Chloroplasts in angiosperms contain at least seven nucleus-encoded members of the DEAD box RNA helicase family. Phylogenetic analysis shows that five of these plastid members (RH22, -39, -47, -50, and -58) form a single clade and that RH3 forms a clade with two mitochondrial RH proteins (PMH1 and -2) functioning in intron splicing. The function of chloroplast RH3 in maize (Zea mays; ZmRH3) and Arabidopsis (Arabidopsis thaliana; AtRH3) was determined. ZmRH3 and AtRH3 are both under strong developmental control, and ZmRH3 abundance sharply peaked in the sink-source transition zone of developing maize leaves, coincident with the plastid biogenesis machinery. ZmRH3 coimmunoprecipitated with a specific set of plastid RNAs, including several group II introns, as well as pre23S and 23S ribosomal RNA (rRNA), but not 16S rRNA. Furthermore, ZmRH3 associated with 50S preribosome particles as well as nucleoids. AtRH3 null mutants are embryo lethal, whereas a weak allele (rh3-4) results in pale-green seedlings with defects in splicing of several group II introns and rRNA maturation as well as reduced levels of assembled ribosomes. These results provide strong evidence that RH3 functions in the splicing of group II introns and possibly also contributes to the assembly of the 50S ribosomal particle. Previously, we observed 5- to 10-fold up-regulation of AtRH3 in plastid Caseinolytic protease mutants. The results shown here indicate that AtRH3 up-regulation was not a direct consequence of reduced proteolysis but constituted a compensatory response at both RH3 transcript and protein levels to impaired chloroplast biogenesis; this response demonstrates that cross talk between the chloroplast and the nucleus is used to regulate RH3 levels.

DEAD box RNA helicases are mainly involved in ATP-dependent rearrangement of intermolecular and intramolecular RNA structures or remodeling of ribonucleoprotein complexes and are found in all eukaryotes and most prokaryotes (Cordin et al., 2006; Banroques et al., 2011; Linder and Jankowsky, 2011). DEAD box RNA helicases have been shown to be involved in RNA synthesis, modification, cleavage, and degradation as well as in ribosome biogenesis and translation initiation (Banroques et al., 2011; Linder and Jankowsky, 2011). DEAD box RNA helicase proteins possess conserved ATP-binding and -hydrolyzing domains, RNA-binding motifs, and a characteristic Asp-Glu-Ala-Asp (DEAD) sequence (as part of the Walker B motif; Cordin et al., 2006). However, in some cases, the Ala in the DEAD domain is not conserved (resulting into DExD). These conserved domains are clustered in the central core region that spans 350 to 400 amino acid residues. The N- and C-terminal extensions are highly variable and are thought to provide additional interactions with substrates or cofactors, or to confer additional activities such as protease activity (for NS3, see Cordin et al., 2006). DEAD box RNA helicase proteins belong to the helicase superfamily 2, which also includes DEAH and DExH proteins.

It was reported previously that there are 56 predicted DEAD box RNA helicases (and two pseudogenes) in Arabidopsis (Arabidopsis thaliana; Mingam et al., 2004), 11 of which have a TargetP-predicted chloroplast transit peptide. In this study and in the Plant Proteome Database (http://ppdb.tc.cornell.edu/), we adopt the numbering for the RNA helicases as established for Arabidopsis (Mingam et al., 2004). Mass spectrometry analyses identified six DEAD box helicases in Arabidopsis chloroplasts, namely RH3, RH22, RH26, RH39, RH47, and RH50. These plastid...
proteins were detected in high-molecular-mass (greater than 0.6–3 MD) chloroplast stromal complexes (Peltier et al., 2006; Olinares et al., 2010). Maize (Zea mays) homologs for these six plastid helicases were found by proteome analysis of maize plastids (Friso et al., 2010; Majeran et al., 2012). Most of these maize proteins were enriched in plastid nucleoids (Majeran et al., 2012) and also in Arabidopsis nucleoids (G. Friso, K. Nishimura, X. Qu, and K.J. van Wijk, unpublished data). The tobacco (Nicotiana tabacum) homolog of RH58, named VDL helicase, was identified in tobacco chloroplasts (Wang et al., 2000) but was not (yet) detected in Arabidopsis, likely because of its low abundance. The maize homolog for RH58 was found in a co-immunoprecipitation (co-IP) with anti-WHY1 serum against maize nucleoids (Majeran et al., 2012), but RH58 abundance levels were very low compared with the other plastid RH helicases.

Mutants for several chloroplast RNA helicases have been characterized to various degrees and exhibit delayed or impaired chloroplast and leaf development and defects in chloroplast RNA metabolism. OsBIRH1, the rice (Oryza sativa) homolog of Arabidopsis RH50, exhibited RNA helicase activities in vitro and helped to confer plant resistance against various stresses (Li et al., 2008). Arabidopsis mutant nar12-1, with reduced accumulation of RH39, showed strongly reduced levels of the photosynthesis machinery due to inefficient translation elongation, most likely due to the loss of RH39-mediated defects in 23S rRNA maturation (formation of the hidden break) affecting the functional state of the ribosome (Nishimura et al., 2010). Complete loss of Arabidopsis RH22 resulted into embryo lethality, whereas reduced RH22 expression resulted in virenscent seedlings in which precursors of 23S and 4.5S rRNA (in the 50S particle) accumulated (Chi et al., 2012). It was concluded that RH22 facilitates the assembly of the 50S ribosomal subunit by interacting with ribosomal protein RPL24 and with the region of 23S RNA encompassing the RPL24 binding site (Chi et al., 2012). Tobacco mutants with loss of the RH58 homolog (VDL helicase) showed pale-green and variegated leaves and abnormal roots and flowers, but no function was determined (Wang et al., 2000).

In this study, we focus on the molecular function of the chloroplast-localized DEAD box RNA helicase AtRH3 in Arabidopsis and its co-orthologs in maize, ZmRH3A and ZmRH3B. Proteome analysis showed that RH3 is the most abundant plastid DEAD box RNA helicase in maize and Arabidopsis (Zybailov et al., 2008; Friso et al., 2010). Our attention was drawn to RH3 for two reasons. First, we found that AtRH3 was strongly (more than 5-fold) up-regulated in the mutants clpr1-1 and clpr4-1, with defects in the chloroplast stroma-localized Caseinolytic protease (Clp) protease core complex (Rudella et al., 2006; Kim et al., 2009; Zybailov et al., 2009). These clpr1-1 and clpr4-1 mutants are chlorophyll-deficient and developmentally delayed mutants and have partial defects in chloroplast rRNA processing (Rudella et al., 2006; Kim et al., 2009; Zybailov et al., 2009), as observed for the Clp core mutant clpr1-1 (Koussevitzky et al., 2007). Second, we detected ZmRH3 in co-IP with several proteins that promote the splicing of chloroplast group II introns (G. Friso, K.P. Watkins, A. Barkan, and K.J. van Wijk, unpublished data). Here, we address the function of RH3 in both maize and Arabidopsis as well as the basis for its up-regulation in Arabidopsis plastid Clp protease mutants. Our results show that RH3 is directly involved in chloroplast intron splicing and possibly also in 50S ribosome biogenesis. RH3 levels peak during early stages of chloroplast biogenesis, consistent with a role in establishing the chloroplast gene expression machinery. Our results suggest that RH3 overaccumulates in Clp mutants not because it is a direct substrate of Clp protease but rather as a consequence of a disruption in chloroplast biogenesis or protein homeostasis. Finally, we use phylogeny and functional domain analysis of the Arabidopsis DEAD box RNA helicase family to compare RH3 with other plastid DEAD box RNA helicases.

RESULTS

Phylogenetic Analysis of Plant DEAD Box RNA Helicases and Architecture of RH3 Orthologs

To establish the phylogenetic relationships among maize, rice, and Arabidopsis DEAD box proteins, we created unrooted phylogenetic trees based on the 56 Arabidopsis DEAD box helicases listed by Mingam et al. (2004), 46 related maize and 47 rice sequences, as well as the five known DEAD box RNA helicases from Escherichia coli (for accession numbers, annotations, and clade assignments, see Supplemental Table S1; for all amino acid sequences, see Supplemental Text S1). If there was more than one protein model per gene, we selected the longest protein. Phylogenetic trees were built from alignments based on full-length sequences as well as after removal of the variable N- and C-terminal extensions and removing gaps within the central conserved core helicase region. Sixteen clades could be distinguished for the plant proteins (using a minimal bootstrap value of 50 to define clades), with proteins for each of the three plant species represented in each clade (Supplemental Fig. S1; Supplemental Table S1). The relationship between protein accessions inferred from the various trees was not affected by the removal of gaps and extensions, indicating robustness of these relationships.

The cladogram showed that AtRH3 (At5g26742) has two co-orthologs in maize, ZmRH3A (GRMZM2G145491_P01) and ZmRH3B (GRMZM2G163072_P01), and one rice ortholog (Os03g61220; Fig. 1A; Supplemental Fig. S1). Furthermore, these RH3 orthologs formed a distinct clade (clade 7) with the closely related pair RH9/RH39 and the more distant RH7 (Fig. 1A; Supplemental Fig. S1). AtRH9 (AT3G22330) and AtRH39 (AT3G22330) are mitochondrial proteins (also named PMH1 and PMH2) and were shown to be part of a large complex in the mitochondrial matrix (Matthes et al., 2007).
Moreover, PMH2 was shown to be involved in group II intron splicing in mitochondria (Köhler et al., 2010). In the same clade, but more distant, was AtRH7, a demonstrated nuclear protein named PRH75 but with unknown function (Lorković et al., 1997). Furthermore, five of the seven known plastid proteins (RH22, RH39, RH47, RH50, and RH58) formed one clade (clade 8), suggesting a common ancestry and likely functions different from RH3 (Supplemental Fig. S1).

To understand the features that are unique to RH3 as compared with other plastid RH proteins, we analyzed the conservation of domains of all 56 Arabidopsis DEAD box RNA helicases and compared that with the conservation in the RH3 clade (Supplemental Table S1). Figure 1B shows the conserved motifs (greater than 60% identity) across all combined maize, rice, and Arabidopsis DEAD box RNA helicase families. The top lines show the conserved motifs (60% minimal conservation threshold) across the 56 Arabidopsis, 46 maize, and 47 rice DEAD box RNA helicases. The bottom lines show the conserved motifs (greater than 90% identity) for the RH3 clade.

**Figure 1.** Phylogenetic and protein domain analyses of the DEAD box RNA helicase family. A, The RH3-containing clade (clade 7) from the phylogenetic tree of all 149 DEAD box helicases in maize, rice, and Arabidopsis and the five *E. coli* DEAD box RNA helicases (see Supplemental Fig. S1). Bootstrap values are indicated. B, Conserved domains of the maize, rice, and Arabidopsis DEAD box RNA helicase families. The top lines show the conserved motifs (60% minimal conservation threshold) across the 56 Arabidopsis, 46 maize, and 47 rice DEAD box RNA helicases. The bottom lines show the conserved motifs (greater than 90% identity) for the RH3 clade.

RH3 Accumulates in Stroma and Nucleoids of Green Tissues, with Peak Accumulation during Chloroplast Biogenesis

To examine the temporal and organ expression patterns of AtRH3, we used an antibody raised to a conserved region of ZmRH3 (Fig. 2A and Supplemental Fig. S2). AtRH3 was found to be predominantly expressed in young leaves, peaking in 2-week-old seedlings and decreasing to very low levels in older leaf tissue (Fig. 2B). In addition, the AtRH3 protein was also detected in...
flowers and siliques, but not in stems or roots, of 6-week-old Arabidopsis plants. Mass spectrometry-based analysis of isolated nucleoids and chloroplasts showed a very strong nucleoid enrichment of ZmRH3 (Majeran et al., 2012) as well as AtRH3 (G. Friso, K. Nishimura, X. Qu, and K.J. van Wijk, unpublished data). Both ZmRH3 homologs were detected by mass spectrometry along the development all leaf gradient to expand maize leaves, with expression peaking in the sink-source transition zone (Fig. 2C), together with many other proteins involved in plastid gene expression and protein homeostasis (Majeran et al., 2010), including the nucleoid proteins. In initial accumulation profiles of chloroplast, ribosomal subunits followed ZmRH3 and nucleoids, but accumulation levels of ribosomes remained high and constant in mature tissue, unlike RH3 and (other) nucleoid proteins (Fig. 2C). This is consistent with their respective functions. Western-blot analysis showed that ZmRH3 and DNA/RNA-binding protein ZmWHY1 were present in chloroplast stroma as well as thylakoid membranes, likely reflecting nucleoids associated to the thylakoids (Fig. 2D). Indeed, western blots of nucleoids extracted from the thylakoid membranes identified both ZmRH3 and ZmWHY1 (Fig. 2D), in agreement with previous mass spectrometry analysis of nucleoids (Majeran et al., 2012). We note that the total amount of nucleoid proteins used for the western blot was lower than the other chloroplast fractions, thus underestimating the nucleoid enrichment for ZmRH3 and ZmWHY1. In contrast, the abundant CPN60α chaperone was only found in the stromal fraction (Fig. 2D).

ZmRH3 Associates with Group II Introns and Pre-50S Ribosomal Particles in Vivo

To determine the distribution of the particles with which ZmRH3 associates, maize chloroplast stroma was fractionated by Suc gradient sedimentation under conditions that dissociate 70S ribosomes into 30S and 50S ribosome particles. Immunoblots and RNA staining showed that RH3 primarily accumulated in particles sedimenting between 30S and 50S ribosomal subunits (1–2 MD), consistent with an association with partially assembled 50S ribosomal particles (Fig. 3A).

To identify chloroplast RNAs that associate with RH3 in vivo, RH3 was coimmunoprecipitated from
maize chloroplast extracts. RNAs in the co-IP pellets were identified by hybridization to a tilling microarray of the maize chloroplast genome. Figure 3B shows the degree to which each chloroplast RNA sequence was enriched in the RH3 co-IP pellets, plotted according to chromosomal position, using the well-established RNA immunoprecipitation (RIP)-chip procedure as described by Barkan (2009). RNAs from five loci, rps12, rpl2, trnA, trnI, and rrn23, were strongly enriched in the RH3 co-IP pellet in comparison with a negative control assay. These loci encode two kinds of large and highly structured RNAs: group II introns in the rpl2, trnA, trnI, and rps12 loci and the 23S rRNA (rrn23). A minor enrichment of RNA from the ycf3 locus, which includes two group II introns, was also suggested by these data.

The rrn23, trnI, and trnA genes are cotranscribed together with rrn16 in the chloroplast rrn operon. The primary transcript is processed via cleaving between coding regions, removal of group II introns in trnI and trnA, and trimming of the termini once ribosome assembly is complete (Stern et al., 2010). Suc gradient fractionation (Fig. 3A) suggested that ZmRH3 is associated primarily with assembling 50S ribosomal subunits (containing incompletely processed 23S rRNA) rather than with mature 50S subunits. To address that possibility, we used poisoned-primer extension to analyze the processing status of the 5' end of the 23S rRNA in the RH3 co-IP pellets (Fig. 3C). This showed a strong enrichment for incompletely processed 23S rRNA in the RH3 co-IP pellet compared with the supernatant (Fig. 3C). These results, in conjunction with the Suc gradient sedimentation data, suggest that RH3 associates with assembling 50S ribosomal subunits and, potentially, with mature 50S ribosomal particles as well. The coprecipitation with assembling 50S ribosomal subunits could be direct, or it could be due to tethering to as-yet-unprocessed trnI and trnA, whose introns are associated with RH3. However, the cosedimentation of RH3 with pre-50S subunits favors a direct association, as group II intron particles analyzed to date are considerably smaller (approximately 700 kD) than those observed here (Till et al., 2001; Asakura and Barkan, 2007).

AtRH3 Is Required for Embryogenesis, Chloroplast Development, and Photosynthesis

To further assess how RH3 affects the RNAs with which it associates, three T-DNA insertion lines in the single RH3 gene in Arabidopsis were obtained from

Figure 3. RH3 cosediments with pre-50S ribosomal subunits and interacts with specific plastid RNA. A, Maize stroma were sedimented through Suc gradients under the condition that dissociates 30S and 50S subunits. An equal proportion of each fraction was analyzed by probing an immunoblot with anti-RH3 antiserum (top panel). The same blot stained with Ponceau S is shown in the bottom panel and also visualizes the large subunit (RBCL) of the Rubisco holocomplex at 550 kD. RNA extracted from gradients was analyzed by RNA gel blotting and methylene blue staining (middle panel). 16S rRNA marks 30S ribosomal subunits, and 23S rRNA marks 50S ribosomal subunits. B, Identification of RNA ligands of RH3 using the co-IP assay. The RIP-chip assay revealed the enrichment of several RNAs (as indicated) in the RH3 co-IP pellets. C, RH3 associates with the precursor of 23S RNA determined by poisoned-primer extension assays. Reverse transcriptase reactions were initiated with a radiolabeled primer complementary to the mature and precursor forms of 23S RNA; a dideoxy nucleotide that terminates the reverse transcription after different distances on mature and precursor forms of 23S was included in the reactions. Pre 23S, Precursor form of 23S RNA. To, Total; sup, supernatant; ppt, pellet.
the SALK and SAIL T-DNA insertion collections (Sessions et al., 2002; Alonso et al., 2003). We verified the positions of the insertions and found that \( rh3-2 \), \( emb1138 \), and \( rh3-4 \) mutants have T-DNA insertions in intron 2, exon 3, and intron 9, respectively (Fig. 4A).

The different AtRH3 alleles conditioned distinct phenotypes. Self-pollination of heterozygous \( rh3-2 \) and \( emb1138 \) plants yielded developing siliques with green and white seeds in an approximately 3:1 ratio; these white seeds shriveled after seed maturation (Fig. 4B). We analyzed over 80 progeny of self-pollinated plants for each allele that were germinated on agar plates with Suc at low light intensity (20 \( \mu \text{mol} \text{ photons m}^{-2} \text{ s}^{-1} \)) but recovered no homozygous mutants. These results strongly suggest that null mutants for RH3 are embryo lethal (Fig. 4B), consistent with the identification of \( emb1138 \) in a large-scale screen for embryo-lethal mutants (Tzafrir et al., 2004). In contrast, an insertion in intron 9 (\( rh3-4 \)) resulted in seedlings with small chlorotic cotyledons and pale-green leaves with delayed growth (Fig. 4C). Homozygous \( rh3-4 \) mutants were able to grow on soil, set viable seeds, and be maintained as a homozygous population.

RH3 mRNA was strongly reduced in \( rh3-4 \) homozygotes (Fig. 4D), whereas RH3 protein was reduced to approximately 20% of the wild-type level (Fig. 5A). The heteroallelic progeny of a complementation cross between \( rh3-4 \) and \( emb1138 \) germinated as albino seedlings, which was an intermediate phenotype with respect to those of the parental lines (Supplemental Fig. S3A). Moreover, the AtRH3 complementary DNA (cDNA) complemented the \( rh3-4 \) phenotype (Fig. 4C). Collectively, these results confirm that a reduction in RH3 function causes a pale-green seedling phenotype, whereas complete loss leads to embryo lethality.

Immunoblot analysis of total leaf extracts was used to quantify effects on the thylakoid-localized photosynthetic machinery in \( rh3-4 \) homozygotes (Fig. 5B). Accumulation of protein subunits of PSI (psaD/subunit II and psaF/subunit III), PSII (D1, D2, and OEC23), as well as the cytochrome b_{6f}(cytb_{6f}) complex (PetD/subunit IV and PetA/cytf) was 2- to 3-fold reduced in \( rh3-4 \). In contrast, the \( \alpha \) and \( \beta \) subunits of the ATP synthase increased severalfold. For comparison, we also included an analysis of clpr2-1 mutant thylakoids. The reduction of PSI, PSII, and cytb_{6f} subunits was 2- to 3-fold stronger than in \( rh3-4 \) mutants, consistent with the stronger phenotype of clpr2-1 (Rudella et al., 2006). clpr2-1 accumulated the precursor form of the PSI subunit III (PsaF), as observed previously.
(Rudella et al., 2006), but *rh3-4* did not. Similar to *rh4-1*, ATP synthase levels increased in *clpr2-1*. This differential down-regulation was surprising to us, but verification of studies on other chloroplast biogenesis mutants showed that it is frequently observed that the thylakoid ATP synthase is less affected (or even increased), whereas the thylakoid electron transport chain complexes are down-regulated. For instance, the *apo1* mutant involved in intron II splicing also shows unchanged levels of ATPase but very strong decreases of PSI, PSII, and the cyt*b*$_6$f$_1$ complex (Watkins et al., 2011). Arabidopsis *lpa2*, with defects in PSII assembly, shows a decrease of PSII and PSI but not of ATP synthase (Ma et al., 2007). This shows that chloroplast and thylakoid biogenesis is a complex process. Chloroplast chaperone CPN60 was slightly (approximately 2-fold) up-regulated in both single mutants (Fig. 5B), similar to what we observed previously for *clpr2-1* (Rudella et al., 2006).

**AtRH3 Is Required for the Splicing of Group II Introns That Coimmunoprecipitated with ZmRH3**

The splicing of each intron whose ortholog coimmunoprecipitated with ZmRH3 (Fig. 4B) was analyzed in the Arabidopsis *rh3-4* mutant. For comparative purposes, we included Arabidopsis *cfm2-2* mutants that have reduced levels of the plastid-localized splicing factor CFM2 (AT3G01370). *cfm2-2* mutants are pale green and have chloroplast biogenesis defects resulting from defects in the splicing of several group II introns, but not in any of the RH3 intron ligands detected here (Asakura and Barkan, 2007). *rh3-4* mutants accumulated markedly reduced levels of mature *trnA* and *trnl* but accumulated increased levels of their unspliced precursors (Fig. 6A; compare exon and intron levels), indicating a reduction in *trnl* and *trnA* splicing efficiency. Poisoned-primer extension assays revealed a clear decrease in the ratio of spliced to unspliced RNA for the trans-spliced intron *rps12-int1* (Fig. 6B). The ratio of spliced to unspliced *rpl2* and *rps12-int2* was also reduced in *rh3-4* mutants, but more modestly (Fig. 6B). The fact that effects on the splicing of *trnI*, *trnA*, *rps12-int1*, *rps12-int2*, and *rpl2* were detected even in the weak *rh3* mutant allele, in conjunction with the RNA co-IP data, strongly suggests that RH3 directly promotes the splicing of these introns. The splicing of three chloroplast introns that are found in Arabidopsis but not in maize (*clpP-int1*, *clpP-int2*, and *rpoC1*) and introns found in minor peaks in the maize RIP-chip experiments in Figure 3 (*ycf3-int1* and *ycf3-int2*) was unaffected in *rh3-4* (Fig. 7). These results suggest that RH3 does not contribute to the splicing of these introns, but analysis of stronger mutant alleles would be required to firmly address this point.

RH3 coimmunoprecipitates immature and mature 23S rRNA (Fig. 3C). This could indicate that RH3 also promotes the assembly of the 50S ribosomal subunit, a defect that is expected to be reflected by a decrease in

**Figure 5.** Protein expression patterns in pale-green mutants. A, RH3, ClpR2, and cpHSP70 protein levels in *rh3-4*, *clpr2-1*, and *rh3-4/clpr2-1* mutants. Total leaf proteins (30 μg of protein or dilutions as indicated) from seedlings at leaf stage 1.07 were analyzed on immunoblots by probing with anti-RH3, anti-ClpR2, and anti-cpHSP70 antisera. The Ponceau S-stained membrane at the bottom shows sample loading and abundance. LHCP, Light-harvesting chlorophyll a/b-binding protein; RBCL, large subunit of Rubisco; WT, wild type. B, Immunoblot analysis of subunits of chloroplast protein complexes in *rh3-4* and *clpr2-1* mutants. Total leaf extracts (20 μg of protein or dilutions as indicated) from seedlings shown at the top at leaf stage 1.07 planted on one-half-strength Murashige and Skoog medium with 2% Suc were analyzed by immunoblotting probing with antibodies for the proteins named at the right. The blot stained with Ponceau S illustrates sample loading and the abundance of the large subunits of Rubisco.
23S rRNA processing (for references and discussion, see Nishimura et al., 2010; Chi et al., 2012). To further address the role of RH3 in rRNA metabolism, we analyzed the four rRNAs by RNA gel-blot hybridization (Fig. 8A). We observed that the accumulation of each rRNA in the 50S ribosomal subunit (23S, 5S, and 4.5S) was slightly reduced in \( rh3-4 \) mutants, whereas 16S rRNA of the 30S particle was not affected.

RH3 and ClpR2 Interact Genetically, But RH3 Is Unlikely To Be a Substrate for the Clp Protease

RH3 was 5- to 10-fold up-regulated in the two ClpPR protease mutants \( clpr2-1 \) and \( clpr4-1 \) (Kim et al., 2009; Zybailov et al., 2009). This could suggest that RH3 is a substrate of the Clp protease, with the increased accumulation of RH3 due to reduced proteolytic capacity in the chloroplast. Alternatively, the increase in RH3 might be an indirect compensatory or pleiotropic response to
the loss of Clp protease capacity. To explore the relationship between RH3 and ClpPR protease, a double homozygous mutant was generated between rh3-4 and clpr2-1 (Fig. 4C; Supplemental Fig. S4). The double-homozygous seedlings were albino and very slow growing; this phenotype was much stronger than the phenotype of either of its parents (Fig. 4C). However, viable seed could eventually be obtained from the homozygous rh3-4 × clpr2-1 double mutants (see “Materials and Methods”).

Immunoblot and RNA gel-blot analyses were carried out to compare levels of RH3 and ClpR2 mRNA (Fig. 4D) and protein (Fig. 5A) in rh3-4 and clpr2-1 single and double mutants. RH3 mRNA was reduced to near undetectable levels in both lines that were homozygous for rh3-4 but was increased severalfold in homozygous clpr2-1 mutants. CLPR2 mRNA levels were unaffected in rh3-4 mutants but strongly reduced in the clpr2-1 background (Fig. 4D). Consistent with our previous mass spectrometry-based analysis (Zybailov et al., 2009), RH3 protein abundance strongly increased (more than 10-fold) in clpr2-1 seedlings (Fig. 5A). Whereas RH3 protein was strongly reduced in the rh3-4 mutant, RH3 protein levels were approximately 2-fold increased in the double mutant as compared with the wild type, indicating that the reduced ClpR2 levels up-regulated RH3 even in the rh3-4 background. The ClpR2 protein level in clpr2-1 was about 20% of the wild type, as reported previously (Rudella et al., 2006), and remained at that level in the double mutant (Fig. 5A).

Consistently, ClpR2 protein levels were not significantly affected in the rh3-4 seedlings. Chloroplast cpHSP70 levels were used as an internal control and were slightly increased in the single and double mutants (Fig. 5A).

The expression of RH3 is under strong developmental control, with highest expression levels in young, developing leaf tissue (Fig. 2, B–D). To better resolve the developmental kinetics and gain insight into why RH3 protein overaccumulated in the clpr2-1 mutant, we determined RH3 mRNA and protein levels in different leaf rosette developmental stages (stages 1.04–1.15). RH3 mRNA levels decreased during the development of the leaf rosette in both the wild type and clpr2-1 (Fig. 9A); in the oldest rosette, transcript levels were identical in the wild type and clpr2-1. However, RH3 mRNA levels were severalfold higher in the clpr2-1 mutant during early leaf development (stages 1.04 and 1.08), indicating delayed transcriptional down-regulation. A similar but more dramatic pattern was observed at the RH3 protein level (Fig. 9B): RH3 levels clearly decreased with progressive leaf rosette development, but RH3 levels were consistently higher in the clpr2-1 mutant (Fig. 9B).

To determine whether RH3 up-regulation is specific for ClpPR core protease mutants or is a general result of defects in chloroplast biogenesis, we determined RH3 protein levels for two other pale-green chloroplast mutants, ffc1-2 and tics40, at leaf stages 1.07 and 1.14 (Fig. 9C). ffc1-2 is deficient for chloroplast signal recognition particle subunit SrpS4, involved in the
targeting of various proteins to the thylakoid membrane (Rutschow et al., 2008). The mutant tic40 is deficient for the inner chloroplast envelope protein TIC40, involved in the import of nucleus-encoded proteins (Kovacheva et al., 2005). At leaf stage 1.07, RH3 levels increased about 2-fold in the ffc1-2 and tic40 mutants, compared with approximately 5-fold in clpr2-1. At least stage 1.14, RH3 levels in ffc1-2 and tic40 mutants increased to nearly the level as in the clpr2-1 mutant. Together with the increased RH3 mRNA levels, this indicates that the increase in RH3 levels is not specific to the ClpPR core mutants, even if up-regulation in the early stages of leaf development is stronger in clpr2-1.

If RH3 were a substrate for degradation by the ClpPR core complex, RH3 or ClpR3 fragments might be found in association with the ClpPR complex. Therefore, we carried out co-IP experiments against stroma from isolated chloroplasts of wild-type and clpr2-1 plants using our anti-ClpR2 and anti-RH3 antisera. Whereas co-IP using anti-ClpR2 and anti-RH3 successfully precipitated ClpR2 and RH3, respectively, anti-ClpR2 did not coimmunoprecipitate with RH3, nor did anti-RH3 coimmunoprecipitate with ClpR2 (Fig. 10). Furthermore, fractionation of stromal extracts from Arabidopsis wild-type and clpr2-1 mutant plants on Suc gradients identified RH3 in high-molecular-mass fractions over 550 kD but never in 350-kD ClpPR complexes (Supplemental Fig. S5). Similarly, tandem mass spectrometry analysis of tagged ClpPR core complexes, such as His6-tagged ClpPR cores (Rudella et al., 2006) or StrepII-tagged ClpPR cores (Olinares et al., 2011), did not find RH3 protein, unlike other putative substrates (data not shown).

Comparison of RNA Metabolism in rh3-4 and clpr2-1 Mutants

So far, we determined that RH3 associates with and promotes the splicing of several group II introns (rps12-int1, rpl2-int, trnA, and trnI) and also coimmunoprecipitates with rrn23 (Fig. 3B). Moreover, we showed that mature forms of rrn4.5 and rrn5 as well as the 0.5-kb fragment of 23S rRNA accumulated at lower levels in the rh3-4 mutant (Fig. 8), suggesting a reduced accumulation of the assembled 50S ribosome particles. In contrast to rh3-4, the ratio of spliced to unspliced RNAs from the RH3-dependent introns is nearly normal in clpr2-1 mutants (Figs. 6 and 7). The metabolism of rRNAs also differed between the two mutants: various precursors overaccumulated in the clpr2-1 background, but the fully processed rRNAs accumulated to wild-type levels. Thus, the abundance of assembled 50S ribosomes was unaffected in the clpr2-1 mutant but was reduced in the rh3-4 mutant.

DISCUSSION

RH3 Is a Group II Intron-Splicing Factor in Plastids and May Also Contribute to 50S Ribosomal Subunit Assembly

In this study, we have shown that RH3 is a plastid-localized DEAD box RNA helicase in both maize and
defects in the chloroplast type II introns (Fig. 3), and (2) specific splice factors (Supplemental Fig. S6). This was based on a number of complementary measurements, namely (1) the interaction of RH3 with a subset of plastid splice factors, and (2) specific splice defects in the rh3-1 mutant (Figs. 6 and 7). Furthermore, sequence analysis of RH3 clearly shows that RH3 contains the conserved domains for ATP-dependent RHA helicase activity and also has the conserved DEAD domain. Thus, our analysis clearly indicates that RH3 belongs to the class of DEAD box RNA helicase proteins. In contrast, some of the other chloroplast helicases do not have a strictly conserved DEAD domain (but have a DEXD domain), whereas others lack some of the key residues involved in ATP binding and hydrolysis. Strikingly, the role of RH3 in intron slicing is also consistent with the phylogenetic analysis of the RH3 family, in that RH3 is part of a clade with two closely related mitochondrial RH proteins (RH9 and RH35; Matthes et al., 2007), one of which (RH35) has been demonstrated to function in intron II splicing in mitochondria (Köhler et al., 2010).

Other results suggest that RH3 may have an additional function in the assembly of 50S ribosomal subunits. First, the abundance of mature 50S ribosomal subunits is reduced in rh3 mutants, as determined by the significant decrease in the accumulation of 23S, 4S, and 5S rRNA but not the 16S RNA of the 30S particle (Fig. 8). In addition, RH3 is found in particles of a suitable size (approximately 45S; Fig. 3A), and it coimmunoprecipitates with pre-23S rRNA (Fig. 3C). Such a role would be in keeping with the fact that other DEAD box proteins promote ribosome assembly in plastids and in other systems (Nishimura et al., 2010; Linder and Jankowsky, 2011; Chi et al., 2012). However, we cannot rule out the possibility that the decrease in ribosome abundance in rh3 mutants is a secondary effect of the splicing defects that impacts several components of the translation machinery (trnI, trnA, and rps12). The only viable rh3 allele was quite weak, and it is possible that a ribosome assembly defect would be revealed more clearly with a stronger (but not total) loss of function. The fact that RH3 is essential for embryogenesis and viability, based on the phenotypes of the two null RH3 alleles, is consistent with the need for functional plastid ribosomes and translation during embryogenesis in Arabidopsis (Stern et al., 2010).

RH3 Increase in Clp Protease Mutants Constitutes a Compensatory Effect and Must Involve Retrograde Signaling from the Plastid to the Nucleus

Both RH3 mRNA transcripts and protein levels of RH3 were severalfold increased in the clpr2-1 background. The strong increase in RH3 is not specific for clpr2-1 but applies also to pale-green mutants in plastid ClpPR genes, such as clpr4-1 (Kim et al., 2009) and clpp3 (J. Kim, P.D. Olinares, and K.J. van Wijk, unpublished data). Because these ClpPRs are subunits of the same Clp core protease complex, this shows that RH3 levels increase in response to the loss of ClpPR protease capacity. However, we did not find evidence that RH3 increases are directly due to the loss of degradation capacity; rather, our data suggest that the simultaneous increase in RH3 mRNA and protein constitutes a compensatory effect to the Clp mutant phenotype. The increase in RH3 mRNA level suggests a transcriptional response due to the loss of Clp core activity and must involve retrograde signaling from plastid to nucleus; the nature of this signal is still unclear (Sun et al., 2011), but many studies have indicated that inhibition of chloroplast homeostasis does contribute to the signal (Chan et al., 2010; Šimková et al., 2012). Indeed, multiple proteins involved in regulating chloroplast biogenesis and homeostasis, such as nucleoid-associated proteins (e.g. pTAC1/Why1), translation factors (e.g. EF-Tu-1, EF-Tu-G/Sco1, and EF-Tu-TypeA/BipA), as well as the major chaperones (CPN60, HSP70, HSP90, and ClpB3), were up-regulated in the Clp mutants (Kim et al., 2009; Zybaliov et al., 2009), indicative of significant protein stress.

The Difference between RH3 and Other Plastid-Localized DEAD Box Helicases

Out of the seven identified plastid-localized DEAD box RNA helicases (RH3, -22, -26, -39, -47, -50, and -58), only the functions of RH39 and RH22 were previously studied in detail in Arabidopsis (Nishimura et al., 2010; Chi et al., 2012). Our phylogenetic analysis showed that five of these plastid helicases (RH22, -39, -47, -50, and -58) form a separate clade, distinct from the clade with RH3 and its mitochondrial and nuclear homologs, and that RH26 is in a clade with extraplastidic proteins with unknown functions. The five plastid RH proteins in clad 8 lack several conserved motifs likely reducing RNA helicase activity, whereas plastid RH3 and RH26 show full conservation of the
DEAD box RNA helicase motifs. Recently, we provided indirect evidence that RNA metabolism and ribosome assembly occur in association with nucleoids (Majeran et al., 2012). The identified plastid RH helicases were found in high-mass complexes (greater than 1 MD) in Arabidopsis stroma (Oliinares et al., 2010) or present in maize nucleoids (Majeran et al., 2012), consistent with a role in RNA metabolism and ribosome assembly. Future experiments should focus on the identification of functions of plastid-localized RH26, -47, -50, and -58.

MATERIALS AND METHODS

Phylogenetic and Functional Domain Analysis

Selected proteins were aligned using MUSCLE (http://toolkit.tuebingen.mpg.de/muscle) using 15 to 25 iterations, and the aligned sequences in Clustal format were viewed and edited (removal of gaps and variable extensions) in Jalview. Sequences were then converted into PHYLIP format using http://seqalignment.ucm.tmc.edu/seq-util/readseq.html. Phylogenetic trees were generated (1,000 iterations) using the CIPRES Web portal (http://www.phylo.org/sub_sections/portal/) using the RAxML HPC Blackbox, with the general time reversal model selected as the protein substitution matrix. The resulting phylogenetic trees were annotated in FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Plant Materials and Growth Conditions

Maize (Zea mays inbred line B73) seedlings for stroma and cDNA preparations were grown in soil in a growth chamber under a 16-h-light (28°C, 400 μmol photons m⁻² s⁻¹) and 8- and dark (26°C) cycle. They were harvested between 7 and 10 d after planting and used for preparation. Maize seedlings for chloroplast and nucleoid preparations were grown in a growth chamber under a 12-h-light (31°C, 400 μmol photons m⁻² s⁻¹) and 12- and dark (22°C) cycle. They were harvested 7.5 d after planting for the nucleoid isolation or 8.5 d for chloroplast isolation.

Arabidopsis (Arabidopsis thaliana) T-DNA insertion lines SALK_025572 (rh3-2), SALK_009202 (rh3-4), and CS16011 (emb1138) were obtained from the Salk Institute Genomic Analysis Laboratory. eml1138, SALK_046378 (clpr-2), SAIL_6E7_05 (Atcfm2-2), and fj1-1 mutants were described previously (Amin et al., 1999; Tazvir et al., 2004; Asakura and Barkan, 2007; Rutschow et al., 2008). The fj1-40 mutant (SAIL_92_C10) was described previously (Kovacheva et al., 2005). Arabidopsis plants used for protein, DNA, and RNA extraction were grown on one-half-strength Murashige and Skoog plates supplemented with 2% Suc under a 10-h-light (40 μmol photons m⁻² s⁻¹, 23°C) and 14-h-dark (21°C) cycle unless otherwise indicated. Arabidopsis seedlings for stroma, protein, and RNA isolation for different stages and organs (wild type and clpr-2-1) were grown on soil under a 10-h-light (100 μmol photons m⁻² s⁻¹, 23°C) and 14- and dark (21°C) cycle and were harvested after 4 weeks (wild type) or 5 to 6 weeks (clpr-2-1) after planting. Seeds from the double homozygous clpr-2-1 × rh3-4 mutant was obtained by growing seedlings first for 2 months on agar plates supplemented with 2% Suc under short-day and low-light (60 μmol m⁻² s⁻¹) conditions followed by transfer to soil and growth for 6 months under long days at low light intensity (20 μmol photons m⁻² s⁻¹).

Chloroplast Isolation and Fractionation

Maize stroma for RIP-chip and Suc gradient were isolated as described previously (Voelker and Barkan, 1995). Maize chloroplasts for subfractionation and Arabidopsis stroma were isolated essentially as described (Majeran et al., 2005; Oliinares et al., 2010). RNase and DNase treatments of thylakoid membranes were performed as described previously (Prikryl et al., 2008). Chloro-}

Production of Maize Anti-RH3 and Arabidopsis Anti-ClpR2 Antisera

ZmRH3A (amino acids 531–691) and the C-terminal end of Arabidopsis ClpR2 (amino acids 189–279) were expressed in Escherichia coli as 6× His-

Tagged fusion proteins from pET28a(+) or pET21a(+) vectors (Novagen), respectively. A ZmRH3A recombinant protein was expressed in BL21(DE3) Star (Novagen) and induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h. AntiClpR2 recombinant protein was expressed in Rosetta (DE3) cells (Novagen) and introduced by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. Both proteins were recovered in a soluble fraction, purified with nickel-nitrilotriacetic acid agarose beads (Qia-}

gen) according to the manufacturer’s protocol, and equilibrated with phos-}

phate-buffered saline. Polyclonal antisera from 10 mg of antigens were generated in rabbits at the University of Oregon antibody facility for anti-}

ZmRH3A or at Alfa Diagnostic International for anti-ArClpR2. Antisera were affinity purified against the same antigen that had been used for the im-

munizations coupled to a HiTrap N-hydroxysuccinimide (NHS) ester-activated column (GE Healthcare Life Science) or cyagen bromide-activated Sephar-

ose (Sigma).

Protein Extraction and Immunoblot Analysis

Arabidopsis seedlings from six to 10 plants at leaf stage 1.07, unless oth-

erwise mentioned, were ground with a mortar and pestle in liquid nitrogen by adding an extraction buffer (2% SDS, 50 mM Tris-HCl, pH 8.8, and 5 mM EDTA) with protease inhibitors as described above and vortexed for 30 s to solubilize proteins. Insoluble materials were removed by centrifugation (0.8-mL column, 30 μm; Pierce) for 1 min at 10,000 rpm. Protein concentrations were deter-

}
Arabidopsis RNA Analysis

RNA was extracted from the Arabidopsis leaf tissues by using Trizol reagent (Invitrogen) according to the manufacturer’s instruction. RNA gel-blot hybridizations and probes for chloroplast ribosomes were as described previously (Zybailov et al., 2009). Other probes were amplified by PCR and reverse transcription-PCR from Arabidopsis genomic DNA or RNA with the primers listed in Supplemental Table S2. Poisson–primer extension assays were performed using 10 μL of leaf RNA as described previously (Asakura and Barkan, 2006).

Maize Stroma Suc Gradient to Dissociate 50S and 30S Ribosomes

A maize stroma Suc gradient was carried out as described with minor modifications (Barkan et al., 2007). A total of 100 μL of maize stroma (approximately 1 mg of protein) was diluted with 400 μL of dilution buffer (20 mM Tris-4HCl, pH 7.8, 100 mM ammonium chloride, and 5 mM 2-mercaptoethanol) to reduce the magnesium concentration to 2 mM in maize stroma. A total of 500 μL of diluted stroma was layered onto a 10% to 40% Suc gradient in dissociation buffer (20 mM Tris-HCl, pH 7.8, 1 mM MgCl2, and 100 mM ammonium chloride) and was ultracentrifuged in a Beckman SW41 rotor at 35,000 rpm for 7 h at 4°C. RNA was extracted from 300 μL of each fraction by adding 50 μL of 5% SDS and 0.2 M EDTA, followed by phenol/chloroform extraction and ethanol precipitation. An equal proportion of each fraction was used for immunoblot and RNA gel-blot analyses.

Arabidopsis Stroma Suc Gradient

A total of 500 μL of Arabidopsis stroma (1 mg of protein) from the wild type and clpR2-1 with 120 units of RNasIn (Promega) was fractionated by a 10% to 40% Suc gradient containing KEX buffer [30 mM HEPES-KOH, pH 8.0, 100 mM KOAc, 1 mM Mg(OAC)2, and 5 mM dithiothreitol] and was ultracentrifuged in a Beckman SW41 rotor at 35,000 rpm for 3 h at 4°C. An equal proportion of each fraction was used for immunoblot analysis.

Supplemental Data

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

Supplemental Figure S1. Phylogenetic tree of the RH3 DEAD box helicase family in Arabidopsis, maize, rice, and E. coli based on the core domains with major gaps removed.

Supplemental Figure S2. Multiple sequence alignment of ZmRH3A, ZmRH3B, and ARH3.

Supplemental Figure S3. An albino seedling phenotype of heteroalleleic emb1138/h3-4.

Supplemental Figure S4. PCR genotype of the double knockdown mutant nh3-4/clpR2-1.

Supplemental Figure S5. RH3 in the wild type and the clpR2-1 mutant is in the same fraction in Suc gradients.

Supplemental Figure S6. RH3 functions and chloroplast splicing factors and their intron target in land plants (updated from Barkan, 2011).

Supplemental Table S1. Protein accession numbers, nomenclature, annotation, and clade assignments for the RH3 DEAD box helicase family in Arabidopsis, maize, and rice used for phylogenetic analysis.

Supplemental Table S2. Primer sequences used for the verification of Arabidopsis T-DNA insertions and RNA analysis.

Supplemental Text S1. All 154 DEAD box RNA helicase sequences (all with DEAD or DEaD motifs) used for the phylogenetic analysis.

ACKNOWLEDGMENTS

We thank Masato Nakai for providing the hic40 mutant and antibodies to CPN60a and cHP570 and Hendrik Scheller for antibody to Psaf. We also thank Rena Shinmizu, Paul Dominic B. Olinares, Susan Belcher, Rosalind E. Williams-Carrier, Arthea Vichas, and Giulia Friso for help.

Received March 20, 2012; accepted May 8, 2012; published May 10, 2012.

LITERATURE CITED


Lorkovič ZJ, Herrmann RG, Oelmüller R (1997) PRH75, a new nucleus-localized member of the DEAD-box protein family from higher plants. Mol Cell Biol 17: 2237–2245


Till B, Schmitz-Linneweber C, Williams-Carrier R, Barkan A (2001) CRS51 is a novel group II intron splicing factor that was derived from a domain of ancient origin. RNA 7: 1227–1238


