The Function of RH22, a DEAD RNA Helicase, in the Biogenesis of the 50S Ribosomal Subunits of Arabidopsis Chloroplasts

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The chloroplast ribosome is a large and dynamic ribonucleoprotein machine that is composed of the 30S and 50S subunits. Although the components of the chloroplast ribosome have been identified in the last decade, the molecular mechanisms driving chloroplast ribosome biogenesis remain largely elusive. Here, we show that RNA helicase 22 (RH22), a putative DEAD RNA helicase, is involved in chloroplast ribosome assembly in Arabidopsis (Arabidopsis thaliana). A loss of RH22 was lethal, whereas a knockdown of RH22 expression resulted in virecent seedlings with clear defects in chloroplast ribosomal RNA (rRNA) accumulation. The precursors of 23S and 4.5S, but not 16S, rRNA accumulated in rh22 mutants. Further analysis showed that RH22 was associated with the precursors of 50S ribosomal subunits. These results suggest that RH22 may function in the assembly of 50S ribosomal subunits in chloroplasts. In addition, RH22 interacted with the 50S ribosomal protein RPL24 through yeast two-hybrid and pull-down assays, and it was also bound to a small 23S rRNA fragment encompassing RPL24-binding sites. This action of RH22 may be similar to, but distinct from, that of SmB, a DEAD RNA helicase that is involved in the ribosomal assembly in Escherichia coli, which suggests that DEAD RNA helicases and rRNA structures may have coevolved with respect to ribosomal assembly and function.

Given the prokaryotic origin of chloroplasts, the protein-synthesizing systems of chloroplasts are often considered to be very similar to those of bacteria. Chloroplasts contain 70S ribosomes, which are similar to prokaryotic ribosomes and distinct from their cytosolic counterparts, 80S ribosomes. The ribosomal RNAs (rRNAs) of higher plant chloroplasts are highly conserved and are most closely related to bacterial sequences (Branlant et al., 1981; Harris et al., 1994). The 30S subunit comprises 16S rRNA in chloroplasts and Escherichia coli. However, the 50S subunit of chloroplast ribosomes is composed of three rRNAs (23S, 4.5S, and 5S rRNAs), rather than the 23S and 5S rRNAs of E. coli (Harris et al., 1994). In chloroplasts, the 16S, 23S, 4.5S, and 5S rRNAs are encoded in an operon and are synthesized as a single large precursor (Edwards and Kössel, 1981). Maturation of the primary transcript occurs via multiple steps, including nucleotide modifications as well as endonucleolytic and exonucleolytic cleavages that sequentially remove the precursor sequences (Strittmatter and Kössel, 1984; Walter et al., 2002; Bollenbach et al., 2005).

Although the overall structural organization of the ribosome has been described in great detail (Ban et al., 2000; Schluenzen et al., 2000; Yusupov et al., 2001), the molecular mechanisms involved in ribosomal biogenesis have not yet been resolved. In E. coli, the biogenesis of ribosomal subunits is a stepwise process that comprises the processing and folding of the pre-rRNA and its concomitant assembly with the ribosomal proteins (Gourse et al., 1996; Cheng and Deutscher, 2003; Holmes and Culver, 2005). Precursor particles that consist of a subset of ribosomal proteins, as well as rRNA precursors, are formed during this process (Lindahl, 1975). The isolation and characterization of these precursor particles enabled mapping of the basic series of events of ribosome assembly in E. coli (Nierhaus, 1991; Williamson, 2003). In chloroplasts, however, the existence of such precursor particles has not been fully proven (Dorne et al., 1984). Thus, our knowledge of many important aspects of chloroplast ribosome biogenesis is still limited, especially regarding the coordination of the processing and modification of the rRNA to ensure its correct structural assembly with ribosomal proteins.

Ribosomal biogenesis depends on a large number of nonribosomal factors that confer directionality and accuracy in this process (Fromont-Racine et al., 2003; Henras, et al., 2008; Shajani et al., 2011). Genetic approaches have been used to identify mutants that...
are defective in ribosome biogenesis in E. coli, and these studies have uncovered a considerable number of nonribosomal factors that are involved in the process, including AAA-ATPases, GTPases, kinases, chaperone proteins, and RNA helicases (Kaczanowska and Rydén-Aulin, 2007; Shahani et al., 2011). These factors play important roles in the transcription and processing of preribosomal RNAs as well as in their proper folding and assembly with ribosomal proteins (Kaczanowska and Rydén-Aulin, 2007). In higher plants, several mutants that are defective in chloroplast ribosome biogenesis have been isolated, and most of these are defective in rRNA processing (Barkan, 1993; Walter et al., 2002; Bellaoui et al., 2003; Bisanz et al., 2003; Kishine et al., 2004; Bollenbach et al., 2005; Schmitz-Linneweber et al., 2006; Koussevitzky et al., 2007; Watkins et al., 2007; Beick et al., 2008; Beligni and Mayfield, 2008; Yu et al., 2008; Zybailov et al., 2009; Nishimura et al., 2010). However, the molecular mechanisms responsible for regulating rRNA processing and ribosomal biogenesis in most of the identified mutants are not fully understood.

DEAD-box family RNA helicases are characterized by the presence of at least nine conserved motifs and are named for the highly conserved Asp-Glu-Ala-Asp residues in motif II (Caruthers and McKay, 2002; Cordin et al., 2006). These proteins are generally believed to unwind or rearrange local RNA secondary structures using energy released from ATP hydrolysis (Caruthers and McKay, 2002; Cordin et al., 2006). However, it was recently shown that in several cases, these enzymes may assist in the correct folding of RNA by acting as RNA “chaperones” or promoting the dissociation of RNA and protein molecules (Jankowsky and Fairman, 2007). The DEAD RNA helicases participate in nearly every process that involves RNA synthesis, RNA modification, RNA cleavage, RNA degradation, ribosome biogenesis, and translation initiation (Silverman et al., 2007; Watkins et al., 2007; Beick et al., 2008; Beligni and Mayfield, 2008; Yu et al., 2008; Zybailov et al., 2009; Nishimura et al., 2010). Herein, we demonstrate that one of these chloroplast DEAD RNA helicases, RH22, plays an important role in the biogenesis of the chloroplast ribosome.

RESULTS

RH22 Is a Chloroplast DEAD Box Protein

RH22 is a putative DEAD-box RNA helicase with a 58-amino acid transit peptide that is predicted to be targeted to the chloroplast, according to the TargetP program. To confirm the chloroplast localization of RH22, a chimeric construct consisting of the coding region of RH22 fused to GFP and expressed under the control of the 35S cauliflower mosaic virus promoter was generated and transiently expressed in the protoplasts of Arabidopsis mesophyll cells. We observed GFP signals by confocal laser-scanning microscopy and found that the fusion protein colocalized with chloroplastic chlorophyll in the mesophyll cells, consistent with the results obtained for GFP fused to the transit peptide of the small subunit of Arabidopsis ribulose bisphosphate carboxylase (Fig. 1A). The fluorescence signals were dispersed in the cytosol and the nucleus when protoplasts were transformed with a control vector without a specific targeting sequence (Fig. 1A). Thus, these findings indicate that RH22 localizes to the chloroplast.

RH22 is widely expressed in most Arabidopsis tissues, including leaves, stems, roots, siliques, and flowers, as revealed by RNA-blot analysis (Fig. 1B). However, RH22 transcript accumulation was relatively higher in leaves than in other tissues. The expression of RH22 appeared to be closely associated with the age and developmental stages of the Arabidopsis leaves, occurring primarily during early stages of development. The RH22 transcripts accumulated at the highest levels in 4-d-old seedlings and were significantly reduced in 8-d-old seedlings (to approximately 20% of the level detected in 4-d-old seedlings; Fig. 1C). Nevertheless, the levels of RH22 transcript in 12-d-old seedlings were only slightly reduced compared with those in 8-d-old seedlings (Fig. 1C). To examine the effects of light on the expression of RH22 in Arabidopsis, the levels of RH22 transcripts in the leaves of dark-adapted plants were compared with those in plants illuminated with white light after dark adaptation. As shown in Figure 1D, RH22 transcripts accumulated at very low levels in dark-adapted plants and increased upon illumination, similar to the pattern in plants under continuous light.

Knockdown of RH22 Leads to a Delayed-Greening Phenotype in Arabidopsis

We obtained three T-DNA insertion alleles of RH22 from the Arabidopsis Biological Resource Center (ABRC) and named them rh22-1 (Salk_032399), rh22-2 (Salk_065388C), and rh22-3 (CS856759). The T-DNA
insertions in rh22-1 and rh22-2 were in introns 6 and 7, respectively, and rh22-3 was in exon 1 (Fig. 2A). The homozygous rh22-1 and rh22-2 mutant plants exhibited a high-chlorophyll-fluorescence phenotype in a developmentally regulated manner, similar to that of dg1 (Chi et al., 2008). As shown in Figure 2B, the growth of rh22-1 was retarded, and 10-d-old cotyledons displayed an obvious pale-green phenotype under normal growth conditions. The analysis of the chlorophyll fluorescence kinetics showed that the ratio of the variable fluorescence to the maximum fluorescence ($F_v/F_m$), which reflects the maximum potential of PSII photochemical reactions, was significantly lower in the rh22-1 cotyledons than in wild-type plants (Fig. 2B). After growth for 20 d, the cotyledons of rh22-1 gradually turned green, and the $F_v/F_m$ ratios gradually increased and approached the wild-type levels (0.8), but the newly emerging true leaves showed the pale-green phenotype (Fig. 2B). The rh22-2 mutant had a phenotype similar to that of rh22-1, but its chlorosis and growth retardation were less severe. After growth for 10 d, only the newly emerging true leaves following the cotyledons of rh22-2 were pale green; the cotyledons of rh22-2 were restored to wild-type levels (Fig. 2B). Electron microscopic analysis of chloroplasts from wild-type and mutant plants of 10-d-old seedlings showed that the chloroplasts of wild-type cotyledons had well-structured thylakoid membranes that were composed of grana connected by stroma lamellae, whereas those of rh22-1 cotyledons had fewer stacked thylakoids and exhibited a disturbed thylakoid membrane organization (Supplemental Fig. S1). These data suggest that the rh22 mutation affects chloroplast development.

The T-DNA insertions did not completely suppress RH22 gene expression in the rh22-1 and rh22-2 mutants, as reverse transcription (RT)-PCR analysis showed that At1g59990 (RH22) remained expressed at low levels in both of them (Fig. 2C). Using quantitative (q)RT-PCR analysis, we found that RH22 RNA was reduced to approximately 16% and 45% of the wild-type levels in rh22-1 and rh22-2, respectively (Fig. 2D). Immunoblot analysis using a specific antibody raised against RH22 also showed that the mutants had reduced levels of RH22 protein (Fig. 2E).

To obtain potential null alleles of RH22, we analyzed the rh22-3 mutant line, in which the T-DNA was inserted into the first exon of the At1g59990 gene. However, no homozygous rh22-3 plants were identified among the progeny in the crossing of heterozygous rh22-3/RH22 plants, implying that a complete disruption of the RH22 gene is lethal. The siliques of the rh22-3/RH22 plant contained approximately 25% aborted seeds (Supplemental Fig. S2), indicating that rh22-3 was a single-gene mutant. Thus, the complete absence of RH22 may result in the embryonic lethality of abnormal seeds. Consistent with this analysis, the RH22 locus was recently designated EMBRYO DEFECTIVE3108 (EMB3108) in the database of an Arabidopsis knockout collection that includes lethal alleles (http://www.seedgenes.org/SeedGeneProfile).

Chloroplast Proteins Are Reduced, but Their mRNA Transcripts Accumulate Normally, in rh22 Mutants

To investigate whether the accumulation of chloroplast proteins was also affected in the rh22 mutants, the
levels of representative chloroplast-encoded protein components of each of the thylakoid protein complexes in the pale-green leaves were analyzed by immunoblotting (Fig. 3). In the rh22-1 mutant, the levels of the photosynthetic D1 and D2 reaction center proteins of PSII, the β-subunit of ATP synthase, and the cytochrome f protein of the cytochrome b, f complex were reduced to approximately 25% of the wild-type levels (Fig. 3). The levels of PsA/B, the reaction center proteins of PSI, were decreased to approximately 50% of wild-type levels (Fig. 3). The levels of these chloroplast proteins were also reduced in plants with the weaker allele, rh22-2, but to a lesser extent than in rh22-1.

The deficiencies in the levels of chloroplast-encoded proteins may be the result of defects in chloroplast transcript accumulation. To test this possibility, RNA-blot analysis of the wild-type and mutant plants was performed. No obvious differences were observed in the levels of psbA (encoding D1), psbB (encoding CP47), psbD (encoding D2), atpB (encoding CF1β), psaA (encoding PsaA), or petA (encoding cytochrome f) transcripts between rh22-1 and wild-type plants (Supplemental Fig. S3).

Chloroplast rRNA Processing Is Affected in rh22

Although the chloroplast mRNAs examined above accumulated normally in rh22-1, we could not exclude the possibility that the accumulation of other chloroplast gene transcripts was affected, because these DEAD proteins are involved in RNA metabolism. To address this possibility, the abundance of global chloroplast transcripts in the rh22-1 mutant and wild-type plants was analyzed using qRT-PCR (Supplemental...
expression of (Supplemental Fig. S4), which indicates that the ex-
approximately 9-fold compared with the wild type

As described above, the 23S-4.5S precursor rRNA undergoes further processing to generate mature forms of 23S rRNA and 4.5S rRNA, and this processing is believed to occur on the ribosome (Keus et al., 1984; Strittmatter and Kössel, 1984; Leal-Klevezas et al., 2000). Because RH22 appears to be involved in the rRNA maturation process, we next tested whether RH22 was associated with chloroplast ribosomes. First, we performed an immunoblot analysis to examine whether RH22 cosedimented with chloroplast ribosomes. The primary RNA transcripts in Arabidopsis, the 23S-4.5S bicistronic RNA (3.2 kb) undergoes endonucleolytic cleavage to produce a mature 4.5S rRNA and a 23S precursor (2.9 kb). This 23S precursor undergoes fur-

RH22 Associates with Precursors of the 50S Ribosomal Subunit

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Figure 3. The accumulation of chloroplast proteins in rh22 and wild-type (WT) plants. Immunoblot analysis of chloroplast-encoded proteins in wild-type and rh22 mutant plants is shown. Leaf extracts were separated by SDS-PAGE and immunodetected with specific antibodies directed against D1, CP47, D2, CF1β, PsaAB, and cytochrome f. A replicate gel stained with Coomassie blue is shown below to provide an estimate of gel loading.

Fig. S4). We observed no obvious differences in the levels of most of the chloroplast gene transcripts between rh22-1 and the wild-type plants. However, the rrn23 (23S rRNA) transcript level was increased by approximately 9-fold compared with the wild type (Supplemental Fig. S4), which indicates that the expression of rrn23 is affected in the rh22 mutant.

The chloroplast rRNAs are transcribed as polycistronic transcripts that undergo extensive splicing and processing to produce the mature forms. The accumulation and processing of chloroplast rRNAs was further examined by RNA gel blotting using probes specific for individual mature rRNAs (Fig. 4). When the blots were probed with the 2.9-kb 23S rRNA sequence (probe b) showed that, under these conditions, the levels of the 3.2-, 2.9-, and 2.4-kb precursors were increased, but the levels of the 1.7-, 1.2-, 1.0-, and 0.5-kb bands were greatly reduced in the rh22 mutant compared with the wild type (Fig. 4B). To confirm the altered patterns of 23S rRNA, RNA gel blots with a 2.0-kb probe targeting the 5’ end (probe c) and a 0.9-kb probe targeting the 3’ end (probe d) of the fragment were also analyzed. The mutants showed drastically increased levels of 3.2-, 2.9-, and 2.4-kb precursors. The detected PCR product spanned the hidden break at the 3’ end of the 23S rRNA; thus, this result may explain the increase in the rrn23 transcript of the rh22-1 mutant shown in the qRT-PCR data. Consistent with the blots utilizing the b, c, and d probes, analysis of the rh22 mutants with a 4.5S-specific probe (probe e) showed that the levels of the 3.2-kb precursor were increased and those of mature 4.5S rRNA were reduced (Fig. 4B). The amounts of mature 5S rRNA were also reduced in rh22, but accumulation of the 5S RNA precursor was not detected (probe e). The precursor forms of the 23S and 4.5S rRNAs increased, whereas their mature forms decreased, indicating that processing of the 23-4.5S bicistronic RNA and its further maturation were affected in rh22 mutants.

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peak did not correspond to the mature 50S subunit (Fig. 5B), showing that RH22 sediments more slowly than the mature 50S subunit.

The sedimentation behavior of RH22 in the Suc gradient raised the possibility that RH22 may be associated with 50S ribosomal subunit precursors. To test this possibility, RNAs that coimmunoprecipitated with the anti-RH22 antibody were analyzed by slot-blot hybridization using probes corresponding to the different regions of the rrn operon in chloroplasts (Fig. 5C). The rrn23, rrn4.5, and rrn23-rrn4.5 intergenic spaces were also detected in substantial amounts in the precipitate, whereas rrn16 and rrn5 were not detected (Fig. 5C). This analysis suggests that RH22 may be bound to the 23S-4.5S precursors. Subsequently, an RNase-protection assay was used to map the 3' end of the 23S rRNA that coimmunoprecipitated with RH22. Our results showed that the 23S-4.5S rRNA was enriched with respect to mature 23S rRNA in the RH22 immunoprecipitation pellets (Fig. 5D). The sedimentation behavior of RH22 and the coimmunoprecipitation of RH22 with the pre-23S rRNA indicate that RH22 is associated with an assembly intermediate of the 50S ribosomal subunit.

**RH22 Interacts with the 50S Ribosomal Protein RPL24**

The association of RH22 with the 50S ribosomal subunit may be mediated by the interaction of RH22 with the ribosomal protein. To address this possibility, we cloned RH22 lacking a signal peptide into the bait vector and then tested for its interaction with 59 distinct chloroplast ribosomal proteins that were fused to the GAL4-DNA-active domain in the prey vector. As shown in Figure 6A, RH22 specifically interacted with RPL24, one of the chloroplast 50S ribosome proteins. Coimmunoprecipitation analysis also showed that RPL24 was precipitated by an anti-RH22 antibody but not by preserum. As a control, we tested one of the 30S ribosome proteins, RPS1, and showed that it was not precipitated by the anti-RH22 antibody (Fig. 6B).

In *E. coli*, L24 and L4 are essential to generate the first intermediate during 50S assembly in vitro.
RH22 Can Bind to a 5′ Fragment of 23S rRNA

L24 is thought to be the first protein to bind 23S rRNA, thereby initiating the cooperative assembly process of the larger ribosome in *E. coli* (Egebjerg et al., 1987; Nierhaus, 1991; Tumminia et al., 1994). RH22 interacted with PRL24 in chloroplast ribosomes, raising the question of whether RH22 can bind to 23S rRNA. To answer this question, RH22 was generated from the recombinant protein by protease cleavage of the MBP moiety (Supplemental Fig. S5) and used for electrophoretic mobility shift assays (EMSAs) to detect its binding to RNA molecules. Two RNA oligonucleotides were used for the binding assay. One fragment was from the 5′ end of the 23S RNA (Oligo5), which encompassed the potential PRL24-binding site, as revealed by comparison with *E. coli* 23S rRNA; the other fragment was from the 3′ end of the 23S rRNA (Oligo3; Fig. 7A). EMSA revealed the formation of an RNA/protein complex when RH22 was incubated with Oligo5 but not with Oligo3 (Fig. 7B) under the conditions tested. The formation of the RNA/protein complex was increased with increasing protein concentrations (Fig. 7B). Complex formation was reduced when a 20- or 200-fold excess of unlabeled Oligo5 was added (Fig. 7C), whereas there was no change when unlabeled Oligo3 was added (Fig. 7D). This competition assay demonstrates that RH22 can specifically bind to Oligo5 RNA.

Polysomal Loading of mRNA in *rh22*

Abnormal ribosome assembly or rRNA processing may affect the polysomal loading of chloroplast mRNA (Barkan, 1993; Beligni and Mayfield, 2008), and defects in polysomal loading are usually observed in mutants with impaired chloroplast ribosome function (Fleischmann et al., 2011). The defects in rRNA accumulation observed in the *rh22* mutants prompted us to examine whether this characteristic affected the polysomal loading of chloroplast mRNAs. Extracts from wild-type or *rh22-1* seedlings were fractionated on 15% to 55% Suc density gradients and analyzed by RNA gel blot using probes targeting *rrn23*, *rrn16*, or the transcripts of several other chloroplast-encoded proteins (Fig. 8). In the wild-type plant, the 1.7-, 1.2-, 1.0-, and 0.5-kb components of the 23S rRNA were the major entities in the polysome fractions, but considerable amounts of 3.2-, 2.9-, and 2.4-kb precursors were also detected. In the *rh22-1* mutant, the polysome fractions contained all of these components, but the 3.2-, 2.9-, and 2.4-kb precursors were significantly enriched concomitant with the reduction of mature transcripts. The overall 16S rRNA polysome patterns of wild-type and *rh22-1* mutant plants were similar, but the levels of the 16S mature transcript were reduced in the *rh22-1* mutant. The lower levels of mature 23S and 16S rRNA components in the polysomal fractions indicated that the *rh22* chloroplasts contained fewer polysomes than the chloroplasts of wild-type

Figure 5. The association of RH22 with the precursor of the 50S ribosomal subunit. A, *A*$_{260}$ profiles of fractions 35 to 52 from the Suc gradient sedimentation of stromal extracts. B, Immunoblot analyses of each fraction probed with antibodies against RPS1, RPL4, RPL32, and RH22. C, Coimmunoprecipitation of RH22 and rRNAs. The RNA from each immunoprecipitation (P) and supernatant (S) was applied to a nylon membrane with a slot-blot manifold and hybridized with probes as indicated. Immunoprecipitation with preimmune serum (PRE) was conducted as a negative control. D, RNase protection analysis of the 3′ end of the 23S rRNA in the immunoprecipitation pellet (Pel) and supernatant (Sup) samples. The probe is indicated below the diagram of the 23S-4.5S region of the *rrn* operon. The positions of the protected bands, corresponding to the mature rRNA and the 23S-4.5S precursor, are indicated.

(Trubetskoy et al., 2009). Because the chloroplast ribosome is similar to that of *E. coli*, we hypothesized that RH22 might interact with RPL4 as well. However, this interaction was not detected in our yeast two-hybrid assay (Fig. 6A). To further address this issue, we also performed a pull-down assay to investigate the interaction of RH22 with ribosomal proteins in vitro (Fig. 6C). Consistent with the yeast two-hybrid results, maltose-binding protein (MBP)-RH22 was able to pull down RPL24 but not RPL4. Based on these analyses, it is possible that RH22 interacts with RPL24, although an interaction between RH22 and RPL4 in vivo could not be excluded.
plants. To determine whether RH22 is associated with polysomal fractions, proteins from each of the gradient fractions were analyzed by immunoblotting with anti-RH22 antibodies. RH22 was absent from the polysomal fractions but was easily detected in nonpolysomal regions of the gradient (Fig. 8).

The rh22-1 seedlings exhibited reduced levels of chloroplast-encoded thylakoid proteins (Fig. 3); however, the polysomal loading of the corresponding transcripts (psbA, psbB, psbD, psaA, petB, and atpB) was not affected (Fig. 8). Because the polysomal loading of rbcL, which encodes the large subunit of Rubisco, has been shown to be more sensitive to mild defects in chloroplast translation than psbA (Rogalski et al., 2008), its polysomal loading was also investigated (Fig. 8). No substantial change in polysomal loading of rbcL was detected in rh22-1, and similar results were observed in rnr1 and rh39, two other ribosomal biogenesis mutants (Bollenbach et al., 2005; Nishimura et al., 2010). Defects in the initiation step of chloroplast translation usually lead to the alteration of ribosomes associated with mRNAs (Barkan, 1993). There was no significant difference in the polysomal loading of these transcripts between the wild type and rh22-1, suggesting that the initiation step of the chloroplast translation is not affected. Instead, other translation steps might be impaired in this mutant.

**DISCUSSION**

The components of chloroplast ribosomes have been determined in spinach (Spinacia oleracea; Yamaguchi and Subramanian, 2000, 2003; Yamaguchi et al., 2000), barley (Hordeum vulgare; Maki et al., 2000), and Chlamydomonas reinhardtii (Yamaguchi et al., 2003). However, the mechanism by which this discrete set of components assembles into this large, functional complex and coordinates with rRNA molecules still remains largely unknown. In this study, we reported the functional characterization of the chloroplast DEAD RNA helicase RH22 from Arabidopsis and investigated its roles in ribosomal biogenesis.

**RH22 Is Involved in the Biogenesis of the 50S Ribosome**

The maturation of chloroplast rRNAs involves multiple exonucleolytic and endonucleolytic events, similar to the process in bacteria (Strittmatter and Kössel, 1984). Indeed, the components of the enzymatic rRNA metabolism machinery have been implicated in the maturation of rRNA transcripts. RNRI, an RNase R
homolog, acts as a 3'-to-5' exoribonuclease and is involved in trimming the 3' ends of 23S, 16S, and 5S rRNAs (Bollenbach et al., 2005). The Arabidopsis rnrl mutant is impaired in the processing of 23S-4.5S precursors and has a defect that affects the accumulation of mature rRNAs (Bollenbach et al., 2005). The chloroplast polynucleotide phosphorylase (PNPase) is a 3'-to-5' exoribonuclease that mediates efficient 3' end trimming of chloroplast mRNAs and 23S rRNA via its phosphorolytic activity (Walter et al., 2002). 23S rRNA with 3' extensions accumulates in Arabidopsis plants with reduced or absent chloroplast PNPase (Walter et al., 2002; Marchive et al., 2009; Germain et al., 2011). CSP41a/b, two ribosome-associated endonucleases, can alter the balance between 23S precursor and mature rRNA in vitro, and these endonucleases likely act in the final steps of 23S rRNA maturation (Beligni and Mayfield, 2008). No RNase activity has been documented for any DEAD RNA helicase thus far; therefore, it is unlikely that RH22 functions as an RNase. However, DEAD RNA helicases may act as auxiliary and accessory proteins to facilitate rRNA metabolism in combination with RNases. For instance, the DEAD RNA helicase RhlB is present in the E. coli degradosome (Coburn et al., 1999). The chloroplast DEAD RNA helicase RH39 has been suggested to be involved in the introduction of the hidden break into the 23S rRNA in combination with an unknown endorNase (Nishimura et al., 2010). RH22, similar to RH39, may be implicated in the regulation of chloroplast rRNA maturation. However, the behavior of RH22 is distinct from that of RH39, as the contents of one mature form of 23S rRNA, the 0.5-kb fragment, were not affected in rh39 mutant plants (Nishimura et al., 2010).
and/or function. Thus, defects in rRNA processing or maturation could be caused by defects in the RNA processing itself or in ribosome assembly (Granneman and Baserga, 2005). Although the ribosomal assembly process in chloroplasts is not well understood, the principles governing *E. coli* ribosome assembly may be applicable to chloroplast ribosome assembly as well. Therefore, the aberrant processing and maturation of chloroplast rRNA in some mutants may result from a defect in ribosomal assembly. For example, in the *ppr4* mutant, the 16S rRNA-processing defect might be a consequence of incomplete 30S subunit assembly, which in turn may result from an impairment of RPS12 synthesis (Schmitz-Linneweber et al., 2006).

In this study, Suc gradient analyses and RNA coimmunoprecipitation assays produced strong evidence that RH22 is associated with the pre-50S subunit, which suggests that RH22 is involved in 50S ribosomal subunit assembly (Fig. 5). Thus, we propose that the rRNA maturation defect in the *rh22* mutants may be caused by defects in the assembly of the 50S ribosomal subunits rather than by defective processing per se. Indeed, further analysis showed that RH22 could specifically bind to a fragment of domain I of 23S rRNA (Fig. 7), around which no cleavage sites for 23S rRNA processing exist. Various steps in rRNA processing depend on ribosome assembly. In *E. coli* cell extracts, pre-23S rRNA in free ribosomes did not mature; however, this RNA was efficiently processed in polysomes, which suggests that formation of the polysomal complex is required for rRNA processing (Srivastava and Schlessinger, 1988). Similarly, the formation of a functional translation apparatus may be required for the normal maturation of chloroplast rRNAs (Barkan, 1993). However, RH22 does not seem to be associated with the polysomal fractions (Fig. 8) and is thus unlikely to be involved in polysome assembly.

The *rh22* mutants also showed a reduction in the mature forms of 16S rRNA, whereas an accumulation of the precursor rRNA was not observed (Fig. 4). The decrease in mature 16S rRNA in *rh22* appeared to represent an indirect consequence of the defect in 50S subunit biogenesis, because RH22 was not found to be associated with the 30S particle (Figs. 5 and 6). A similar phenomenon has been observed in the *E. coli srmB* mutant. In *srmB*, assembly of the 50S ribosomal subunit is affected, but incorporation of the 30S subunit into the 70S ribosome was also impaired, delaying the maturation of 16S rRNA (Charollais et al., 2003). How-

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**Figure 8.** Polysomal loading in *rh22* and wild-type (WT) plants. Twelve fractions of equal volume were collected from top to bottom of 15% to 55% Suc gradients, and equal proportions of the RNA purified from each fraction were analyzed by gel-blot hybridization with the probes indicated at the right of each panel. The positions of the ribosome (less than 80S) and the polysomal fractions were determined by running puromycin-treated samples in parallel with the experimental samples, as indicated at the bottom. The sedimentation of RH22 in the wild-type extracts was followed by an immunoblot analysis of the proteins from the same gradient fractions.
ever, Tiller et al. (2011) analyzed the rRNA-processing defects of Arabidopsis ribosomal mutants in RPS17, RPL24, and five plastid-specific ribosomal proteins. They found that defects in these mutants seem to be mainly specific to either the large or small subunit of the chloroplast ribosome (Tiller et al., 2011), in contrast to our results. Another possible reason for the decrease of mature 16S rRNA in rh22 is that RH22 has other functions in addition to its role in ribosome assembly. RH22 may be involved in the regulation of other processes associated with rRNA gene expression, such as RNA transcription or the stabilization of rRNA transcripts. For example, CSP41a/b has roles not only in the assembly of the 50S subunit of the chloroplast ribosome but also in the activation of chloroplast-encoded RNA polymerase (Beligni and Mayfield, 2008; Bollenbach et al., 2009).

Potential Roles of RH22 in Ribosomal Assembly

Using yeast two-hybrid analysis and immunoprecipitation assays, we showed that RH22 can interact with the L24 protein of the chloroplast 50S ribosomal subunit (Fig. 6). Using EMSA analysis, we also showed that RH22 can bind to a 5′ fragment of 23S rRNA that belongs to domain I of the 23S rRNA and includes putative PRL24-binding sites (Fig. 7). An RNA/protein complex containing RH22 appears to be formed during assembly of the 50S ribosomal subunit in chloroplasts. A similar RNA/protein complex involving SmB, the DEAD RNA helicase involved in 50S assembly, was identified in E. coli; this complex comprises the ribosomal proteins L24 and L4 and a small 23S rRNA fragment that encompasses their binding sites (Trubetskoy et al., 2009). Phylogenetic analysis of the DEAD RNA helicase proteins from bacteria and chloroplasts with the Phylogeny.fr program online (for details, see Supplemental References S1) indicated that RH22, RH39, RH50, and SmB are grouped into one cluster and that RH3, RH26, and other bacterial DEAD-box proteins are grouped into another cluster (Supplemental Fig. S6). This analysis suggested that RH22 may have evolved from the bacterial SmB protein. The reconstitution assay suggested that the L24 and L4 proteins might interact directly with SmB via protein-protein contacts (Trubetskoy et al., 2009). Consistent with this hypothesis, we identified chloroplast RPL24 as an interaction partner for RH22, suggesting that the action of RH22 is similar to that of SmB in E. coli. Based on the data presented here, we could not determine whether RPL4 interacts with RH22 in vivo, but no interaction between RH22 and L4 protein was detected in either the yeast two-hybrid or pull-down assay. However, if RPL4 is truly not a partner of RH22, this would indicate that the functional property of RH22 is not identical to that of SmB. In E. coli, L4, L24, and domain I are presumably among the first components of the 50S subunit to associate with one another, and SmB is involved in the formation of this complex (Trubetskoy et al., 2009). Domain I of the 23S rRNA in chloroplasts is very similar to that in E. coli, although it lacks helix 8 and contains additional sequence between helices 13 and 14 (Branlant et al., 1981; Edwards and Kössel, 1981; Harris et al., 1994). The binding sites of L24 and L4 in the E. coli 23S rRNA are thought to be in the internal loops that are bordered by helices 18, 19, and 20 in domain I (Fig. 7A; Stelzl and Nierhaus, 2001). The chloroplast 23S rRNA has a similar helical structure in domain I (Fig. 8A). However, putative binding sites for RPL24 (GAAG), but not RPL4, were found in chloroplast 23S rRNA (Fig. 7A). This structural difference in the 23S rRNA molecules of E. coli and chloroplasts suggests that chloroplast RPL4 may not bind to this region or that it binds to this region in a manner distinct from that in E. coli. It is likely that the functions and activities of DEAD RNA helicases may coevolve with rRNA structure in the context of ribosome function.

If RH22, PRL24, and the 5′ end of 23S rRNA form a protein/rRNA complex, and this complex is involved in the early assembly of the 50S subunit, how does RH22 participate in this process? Many rRNA-rRNA and rRNA-protein interactions certainly occur during 23S rRNA processing and its assembly with 50S ribosomal proteins. These interactions cause extensive structural rearrangements that ultimately lead to formation of the functional ribosomal complex. The association of RH22 with the 5′ end of the 23S RNA may facilitate its interaction with RPL24 and the initiation of 50S ribosomal assembly. The helicase activity of RH22 may unwind and/or rearrange the structure of the 23S precursor rRNA to allow it to contact RPL24. It has been reported that DpbA, an ATP-dependent helicase in E. coli, was able to bind to hairpin 92 of 23S rRNA to promote a conformational change of the peptidyl transferase center of 23S rRNA and affect its association with several ribosomal proteins (Sharpe Elles et al., 2009). Alternatively, RH22 may simply clamp its RNA-binding track on the 23S rRNA to transiently stabilize the RNA structure and maintain its structure to facilitate the kinetics of ribosome assembly. In eukaryotes, the DEAD-box initiation factor 4AIII can interact with the phosphate-ribose backbone of six consecutive nucleotides and prevent part of the bound RNA from forming a duplex with another strand, which is required for the formation of a multiprotein exon junction complex (Andersen et al., 2006).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Wild-type and mutant Arabidopsis (Arabidopsis thaliana ecotype Columbia) plants were grown in soil under short-day conditions (10-h-light/14-h-dark cycles) with a photon flux density of 120 μmol m−2 s−1 at 22°C. To ensure synchronized germination, the seeds were incubated in darkness for 48 h at 4°C before sowing. For growth on agar plates, the seeds were surface sterilized and sown on Murashige and Skoog medium containing 2% Suc. Three rh22 mutant lines, Salk_032399 (rh22-1), Salk_065388C (rh22-2), and CS856759 (rh22-3), were obtained from the ABRC and identified using PCR analysis according to the methods of Alonso et al. (2003) and Woody et al. (2007).
Chlorophyll Fluorescence Measurements

Chlorophyll fluorescence measurements were performed using CF Imager (Technologica) as described in the manufacturer’s instructions. Before each measurement, leaves were dark adapted for 10 min. The minimum fluorescence yield \(F_o\) was measured under measuring light (650 nm) with very low intensity (0.5 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)). A saturating pulse of white light (3,000 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) for 1 s) was applied to estimate the maximum fluorescence yield \(F_m\). The maximal photochemical efficiency of PSII was calculated from the ratio of \(F_m\) to \(F_v\) \(= \frac{F_m - F_o}{F_o}\). Values of \(F_o\) and \(F_m\) were averaged to improve the signal-to-noise ratio. Image data acquired in each experiment were normalized to a false-color scale, with arbitrarily assigned extreme values of 0.1 (lowest) and 0.8 (highest). This resulted in the highest and lowest \(F_o\), \(F_m\) values being represented by the red and blue extremes of the color scale, respectively.

GFP Assays

The coding sequences of RH22 were amplified by RT-PCR using primers 5' - GTTCGACAGTCATGATATGAAAGAGACGAC-3' and 5' - CATGCTCCTGGAATACCAAGAGATGAGAC-3'. The PCR products were then cloned into SalI/NcoI sites of the transient expression vector pUC18-356-sGFP, which contained the mGFP5 coding sequence under the control of the cauliflower mosaic virus 35S promoter, to generate a RH22 fusion protein with GFP in the C terminus. Similarly, the fragments containing the transit peptide (1-81 amino acids) of the small subunit of ribulose bisphosphate carboxylase were amplified using the primer pair 5' - CAGTCGACAAACCTCAGTCACACAAAGAG-3' and 5' - TCCATGATGGAATACCAAGAGATGAGAC-3', which were subcloned into this vector and used as the chloroplast localization control. The resulting constructs and the negative control vector without additional coding sequence were transfected into Arabidopsis mesophyll protoplasts according to the method described by Kovtun et al. (2000). Fluorescence analysis was performed on a LSM 510 META confocal laser-scanning system (Zeiss).

Protein Isolation and Immunoblotting

Proteins of thylakoid membrane were isolated essentially as described by Zhang et al. (1999). Briefly, 0.1 g of Arabidopsis leaves was homogenized in an ice-cold extraction buffer (400 \(\mu\)l Suc, 50 mM HEPES-KOH, pH 7.8, 10 mM NaCl, and 2 mM MgCl\(_2\)), filtered through two layers of cheesecloth, and centrifuged at 5,000 \(\times\) g for 10 min. The thylakoid pellets were resuspended in 30 mM HEPES-KOH, pH 8, 60 mM KOAc, 10 mM Mg\(_2+\), 2 mM dithiothreitol (DTT), and a protease inhibitor cocktail (2 mg ml\(^{-1}\) aprotonin, 2 mg ml\(^{-1}\) leupeptin, 1 mg ml\(^{-1}\) pepstatin, and 1 mg ml\(^{-1}\) phenylmethylsulfonyl fluoride) with repeated vortexing and incubation on ice for 30 min. The stromal fraction was obtained from lysed plastids by centrifugation at 29,000 rpm for 30 min at 4°C. A layer of 200 \(\mu\)l of stroma extract (about 10 mg ml\(^{-1}\)) was placed on the top of 10% to 40% linear Suc gradients prepared in 30 mM HEPES-KOH, pH 8.0, 10 mM Mg(OAc)\(_2\), 150 mM KOAc, and 5 mM DTT. Centrifugation was carried out in a Beckman SW55.1 rotor at 48,000 rpm for 4 h at 4°C. The gradients were fractionated by carefully pipetting from the top of the gradient, and 100 \(\mu\)l per fraction was collected for further analysis.

RNA Preparation and RT-PCR Analysis

Total leaf RNA was purified from Arabidopsis seedlings using Trizol reagent (Invitrogen). Total RNA (500 ng) was used to generate the first-strand cDNA in a 50-\(\mu\)l reaction with the SuperScript II cDNA synthesis system (Invitrogen) according to the manufacturer’s instructions. The resulting cDNA samples were then used for PCR analysis. The PCR primers for detection of RH22 expression (in fig. 2) were FP 5' - ATGATCTCTCCGAGCTCCTGTC-3' and RP 5' - TTATATCTCATTACAGGCTGC-3'). The specific primers of chloroplast genes for qPCR were adopted from Chatteigner-Boutin et al. (2008). The qRT-PCR was performed using Mx3000P (Stratagene) with SYBR Green I (Invitrogen) according to the manufacturer’s protocols. The amplification of elongation factor1-a was used as an internal control for normalization.

RNA-Blot Analysis

Five micrograms of total RNA from wild-type and rh22 seedlings were separated on a 1.2% to 3.0% (w/v) agarose-formaldehyde gel, blotted to a positively charged nylon membrane by capillary blotting, and fixed by cross-linking. Hybridization was performed with \(^{32}\)P-labeled probes in 5 \(\times\) SSC, 5 \(\times\) Denhardt’s solution, and 0.5% SDS at 65°C overnight. The membranes were washed twice in 2 \(\times\) SSC, 0.1% SDS and once in 1 \(\times\) SSC, 0.1% SDS at 65°C.

Suc Gradient Sedimentation Analysis

The isolation of intact chloroplasts was performed as described previously (Barkan, 1998). The stroma was prepared by hypotonic lysis of purified chloroplasts and used for Suc gradient sedimentation analysis as described by Jenkins and Barkan (2001). Briefly, intact chloroplasts underwent osmotic lysis in 30 mM HEPES-KOH, pH 8.0, 60 mM KOAc, 10 mM Mg\(_2+\), 2 mM dithiothreitol (DTT), and a protease inhibitor cocktail (2 mg ml\(^{-1}\) aprotonin, 2 mg ml\(^{-1}\) leupeptin, 1 mg ml\(^{-1}\) pepstatin, and 1 mg ml\(^{-1}\) phenylmethylsulfonyl fluoride) with repeated vortexing and incubation on ice for 30 min. The stromal fraction was obtained from lysed plastids by centrifugation at 29,000 rpm for 30 min at 4°C. A layer of 200 \(\mu\)l of stroma extract (about 10 mg ml\(^{-1}\)) was placed on the top of 10% to 40% linear Suc gradients prepared in 30 mM HEPES-KOH, pH 8.0, 10 mM Mg(OAc)\(_2\), 150 mM KOAc, and 5 mM DTT. Centrifugation was carried out in a Beckman SW55.1 rotor at 48,000 rpm for 4 h at 4°C. The gradients were fractionated by carefully pipetting from the top of the gradient, and 100 \(\mu\)l per fraction was collected for further analysis.

Pull Down and Coimmunoprecipitation of Proteins and RNA

The pull-down assay was performed according to the method described by Chi et al. (2010) with modifications. MBP only and MBP-RH22 fusion proteins were mixed with testing proteins (His-RPL24 and His-RPL4) in 20 mM Tris-HCl and 25 mM NaCl, pH 8.0, and fractionated on a 1-\(\times\)10 cm DEAE-Sepharose chromatography column (GE Life Sciences) according to the manufacturer’s protocol. The fractions containing proteins were determined by measuring \(A_{280}\) and analyzed by SDS-PAGE and Coomassie blue staining. The fractions containing the RH22 protein free of MBP were pooled and concentrated. Mature RPL24 and RPL4 with an N-terminal 6\(\times\)His tag were expressed from the pET28a vector (Novagen), and a coimmunoprecipitation experiment was purified with the nickel-nitrilotriacetic acid agarose resin (Qiagen). The MBP-RH22 and His-RPL24 fusion proteins were used to generate polyclonal antibodies from rabbits. The antibodies to RPS1, RPL4, and RPL32 were supplied by Uniplastomic of France (www.uniplastomic.com/).

Expression of Recombinant Proteins and Antibody Production

The DNA sequence of mature RH22 was subcloned into the expression vector pMAL-c2X (New England Biolabs) to produce MBP fusions. The MBP-RH22 fusion protein was expressed in Escherichia coli BL21 (DE3) cells. Induction, cell lysis, and purification of the fusion protein using amyllose affinity chromatography were performed according to the manufacturer’s protocol. The fusion protein was cleaved with the specific protease factor Xa, dialyzed with 20 mM Tris-HCl and 25 mM NaCl, pH 8.0, and fractionated on a 1-\(\times\)10 cm DEAE-Sepharose chromatography column (GE Life Sciences) according to the manufacturer’s protocol. The fractions containing proteins were determined by measuring \(A_{280}\) and analyzed by SDS-PAGE and Coomassie blue staining. The fractions containing the RH22 protein free of MBP were pooled and concentrated. Mature RPL24 and RPL4 with an N-terminal 6\(\times\)His tag were expressed from the pET28a vector (Novagen), and a coimmunoprecipitation experiment was purified with the nickel-nitrilotriacetic acid agarose resin (Qiagen). The MBP-RH22 and His-RPL24 fusion proteins were used to generate polyclonal antibodies from rabbits. The antibodies to RPS1, RPL4, and RPL32 were supplied by Uniplastomic of France (www.uniplastomic.com/).
RNA coimmunoprecipitations were performed as described by Barkan (2009). Briefly, RNA was recovered from the precipitated fractions in the presence of 1% SDS, 0.05 μg mL⁻¹ yeast RNA, and 5 μM EDTA. The RNA was isolated by phenol-chloroform isomyl extraction and precipitated with 3 volumes of ethanol. RNAs from the supernatant fractions were extracted as the controls. RNA samples were transferred to a nylon membrane using a slot-blotting apparatus (Bio-Dot SF; Bio-Rad). Subsequently, the membranes were hybridized with radiolabeled DNA probes.

For the RNase-protection assay of the RNA samples, α-32P- radiolabeled RNA probes were synthesized using the MAXIscript kit (Ambion) with templates amplified from Arabidopsis total DNA according to the manufacturer's instructions. The hybridization, the RNase digestion of the unhybridized RNA, and the separation and detection of protected fragments were performed using the RPA III kit (Ambion).

**Yeast Two-Hybrid Analysis**

The protein interactions were detected using the HybrizAP-2.1 two-hybrid system (Stratagene) as described previously (Chi et al., 2010). The cDNAs corresponding to the RH22 protein lacking the N-terminal 58 amino acids of the signal peptide amplified using PCR was cloned into the pSher and SshII sites of the pBD-GAL4 Cam vector and used as the bait. The primers used were as follows: sense (5'-GGGGCCGCACTGTACTAGGAGGAATTG-3') and antisense (5'-GTCGACATATCTCAACGGTGGCCTCTTCT-3'). The cDNAs corresponding to mature ribosomal proteins were amplified using PCR and cloned into the pAD-GAL4-2.1 vector as the prey. The information for all of the ribosomal proteins was obtained from PFDB, a plant proteome database for Arabidopsis and maize (Ze a mays) at Cornell University (Sun et al., 2009). Protein interactions were determined by growing yeast transformants on selective medium plates (without Trp, Leu, and His but containing 10 μg 3-amino-1,2,4-triazole). The β-galactosidase activities were measured using the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside filter assay according to the manufacturer’s instructions.

**EMSAs**

The two oligoribonucleotides used for EMSA were synthesized by Takara, and their sequences were as follows: Oligo3 (5'-UAGCCGAGA-GACGCCGGAUCAUGCCUCGAGCUAGCGAAGCCUUGACUCGAAUGC-3'; 303–352 of rrn23) and Oligo3 (5'-AACAGCGCUAAAACCCCAAAGGAGUCAUACGGCGCGGUGUUGGACC-3'; 2468–2518 of rrn23). The synthetic oligoribonucleotides were 5' end labeled with [γ-32P]ATP and T4 polynucleotide kinase and purified by ethanol precipitation. The binding reaction mixture contained 50 μM Tris-HCl, pH 7.5, 150 μM NaCl, 2 mM DTT, 5% glycerol, 0.1 mg mL⁻¹ bovine serum albumin, 0.5 μg mL⁻¹ heparin, and 5 units of RNAse. The concentrations of RNA and RH22 protein are indicated in the reaction mixture contained 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM DTT, 5 μM MgCl₂, 0.1 mg mL⁻¹ bovine serum albumin, and 5 μg mL⁻¹ cycloheximide). After incubation for 10 min on ice, the nuclei and the insoluble materials were removed by centrifugation at 12,000 rpm for 5 min at 4°C. Sodium deoxycholate was added to the supernatant at a concentration of 0.5%, and the mixture was then placed on ice for 5 min. The remaining insoluble materials were removed by centrifuging at 12,000 rpm for 15 min at 4°C. The supernatants (0.4 mL) were layered on 4 mL of a 15% to 55% sucrose density gradient. Samples were centrifuged in a SW28 rotor (Beckman) at 45,000 rpm for 65 min at 4°C. After centrifugation, 12 fractions (410 μL each) were collected, and the RNA was isolated from each fraction and used for RNA gel-blot analysis as described above.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers RH22(At1g59990).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Transmission electron microscopic images of chloroplasts in 10-d-old leaves from wild-type and rh22 mutant seedlings.

**Supplemental Figure S2.** Microscopy of the developing embryos in opened developing siliques of wild-type and heterozygous rh22-3/RH22 plants.

**Supplemental Figure S3.** RNA gel-blot analysis of the levels of chloroplast-encoded gene transcripts in wild-type and rh22-2 plants.

**Supplemental Figure S4.** qRT-PCR assay of chloroplast gene expression in wild-type and rh22-2 plants.

**Supplemental Figure S5.** Expression and purification of RH22 protein.

**Supplemental Figure S6.** Phylogenetic analysis of DEAD RNA helicase proteins from the chloroplast and E. coli.

**Supplemental Materials and Methods.**

Supplemental References S1. The phylogeny.fr program.

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**LITERATURE CITED**

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al. (2003) Genome-wide inser
tional mutagenesis of Arabidopsis thaliana. Science 301: 653–657

Andersen CB, Ballut L, Johansen JS, Chamieh H, Nielsen KH, Oliveira CL, Pedersen JS, Séraphin B, Le Hir H, Andersen GR (2006) Structure of the exon junction core complex with a trapped DEAD-box ATPase bound to RNA. Science 313: 1968–1972

Aubourg S, Kreis M, Lechary A (1999) The DEAD box RNA helicase family in Arabidopsis thaliana. Nucleic Acids Res 27: 628–636

Ban N, Nissen P, Hansen J, Moore PB, Steitz TA (2000) The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. Science 290: 905–920

Barkan A (1993) Nuclear mutants of maize with defects in chloroplast polysome assembly have altered chloroplast RNA metabolism. Plant Cell 5: 389–402

Barkan A (1998) Approaches to investigating nuclear genes that function in chloroplast biogenesis in land plants. Methods Enzymol 297: 58–57

Barkan A (2009) Genome-wide analysis of RNA-protein interactions in plants. Methods Mol Biol 553: 13–37

Beick S, Schmitz-Linneweber C, Williams-Carrier R, Jensen B, Barkan A (2008) The pentatricopeptide repeat protein PPR5 stabilizes a specific tRNA precursor in maize chloroplasts. Mol Cell Biol 28: 5337–5347

Beligni MV, Mayfield SP (2008) Arabidopsis thaliana mutants reveal a role for CSP41a and CSP41b, two ribosome-associated endonucleases, in chloroplast ribosomal RNA metabolism. Plant Mol Biol 67: 389–401

Beliaeu M, Keddie JS, Gruissem W (2003) DCL is a plant-specific protein required for plastid ribosomal RNA processing and embryo development. Plant Mol Biol 53: 531–543

Bisanc B, Bégot I, Carol P, Perez P, Bligny M, Pese E, Galloux JL, Lerbs-Mache S, Mache R (2003) The Arabidopsis nuclear DCL gene encodes a chloroplast protein which is required for the maturation of the plastid ribosomal RNAs and is essential for chloroplast differentiation. Plant Mol Biol 51: 651–663

Bollenbach TJ, Lange H, Gutierrez R, Erhardt M, Stern DB, Gagliardi D (2003) RNR1, a 3'-5' exoribonuclease belonging to the RNR superfamily, catalyzes 3' maturation of chloroplast ribosomal RNAs in Arabidopsis thaliana. Nucleic Acids Res 33: 2751–2763

**Polysome Analysis**

Polysomes were isolated according to the method of Barkan (1993). Two-tenths of 1 g of leaf tissues was homogenized in 1 mL of polysome extraction buffer (0.2 M Tris-HCl, pH 9.0, 0.2% s KCl, 35 mM MgCl₂, 25 mM EDTA, 0.2% Suc, 1% Triton X-100, 2% polyoxyethylene-10-tridecyl ether, 0.5 mg mL⁻¹ heparin, 100 μM β-mercaptoethanol, 100 μg mL⁻¹ chloramphenicol, and 25 μg mL⁻¹ cycloheximide). After incubation for 10 min on ice, the nuclei and the insoluble materials were removed by centrifugation at 12,000 rpm for 5 min at 4°C. Sodium deoxycholate was added to the supernatant at a concentration of 0.5%, and the mixture was then placed on ice for 5 min. The remaining insoluble materials were removed by centrifuging at 12,000 rpm for 15 min at 4°C. The supernatants (0.4 mL) were layered on 4 mL of a 15% to 55% Suc density gradient. Samples were centrifuged in a SW28 rotor (Beckman) at 45,000 rpm for 65 min at 4°C. After centrifugation, 12 fractions (410 μL each) were collected, and the RNA was isolated from each fraction and used for RNA gel-blot analysis as described above.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers RH22(At1g59990).
Bollenbach TJ, Sharwood RE, Gutierrez R, Lerbs-Mache S, Stern DB (2009) The RNA-binding proteins CSP41a and CSP41b may regulate transcription and translation of chloroplast-encoded RNAs in Arabidopsis. Plant Mol Biol 69: 541–552

Branlant C, Krol A, Machatt MA, Pouyet J, Ebel JP, Edwards K, Kössel H (1981) Primary and secondary structures of Escherichia coli MR 600 235S ribosomal RNA: comparison with models of secondary structure for maize chloroplast 23S RNA and for large portions of mouse and human 16S mitochondrial RNAs. Nucleic Acids Res 9: 4303–4324

Caruthers JM, McKay DB (2002) Helicase structure and mechanism. Curr Opin Struct Biol 12: 123–133

Charollais J, Dreyfus M, lost I (2004) CsdA, a cold-shock RNA helicase from Escherichia coli, is involved in the biogenesis of 50S ribosomal subunit. Nucleic Acids Res 32: 2751–2759

Charollais J, Pfieger D, Vinh J, Dreyfus M, lost I (2003) The DEAD-box RNA helicase SmRb is involved in the assembly of 50S ribosomal subunits in Escherichia coli. Mol Microbiol 48: 1253–1265

Chateigner-Boutin AL, Ramos-Vega M, Guevara-García A, Andrés C, de la Luz Gutiérrez-Nava M, Cantero A, Delamné LF, Jiménez LF, Lurin C, Small I, et al. (2008) CLB19, a pentatricopeptide repeat protein involved in the assembly of 50S ribosomal subunits. Nucleic Acids Res 36: 7217–7225

Cheng ZF, Deutscher MP (2003) Recruitment of a peptidyl-tRNA hydrolase as a factor in the assembly of the 50S ribosomal subunit. Nucleic Acids Res 31: 739–746

Chi et al. (1998) A short fragment of 23S rRNA containing the binding sites for two ribosomal proteins, L24 and L4, is a key element for rRNA folding during early assembly. RNA 4: 758–765

Kishine M, Takabayashi A, Munekage Y, Shikanai T, Endo T, Sato F (2004) Ribosomal RNA processing and an RNase R family member in chloroplasts of Arabidopsis. Plant Mol Biol 55: 595–606

Kossen K, Uhlenbeck OC (1999) Cloning and biochemical characterization of Bacillus subtilis YxiH, a DEAD protein specifically activated by 23S rRNA: delineation of a novel sub-family of bacterial DEAD proteins. Nucleic Acids Res 27: 3811–3820

Kresse S, Stanne TM, Ferrer CA, Giap T, Sjögren LL, Zhao Y, Clarke AK, Chory J (2007) An Arabidopsis thaliana virens mutant reveals a role for ClpR1 in plastid development. Plant Mol Biol 63: 85–96

Koutou V, Chiu W, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci USA 97: 2940–2945

Kressler D, Linder P, de la Cruz J (2004) DExD/H-box proteins and related families. Trends Biochem Sci 29: 17–37

Lindahl L (1975) Intermediate and time kinetics of the in vivo assembly of Escherichia coli ribosomes. J Mol Biol 92: 15–37

Maki Y, Tanaka A, Wada A (2000) Stoichiometric analysis of barley plastid ribosomal proteins. Plant Cell Physiol 41: 289–299

Marchive C, Yehudai-Resheff S, Germaine A, Fei Z, Jiang X, Judkins J, Wu H, Fernie AR, Fait A, Stern DB (2009) Abnormal physiological and molecular mutant phenotypes link chloroplast polynucleotide phosphorylase to the phosphorys deprivation response in Arabidopsis. Plant Physiol 151: 905–924

Nierhaus KH (1991) The assembly of prokaryotic ribosomes. Biochemistry 73: 734–735

Nishimura K, Ashida H, Ogawa T, Yokota A (2010) A DEAD box protein is required for formation of a hidden break in Arabidopsis chloroplast 23S rRNA. Plant J 63: 766–777

Pokhilko A, Karcher D, Bock R (2008) Superwobbling facilitates translation with reduced RNA sets. Nat Struct Mol Biol 15: 192–198

Schulzen F, Tocilj A, Zarivach R, Harms J, Guehmann M, Janell D, Bashan A, Bartels H, Agmon I, Franceschi F, et al. (2000) Structure of functionally activated small ribosomal subunit at 3.3 A˚ resolution. Cell 102: 615–623

Schmitz-Linneweer C, Williams-Carrier RE, Williams-Voelker PM, Kroeger TS, Vichas A, Barkan A (2006) A pentatricopeptide repeat protein facilitates the trans-splicing of the maize chloroplast rps12 pre-rRNA. Plant Cell 18: 2650–2663

Shahani Z, Sykes MT, Williamson JR (2011) Assembly of bacterial ribosomes. Annu Rev Biochem 80: 501–526

Sharpe Elles LM, Sykes MT, Williamson JR, Uhlenbeck OC (2009) A dominant negative mutant of the E. coli rRNA helicase DhpA blocks assembly of the 50S ribosomal subunit. Nucleic Acids Res 37: 6503–6514

Silverman E, Edwards-Gilbert G, Lin RJ (2003) DExD/H-box proteins and their partners: helping RNA helicases unwind. Gene 312: 1–16

Srivastava AK, Schlessinger D (1988) Coregulation of processing and translation: mature 5′ terminal of Escherichia coli 23S ribosomal RNA form in polysomes. Proc Natl Acad Sci USA 85: 7144–7148

Stelzl U, Nierhaus KH (2001) A short fragment of 23S rRNA containing the binding sites for two ribosomal proteins, L24 and L4, is a key element for rRNA folding during early assembly. RNA 7: 598–609

Strittmatter G, Kussel H (1984) Cotranscription and processing of 23S, 5.5S
and SS rRNA in chloroplasts from *Zea mays*. Nucleic Acids Res 12: 7633–7647
Sun Q, Zybailov B, Majeran W, Friso G, Olinares PD, van Wijk KJ (2009) PPDB, the plant proteomics database at Cornell. Nucleic Acids Res 37: D969–D974
Tiller N, Weingartner M, Thiele W, Maximova E, Schöttler MA, Bock R (2011) The plastid-specific ribosomal proteins of *Arabidopsis thaliana* can be divided into non-essential proteins and genuine ribosomal proteins. Plant J 69: 302–316
Trubetskoy D, Proux F, Allemand F, Dreyfus M, Iost I (2009) SrmB, a DEAD-box helicase involved in *Escherichia coli* ribosome assembly, is specifically targeted to 23S rRNA in vivo. Nucleic Acids Res 37: 6540–6549
Tumminia SJ, Hellmann W, Wall JS, Boublik M (1994) Visualization of protein-nucleic acid interactions involved in the in vitro assembly of the *Escherichia coli* 50 S ribosomal subunit. J Mol Biol 235: 1239–1250
Walter M, Kilian J, Kudla J (2002) PNPase activity determines the efficiency of mRNA 3′-end processing, the degradation of tRNA and the extent of polyadenylation in chloroplasts. EMBO J 21: 6905–6914
Wang Y, Duby G, Purnelle B, Boutry M (2000) Tobacco VDL gene encodes a plastid DEAD box RNA helicase and is involved in chloroplast differentiation and plant morphogenesis. Plant Cell 12: 2129–2142
Watkins KP, Kroeger TS, Cooke AM, Williams-Carrier RE, Friso G, Belcher SE, van Wijk KJ, Barkan A (2007) A ribonuclease III domain protein functions in group II intron splicing in maize chloroplasts. Plant Cell 19: 2606–2623
Williamson JR (2003) After the ribosome structures: how are the subunits assembled? RNA 9: 165–167
Woody ST, Austin-Phillips S, Amasino RM, Kysran PJ (2007) The WisDoLox T-DNA collection: an *Arabidopsis* community resource generated by using an improved high-throughput T-DNA sequencing pipeline. J Plant Res 120: 157-165
Yamaguchi K, Beligni MV, Prieto S, Haynes PA, McDonald WH, Yates JR III, Mayfield SP (2003) Proteomic characterization of the *Chlamydomonas reinhardtii* chloroplast ribosome: identification of proteins unique to the 70 S ribosome. J Biol Chem 278: 33774–33785
Yamaguchi K, Subramanian AR (2000) The plastid ribosomal proteins: identification of all the proteins in the 50 S subunit of an organelle ribosome (chloroplast). J Biol Chem 275: 28466–28482
Yamaguchi K, Subramanian AR (2003) Proteomic identification of all plastid-specific ribosomal proteins in higher plant chloroplast 30S ribosomal subunit. Eur J Biochem 270: 190–205
Yamaguchi K, von Knoblauch K, Subramanian AR (2000) The plastid ribosomal proteins: identification of all the proteins in the 30 S subunit of an organelle ribosome (chloroplast). J Biol Chem 275: 28455–28465
Yu F, Liu X, Alsheikh M, Park S, Rodermel S (2008) Mutations in Suppressor of Variation1, a factor required for normal chloroplast translation, suppress var2-mediated leaf variegation in *Arabidopsis*. Plant Cell 20: 1786–1804
Yusupov MM, Yusupova GZ, Baucom A, Lieberman K, Earnest TN, Cate JHD, Noller HF (2001) Crystal structure of the ribosome at 5.5 Å resolution. Science 292: 883–896
Zhang LX, Paakkarinen V, van Wijk KJ, Aro EM (1999) Co-translational assembly of the D1 protein into photosystem II. J Biol Chem 274: 16062–16067
Zybailov B, Friso G, Kim J, Rudella A, Rodríguez VR, Asakura Y, Sun Q, van Wijk KJ (2009) Large scale comparative proteomics of a chloroplast Clp protease mutant reveals folding stress, altered protein homeostasis, and feedback regulation of metabolism. Mol Cell Proteomics 8: 1789–1810
Zybailov B, Rutschow H, Friso G, Rudella A, Emanuelsen O, Sun Q, van Wijk, KJ (2008) Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. PLoS ONE 3: e1994