Mbb1 it has already been shown that this protein is part of a large complex (Vaistij et al., 2000a). Future experiments will need to elucidate the composition of these complexes to explain the mechanisms of function of Mbb1/HCF107.

METHODS

Growth Conditions

Seed of *Arabidopsis thaliana* were surface-sterilized and sown on sucrose-supplemented medium containing 0.3% (w/v) Gelrite (Carl Roth GmbH, Karlsruhe, Germany). Seedlings were grown with a 16-hr photoperiod at a photon flux density of 20 to 50 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ and a temperature of 23°C.

The mutant *hcf107-1* was selected from a collection of mutants induced by ethyl methanesulfonate (Meurer et al., 1996b), whereas *hcf107-2* (originally named DEI117) was selected from a collection of T-DNA lines (Bechtold et al., 1993; Bouchez et al., 1993). Mutant plants that exhibited the phenotype with high-chlorophyll fluorescence were selected in the dark under UV light as described (Meurer et al., 1996b).

Antiserum Production and Immunoblot Analyses

The nucleotide sequence encoding the N-terminal 46 amino acids of the PsbH protein of Arabidopsis was amplified by polymerase chain reaction (PCR). The 5′ and 3′ primers contained a BamHI or a XhoI site, respectively, for in-frame fusion with the glutathione *S*-transferase (GST) sequence of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden). The construction of the recombinant plasmid and the expression in *Escherichia coli* were as described (Meurer et al., 1998). The GST-PsbH fusion protein produced was found to be soluble and was purified by affinity chromatography on glutathione–Sepharose 4B (Amersham Pharmacia Biotech) as recommended by the manufacturer. A polyclonal antiserum was raised in rabbits by BioGenes (Berlin, Germany). The specificity of the antiserum was tested by immunoblotting (Meurer et al., 1996b).

In Vivo Labeling of Proteins

Primary leaves from five to six 12-day-old mutant or wild-type seedlings were incubated for 15 min in 50 μL of double distilled water containing 40 μg/μL cycloheximide. The preincubation medium was then replaced by 50 μL of water containing 250 μCi of ^{35}S-methionine (specific activity >1000 Ci/mmol; Amersham Buchler, Braunschweig, Germany) and 20 μg/μL cycloheximide. Radioactive methionine was allowed to incorporate for varying periods at room temperature in ambient light. After labeling, leaves were frozen immediately at −70°C to block further incorporation. The frozen leaves were processed as described (Meurer et al., 1996a).

In Vitro Phosphorylation of Thylakoid Proteins

In vitro phosphorylation of thylakoid membrane proteins was performed essentially as described (Koivuniemi et al., 1995). Chloroplasts isolated by differential centrifugation (Meurer et al., 1996a) were resuspended in 50 mM Hepes-KOH, pH 7.6, 100 mM sorbitol, 5 mM $MgCl_2$, and 5 mM NaCl at 0.4 mg chlorophyll/mL. Ten microcuries of α-^{32}P-ATP (Amersham-Pharmacia) and NaF (10 mM final concentration) were added to 100 μL of thylakoid membrane suspension, and the assay was incubated for 30 min at 23°C at a photon flux density of 50 $\mu mol\ photons \cdot m^{-2} \cdot sec^{-1}$. The reaction was terminated by centrifugation, and the membranes were washed twice in incubation medium (without NaF). The pelleted membranes were resuspended in SDS sample buffer, electrophoresed on 12.5% polyacrylamide-SDS/urea gels (Schägger and von Jagow, 1987), and finally analyzed by phosphorimaging.

RNA Gel Blot Hybridization Analysis

RNA gel blot analysis of total leaf RNA was performed as described (Meurer et al., 1996a). Hybridization probes were made by cloning ei-

ther PCR fragments or restriction fragments as described by Felder (1999). The probes were labeled by random priming.

S1 Nuclease Protection Mapping

DNA templates for the synthesis of single-stranded DNA probes for S1 mapping of *psbH* and *psbN* RNA 5′ and 3′ ends were prepared by conventional PCR (Dieffenbach and Dveksler, 1995) using the following pairs of primers (5′/3′): psbH 5′, GCGGATCCAAGATGGCGACTAGGGAC/TCTAGACAACAGTTTGTGTAGC; psbH 3′, GGAAGGCCTGTTCTAGATCTGGTC/GGCGGATCCTAAGTATGAATCATAA; psbN 5′, GCGGATCCGGATCTCTTAGTTGTTG/GCTCTAGAACTATCTTCAACAGT; psbN 3′, GCGGATCCACGATCAAATTTATGGA/ACTCTAGAACAGCAACCCTAGTC. The DNA fragments were purified by agarose gel electrophoresis and extracted from the gel using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) according to the suggestions of the manufacturer. Uniformly radioactively labeled probes were then prepared by linear PCR using only the 3′ primer. The 20-μL reactions contained 20 to 50 ng of double-stranded DNA fragment, 20 ng of 3′ primer, 200 μM deoxynucleotide triphosphates, 15 to 20 μCi of α-^{32}P-dATP, and 1.5 units of Taq polymerase (Appligene-Oncor, Heidelberg, Germany) in the appropriate buffer. Standard cycling conditions (35 times) were 94°C for 5 min, 50°C for 1 min, 72°C for 1.50 min, and 72°C for 7 min. The labeled probes were then mixed with loading buffer (0.3% bromphenol blue, 0.3% xylene cyanole FF, 10 mM EDTA, pH 7.5, and 97.5% deionized formamide) and electrophoresed on a 4% polyacrylamide/urea sequencing gel for 1.5 hr. After the gel was exposed to an x-ray film for 5 min, the labeled fragments were excised, crushed, and incubated in 300 μL of elution buffer (0.3 M sodium acetate, pH 6.0, 10 mM EDTA, and 0.2% SDS) at 37°C for 2 hr. The gel slurry was filtered through cotton wool, and the eluted DNA was precipitated with 20 μg of tRNA and 700 μL of ethanol at −20°C for 2 hr. The dried pellet was dissolved in 10 to 20 μL of deionized formamide at −20°C overnight. Twenty micrograms of total leaf RNA was precipitated with sodium acetate and ethanol and dissolved in 14 μL of formamide.

The hybridization reaction was set up by mixing 14 μL of RNA, labeled probe equivalent to 100,000 cpm, and deionized formamide to a final volume of 24 μL. Nucleic acids were denatured at 90°C for 90 sec and then transferred immediately to 48°C. Six microliters of hybridization buffer (2 M NaCl, 5 mM EDTA, and 0.2 M Pipes/NaOH, pH 6.4) was added, and hybridization was allowed to proceed for 7 hr, with the temperature decreased linearly from 48 to 40°C. After hybridization, 300 μL of ice-cold buffer (0.28 M NaCl, 4.5 mM $ZnSO_4$, and 50 mM sodium acetate, pH 4.4) with 150 units of S1 nuclease (Roche Diagnostics, Mannheim, Germany) was added and incubated at 20°C for 1 hr. After phenol/chloroform extraction, nucleic acids were precipitated with sodium acetate/ethanol and dissolved in 3.5 μL of loading buffer. S1-protected fragments were analyzed on a 4% polyacrylamide gel containing 7 M urea. A sequencing reaction was used to determine fragment sizes.

RNA Structure Calculations

Predictions of thermodynamically optimal secondary structures of RNA were made with the software package LinAll (Schmitz and Steger, 1992). Calculations were performed for a temperature of 35°C to extrapolate the ionic strength of the calculations (1 M NaCl) to cellular conditions. This difference is based on systematic studies of the denaturation of potato spindle tuber viroid under different conditions (Riesner and Steger, 1990).

Accession Numbers

The EMBL accession numbers for the proteins described in this article are BAA94982 (HCF107) and CAC19558 (Mbb1).

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