The Conserved Endoribonuclease YbeY Is Required for Chloroplast Ribosomal RNA Processing in Arabidopsis[1]

Jinwen Liu[2], Wenbin Zhou[2,3]*, Guifeng Liu, Chuanping Yang, Yi Sun, Wenjuan Wu, Shenquan Cao, Chong Wang, Guanghui Hai, Zhifeng Wang, Ralph Bock, Jirong Huang, and Yuxiang Cheng*

State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, Harbin 150040, China (J.L., G.L., C.Y., S.C., C.W., G.H., Z.W., Y.C.); College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing 163319, China (J.L.); National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Science, Chinese Academy of Sciences, Shanghai 200032, China (W.Z., Y.S., W.W., J.H.); and Max-Planck-Institut für Molekulare Pflanzenphysiologie, D–14476 Potsdam-Golm, Germany (R.B.)

ORCID ID: 0000-0002-8530-6687 (Y.C.).

Maturation of chloroplast ribosomal RNAs (rRNAs) comprises several endoribonucleolytic and exoribonucleolytic processing steps. However, little is known about the specific enzymes involved and the cleavage steps they catalyze. Here, we report the functional characterization of the single Arabidopsis (*Arabidopsis thaliana*) gene encoding a putative YbeY endoribonuclease. *AtYbeY* null mutants are seedling lethal, indicating that AtYbeY function is essential for plant growth. Knockdown plants display slow growth and show pale-green leaves. Physiological and ultrastructural analyses of *atybeY* mutants revealed impaired photosynthesis and defective chloroplast development. Fluorescent microcopy analysis showed that, when fused with the green fluorescence protein, AtYbeY is localized in chloroplasts. Immunoblot and RNA gel-blot assays revealed that the levels of chloroplast-encoded subunits of photosynthetic complexes are reduced in *atybeY* mutants, but the corresponding transcripts accumulate normally. In addition, *atybeY* mutants display defective maturation of both the 5′ and 3′ ends of 16S, 23S, and 4.5S rRNAs as well as decreased accumulation of mature transcripts from the transfer RNA genes contained in the chloroplast rRNA operon. Consequently, mutant plants show a severe deficiency in ribosome biogenesis, which, in turn, results in impaired plastid translational activity. Furthermore, biochemical assays show that recombinant AtYbeY is able to cleave chloroplast rRNAs as well as messenger RNAs and transfer RNAs in vitro. Taken together, our findings indicate that AtYbeY is a chloroplast-localized endoribonuclease that is required for chloroplast rRNA processing and thus for normal growth and development.

Plastids (chloroplasts) evolved from cyanobacterial ancestors by endosymbiosis. They have their own genome and gene expression system, including bacterial-type transcription and translation machineries (Harris et al., 1994; Dyall et al., 2004). Chloroplast ribosomes (chlororibosomes) are composed of two subunits, designated 30S and 50S subunits. Proteomic and biochemical analysis revealed that the 30S subunit (small ribosomal subunit) contains the 16S ribosomal RNA (rRNA) and about 20 ribosomal protein subunits (Yamaguchi et al., 2000), whereas the 50S subunit (large ribosomal subunit) contains three rRNA species (23S rRNA, 5S rRNA, and 4.5S rRNA) and approximately 30 ribosomal proteins (Yamaguchi and Subramanian, 2000). Chlororibosomes are closely related to and share many common features with bacterial ribosomes due to their common origin (Harris et al., 1994). In addition, it has been demonstrated that chlororibosomes contain a small number of unique proteins that are not found in the *Escherichia coli* ribosome (Yamaguchi and Subramanian, 2000; Yamaguchi et al., 2000; Manuell et al., 2007; Sharma et al., 2007; Tiller et al., 2012).

Although structure and composition of chlororibosomes have been analyzed in great detail, little is known about the biogenesis of chlororibosomes (i.e. ribosome maturation and subunit assembly). Generally, ribosomal biogenesis is initiated with the transcription of a large pre-rRNA precursor, which is subsequently processed, folded, and assembled with ribosomal proteins.

[1] This work was supported by the Program for New Century Excellent Talents in University (grant no. NCET–12–0807), the Natural Scientific Foundation of China (grant no. 31070221), the Program for Changjiang Scholars and Innovative Research Team in University (grant no. IRT13053), and Fundamental Research Funds for the Central Universities (grant no. DL12DA02).

[2] These authors contributed equally to the article.

[3] Present address: Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, D–14476 Potsdam-Golm, Germany.

* Address correspondence to wbzhou@mpimp-golm.mpg.de and chengyuxiang@nefu.edu.cn.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Yuxiang Cheng (chengyuxiang@nefu.edu.cn).

W.Z. and Y.C. conceived the project and designed the experiments. J.L., W.Z., G.L., C.Y., Y.S., W.W., S.C., C.W., G.H., and Z.W. performed the experiments. W.Z., G.L., C.Y., and Y.C. analyzed the data. J.L., W.Z., and Y.C. contributed the reagents/materials/analysis tools. W.Z., R.B., J.H., and Y.C. wrote the article.

www.plantphysiol.org/cgi/doi/10.1104/pp.114.255000

Ribosome biogenesis is a highly complex process requiring the coordinated action of a number of rRNA-processing enzymes and assembly factors (Kaczanowska and Rydén-Aulin, 2007; Shajani et al., 2011). In bacteria, at least five ribonucleases (RNases) are required for processing of the primary rRNA transcripts (Shajani et al., 2011; Stoppel and Meurer, 2012). Similar to the situation in their prokaryotic ancestors, the 16S, 23S, 4.5S, and 5S rRNA genes of chloroplasts are clustered in an operon and transcribed as polycistronic RNAs. The initial step of the processing pathway undergoes endoribonucleolytic cleavage followed by exoribonucleolytic trimming of the 5′ and 3′ ends of the rRNA precursors. To date, several processing factors involved in chloroplast rRNA processing have been described (Barkan, 1993; Bellaoui et al., 2003; Bisanz et al., 2003; Kishine et al., 2004; Schmitz-Linneweber et al., 2006; Koussevitzky et al., 2007; Watkins et al., 2007; Beick et al., 2008; Prikryl et al., 2008; Komatsu et al., 2010; Nishimura et al., 2010; Lu et al., 2011; Park et al., 2011; Bang et al., 2012; Chi et al., 2012; Germain et al., 2013; Fristedt et al., 2014). However, many of these factors have not been characterized biochemically, and in many cases, it has been difficult to distinguish between a primary function in rRNA processing and indirect effects on processing efficiency (Barkan, 1993; Stoppel and Meurer, 2012).

Arabidopsis (*Arabidopsis thaliana*) chloroplasts contain at least two 3′ to 5′ exoribonucleases, a polynucleotide phosphorylase (PNPase), and an RNase R homolog (RNR1). They are involved in the maturation of 23S, 16S, and 5S rRNAs (Walter et al., 2002; Bollenbach et al., 2005). Although PNPase is involved in mRNA and 23S

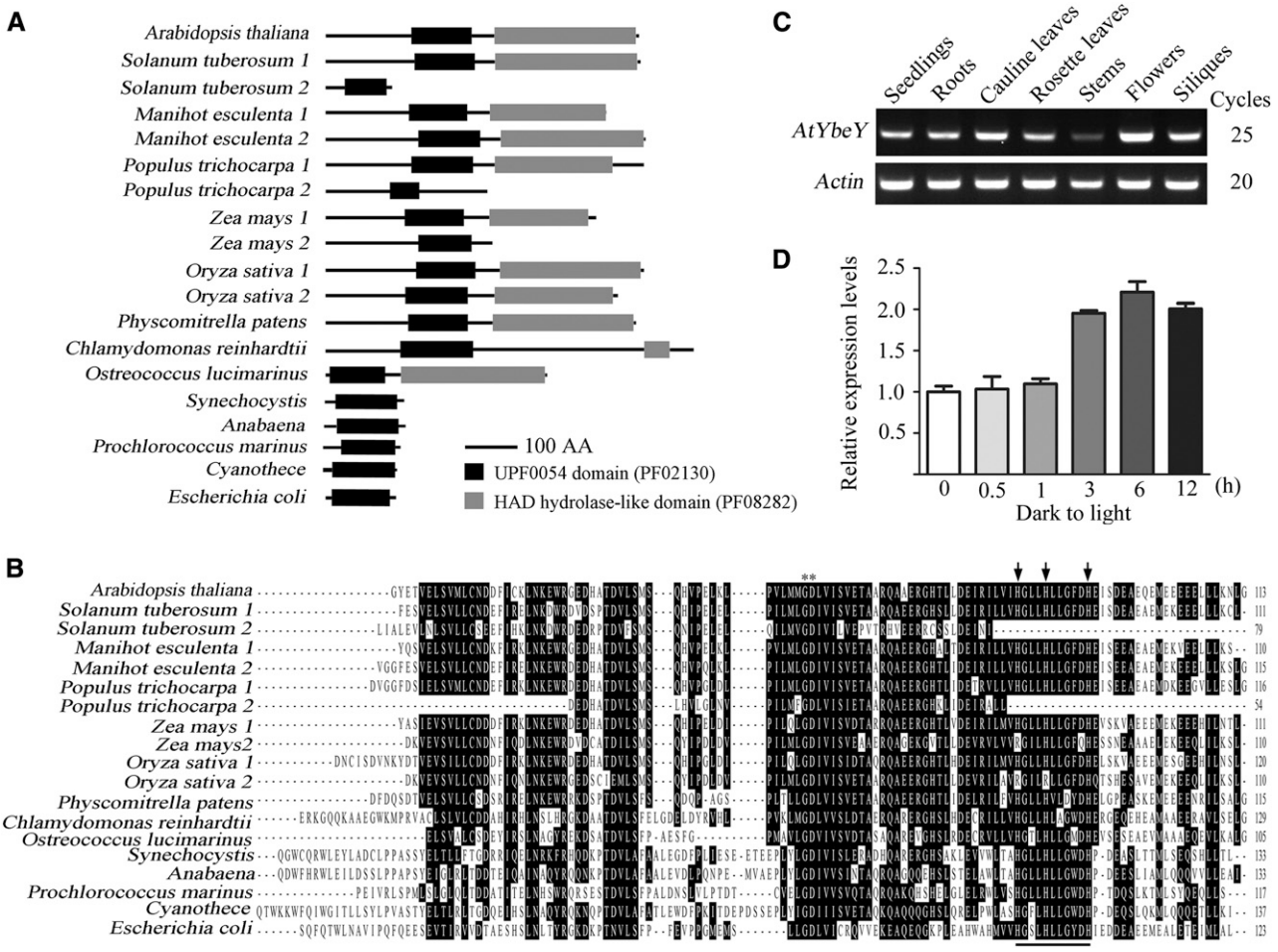


Figure 1. Characterization of plant and cyanobacterial YbeY proteins. A, Domain organization of YbeY proteins in photosynthetic eukaryotes, cyanobacteria, and *E. coli*. The UPF0054 and HAD hydrolase-like domains are shown in black and gray boxes, respectively. Plant species include Arabidopsis, *Solanum tuberosum*, *Manihot esculenta*, *Populus trichocarpa*, *Zea mays*, *Oryza sativa*, *Physcomitrella patens*, *Chlamydomonas reinhardtii*, *Ostreococcus lucimarinus*, *Synechocystis* sp. PCC6803, *Anabaena* sp. PCC7120, *Prochlorococcus marinus* MIT9313, and *Cyanothece* sp. ATCC51142. AA, Amino acid. B, Alignment of the UPF0054 domains of YbeY proteins. The asterisks show identical amino acid residues that are conserved in all species. The conserved H3xH5xH motif is marked by black arrows and underlined. C, Semiquantitative RT-PCR analysis of *AtYbeY* (At2g25870) expression in Arabidopsis. The mRNA of *ACTIN2* is shown as an internal control. D, qRT-PCR analysis of *AtYbeY* transcript levels at indicated time points after etiolated seedlings were exposed to light. Error bars indicate the SD ($n = 3$).

rRNA 3′ processing and RNA degradation, the enzyme is not essential for rRNA maturation and chloroplast translation (Yehudai-Resheff et al., 2001; Germain et al., 2011). RNR1, which is dually targeted to plastids and mitochondria, has been suggested to play a more important role in processing of the 3′ ends of 23S, 16S, and 5S rRNAs (Bollenbach et al., 2005). Moreover, the chloroplast endoribonuclease RNase E and its interacting partner RHON function in RNA degradation and probably also in rRNA processing (Walter et al., 2010; Stoppel et al., 2012). A number of other RNA-processing factors may also be involved in rRNA maturation. For example, the plant-specific endoribonucleases Chloroplast Stem-Loop Binding Protein 41a (CSP41a) and CSP41b have been reported to interact with each other and to be associated with ribosomes (Beligni and Mayfield, 2008; Bollenbach et al., 2009). Chloroplast RNase J, which displays both endoribonucleolytic and exoribonucleolytic activities, plays a major role in RNA surveillance and 5′ mRNA maturation (Sharwood et al., 2011; Luro et al., 2013). In addition, RNase J deficiency results in accumulation of 16S and 23S rRNAs that carry 5′ extensions (Sharwood et al., 2011). However, it was suggested that the impact of CSP41 and RNase J on rRNA maturation is probably due to secondary effects (Qi et al., 2012). Overall, the precise functions of the endoribonuclease required for maturation of chloroplast rRNAs are still unclear.

YbeY is a strand-specific metalloendoribonuclease that belongs to the uncharacterized protein family UPF0054 (accession no. PF02310) and exists in nearly all sequenced bacteria (Zhan et al., 2005; Davies and Walker, 2008; Davies et al., 2010; Jacob et al., 2013). YbeY was first identified in *E. coli* as a heat shock protein with unknown molecular function that is involved in translation (Rasouly et al., 2009). Later, YbeY was shown to be involved in rRNA maturation and ribosome biogenesis (Davies et al., 2010; Rasouly et al., 2010). Deletion of YbeY leads to defects in the processing of 16S, 23S, and 5S rRNAs, with a particularly strong effect on the maturation of both the 5′ and 3′ ends of 16S rRNA as well as the maturation of the 5′ ends of 23S and 5S rRNAs. Moreover, YbeY has genetic interactions with RNase III, RNase R, and PNPase. The YbeY ortholog SMc01113 in *Sinorhizobium meliloti* regulates the accumulation of small RNAs and their target mRNAs (Pandey et al., 2011). Although YbeY is not essential for *E. coli* under normal growth conditions, its function appears to be essential under physiologically diverse stress conditions, especially under high temperature stress (Davies and Walker, 2008; Rasouly et al., 2009; Davies et al., 2010). Recently, YbeY has been reported to control bacterial ribosome quality by degrading defective 70S ribosomes together with RNase R (Jacob et al., 2013). Whether YbeY-like proteins in plants perform similar functions is currently unknown.

In this study, we have characterized the YbeY homolog in the model plant Arabidopsis. We show that YbeY is a chloroplast protein whose loss results in impaired chloroplast development and lethality at the seedling stage. Our findings further reveal that YbeY is an endoribonuclease that is required for chloroplast rRNA processing.

RESULTS

Identification of a Putative YbeY Homolog from Arabidopsis

To investigate the role of putative YbeY homologs in higher plants, we searched for homologs of the *E. coli* YbeY protein in 39 sequenced plant genomes, including photosynthetic eukaryotes and cyanobacteria (Supplemental Tables S1 and S2). Domain analysis revealed that all putative plant YbeYs contain a UPF0054 domain that is similar to bacterial YbeY (Fig. 1). The majority of plant YbeY proteins have an additional haloacid dehalogenase (HAD) hydrolase-like domain, while cyanobacterial YbeY proteins did not contain this domain (Fig. 1A; Supplemental Table S1). The

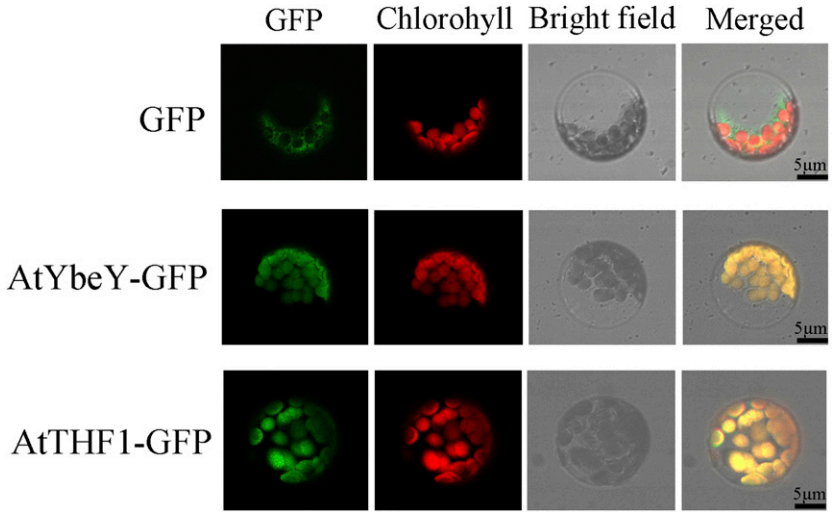


Figure 2. Subcellular localization of the AtYbeY-GFP fusion protein in protoplasts isolated from stably transformed Arabidopsis plants. The GFP fluorescence (GFP), the chlorophyll autofluorescence (Chlorophyll), the bright-field image, and the merged images are shown. Unfused GFP and chloroplast-localized AtTHF1-GFP protein were included as controls. Bars = 5 μm.

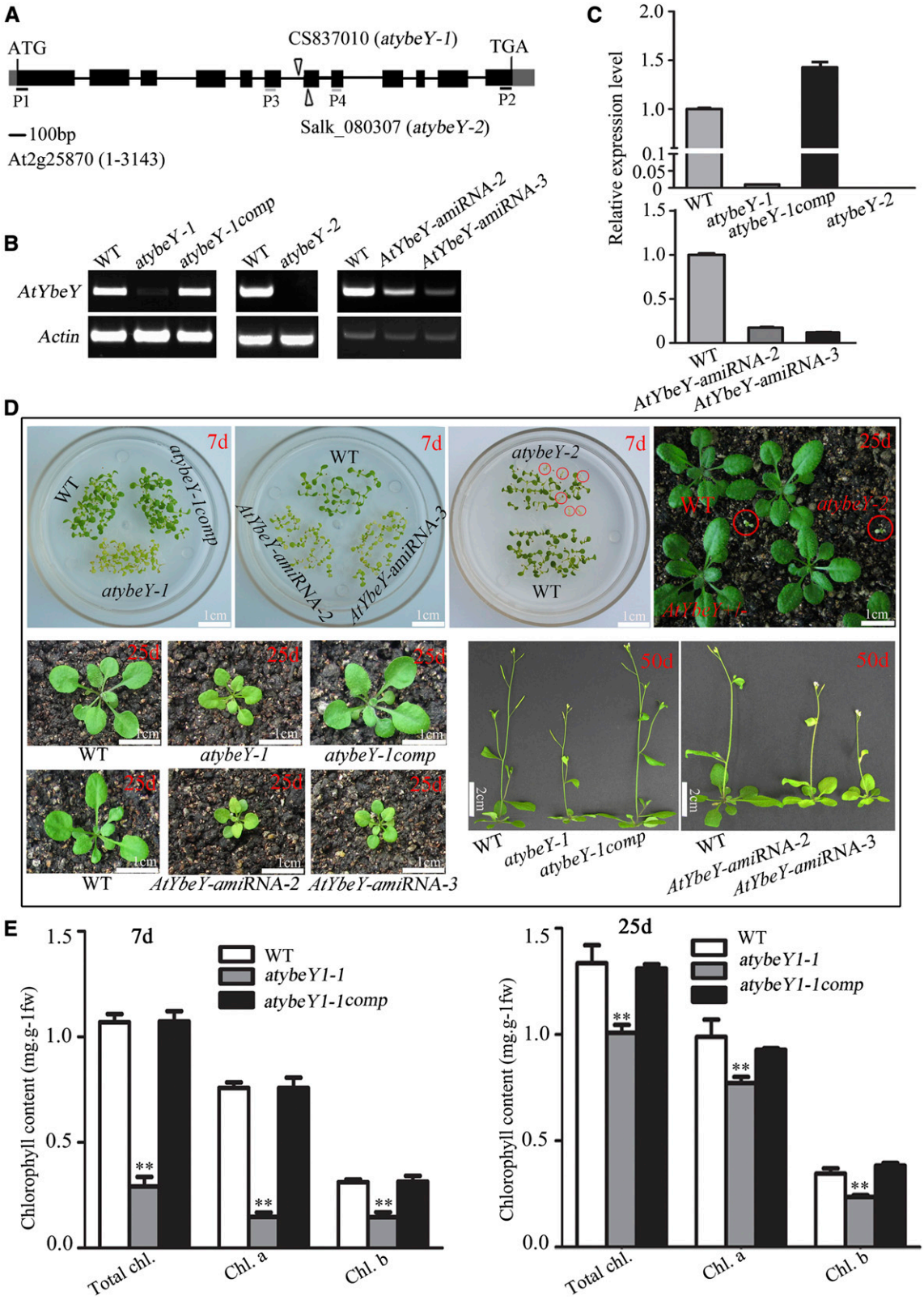


Figure 3. Identification of *atybeY* mutants and their phenotypes. A, Schematic representation of the structure of the *atybeY* gene and location of the T-DNA insertions in the *atybeY-1* and *atybeY-2* mutants (indicated by white triangles). P1 and P2 primers were used for semiquantitative RT-PCR, and P3 and P4 primers were used for qRT-PCR. B, Analysis of *AtYbeY* transcript levels in the wild type (WT), *atybeY* mutants, and *AtYbeY*-amiRNA lines by semiquantitative RT-PCR. C, qRT-PCR analysis of *AtYbeY* transcript levels in the wild

conserved H3xH5xH motif, thought to confer a metal ion-dependent hydrolytic function (Davies et al., 2010), is present in most plant YbeYs with both UPF0054 domain and HAD hydrolase-like domain (Fig. 1B). Phylogenetic analysis showed that these YbeYs can be classified into bacteria, lower plant, or algae, dicot, and monocot groups (Supplemental Fig. S1). In Arabidopsis, there is only one homolog (At2g25870) of the bacterial YbeY (Supplemental Table S1), which subsequently will be referred to as AtYbeY. AtYbeY contains both UPF0054 and HAD hydrolase-like domains (Fig. 1A). Reverse transcription (RT)-PCR analysis showed that the *AtYbeY* gene is expressed in all tissues examined but at a very low level in stems (Fig. 1C). Additionally, the level of *AtYbeY* transcripts was significantly increased when etiolated seedlings were transferred from the dark to the light (Fig. 1D). Bacterial *YbeY* was induced by heat shock (Rasouly et al., 2009). The levels of *AtYbeY* transcripts were not significantly altered upon growth at 8°C, 22°C, 30°C, or 37°C for 1 and 2 h, while high temperature (45°C) increased its transcript abundance at 5 min and decreased subsequently (Supplemental Fig. S2).

To determine the subcellular localization of the AtYbeY protein, we constructed a gene fusion of *AtYbeY* with the gene for the GFP and transformed the chimeric gene into Arabidopsis wild-type plants. Protoplasts were isolated from transgenic plants expressing AtYbeY-GFP and analyzed by fluorescence microscopy. The green fluorescence of the reporter proteins overlapped completely with the red fluorescence of the chlorophyll, strongly suggesting that AtYbeY is localized in chloroplasts (Fig. 2). Chloroplast-localized Arabidopsis Thylakoid Formation1 (AtTHF1)-GFP was used here as a positive control (Huang et al., 2006). This result is consistent with a recently published proteomic study that detected YbeY peptides in the soluble proteome of the chloroplast (Bayer et al., 2011). Taken together, our data indicate that YbeY is a conserved protein in plants and localized in plastids.

Phenotypic Characterization of *atybeY* Mutants

To study the function of *AtYbeY*, we identified two independent mutant alleles for the *AtYbeY* locus, *atybeY-1* (CS830701) and *atybeY-2* (SALK_080307), from the collection of transfer DNA (T-DNA) insertion lines of the Arabidopsis Biological Resource Center. The T-DNA insertions in *atybeY-1* and *atybeY-2* are in intron 6 and exon 7, respectively, of the *AtYbeY* gene (Fig. 3A). Quantitative reverse transcription (qRT)-PCR analysis indicated that expression of *AtYbeY* in *atybeY-1* was extremely low (approximately 2% of the wild-type level) and was undetectable in *atybeY-2* (Fig. 3, B and C). Additionally, we produced *AtYbeY* complemented lines in the *atybeY-1* background and transgenic plants in which expression of *AtYbeY* was down-regulated by an artificial microRNA (amiRNA) approach (Fig. 3, B and C). When compared with the wild type, *atybeY-1* and *AtYbeY-amiRNA* lines exhibited pale-green cotyledons and true leaves and grew slower under greenhouse conditions (Fig. 3D). Consistent with the pale-green color of the leaves, the total chlorophyll content and the levels of both chlorophyll *a* and chlorophyll *b* were significantly reduced in *atybeY-1* and *AtYbeY-amiRNA* seedlings and mature plants compared with wild-type plants (Fig. 3E; Supplemental Fig. S3). The ratio of chlorophyll *a*/*b* was decreased in *atybeY-1* and *AtYbeY-amiRNA* plants (Supplemental Fig. S4), tentatively suggesting that the (largely plastid-encoded) photosystem cores are more severely affected than the (nucleus-encoded) antennae. The mutant phenotype and the pigment deficiency of *atybeY-1* were fully rescued in the *AtYbeY* complemented lines (Fig. 3).

Consistent with *atybeY-2* likely representing a null allele (Fig. 3C), the *atybeY-2* mutant displayed a much more severe phenotype than the *atybeY-1* mutant. Homozygous *atybeY-2* plants are unable to complete their life cycle when grown under our standard greenhouse conditions, while its heterozygous plants displayed no phenotype (Fig. 3D). On the contrary, *AtYbeY* complementation lines in *atybeY-2* background had no defective phenotype and grew normally (Supplemental Fig. S5). In addition, heterozygous *atybeY-2* mutants produced approximately 25% white seeds in young siliques (Supplemental Fig. S6A). However, abnormal or aborted seeds were not observed after seed maturation (Supplemental Fig. S6B). The severe phenotype of the homozygous *atybeY-2* null mutant suggests that the chloroplast-localized AtYbeY protein is very important for plant viability under photoautotrophic growth conditions.

To obtain insights into the physiological role of AtYbeY in photosynthesis, we determined the PSII capacity of *atybeY* mutants. Chlorophyll fluorescence induction experiments showed that the maximum quantum efficiency of PSII and the electron transport rate (ETR) were significantly lower in *atybeY-1* than in the wild type (Fig. 4, A and B). Nonphotochemical quenching (reflecting light energy dissipation) was higher in *atybeY-1* than in the wild type (Fig. 4C). To confirm the defect in chloroplast development suggested by the pale-green leaves of *atybeY-1* mutant plants, cotyledons and true leaves of wild-type plants and

Figure 3. (*Continued.*)
type, *atybeY-1*, *atybeY-2*, the complemented line *atybeY-1* (*atybeY-1comp*), and two *AtYbeY-amiRNA* lines. Error bars indicate the SD ($n = 3$). The expression level in the wild type was set to 100%. The mRNA of *ACTIN2* is shown as an internal control. D, Phenotypes of *atybeY-1*, *atybeY-1comp*, *atybeY-2*, and *AtYbeY-amiRNA* plants grown under long-day conditions. Homozygous individuals are circled. E, Chlorophyll (Chl.) contents of the wild type, *atybeY-1*, and *atybeY-1comp* plants grown for 7 and 25 d under long-day conditions. Error bars indicate the SD ($n = 5$). Asterisks indicate statistically significant differences between the mutant and the wild type (**$P < 0.01$). fw, Fresh weight.

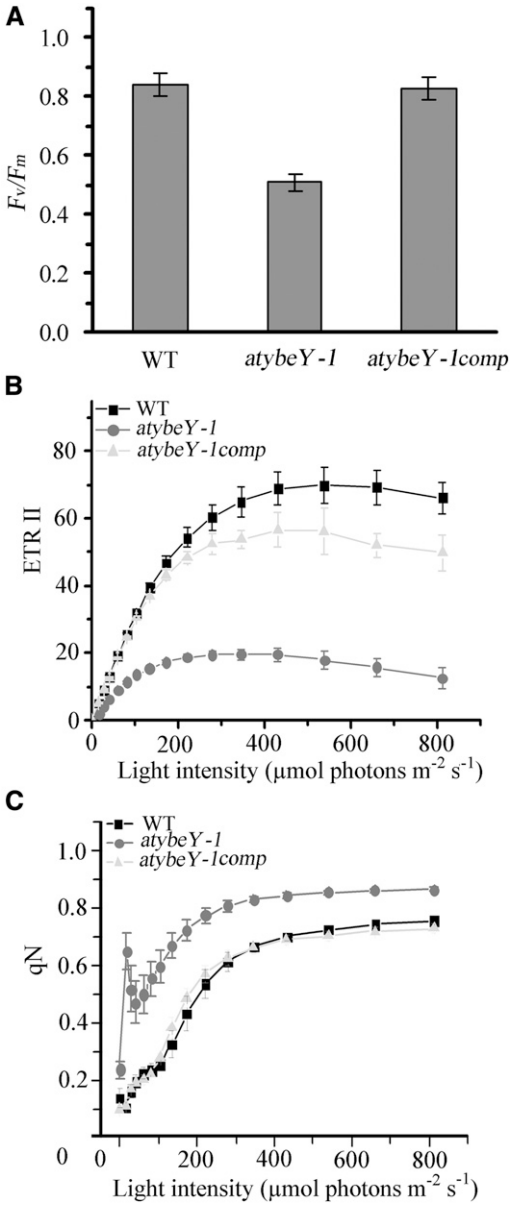


Figure 4. Photosynthetic activity in leaves of wild-type (WT), *atybeY-1*, and complemented *atybeY-1* (*atybeY-1comp*) plants. A, Maximum quantum efficiency of PSII (F_v/F_m). B, Light saturation curve of linear electron flux as calculated from the PSII yield (ETR II). C, Nonphotochemical quenching (qN). Error bars indicate the SD ($n = 4$).

atybeY-1 mutant plants were observed by light microscopy and transmission electron microscopy (TEM). Toluidine blue staining of transverse leaf sections showed that fewer chloroplasts are present in cotyledons of the *atybeY-1* mutant compared with the wild type (Fig. 5A). TEM analysis further revealed that, while chloroplasts in cotyledons and true leaves of wild-type seedlings contained a well-formed thylakoid system of stroma thylakoids and grana thylakoids, thylakoid formation was severely altered in the mutant. No stroma thylakoids and relatively more grana thylakoids were observed in chloroplasts of *atybeY-1* cotyledons, and grana thylakoids overaccumulated in true leaves of *atybeY-1* plants (Fig. 5B). As expected, the defect in thylakoid formation was fully rescued in the complemented line. Together, these results clearly indicate that AtYbeY is required for proper chloroplast development and thylakoid formation in Arabidopsis.

Accumulation of Chloroplast Genome-Encoded Proteins Is Reduced in *atybeY* Mutants

The pale-green phenotype and defective chloroplast development in *atybeY-1* prompted us to examine photosynthetic pigment-containing complexes by blue-native (BN)-PAGE. Compared with the wild type, the accumulation of PSII supercomplexes and PSI monomers/PSII dimers was strongly reduced in *atybeY-1*, while the accumulation of trimeric Light-harvesting complex II (LHCII) was moderately increased (Fig. 6A). SDS-PAGE analysis of extracted total plant protein showed that the abundance of RbcL, the plastid-encoded large subunit of Rubisco, is drastically decreased in *atybeY-1* and *AtYbeY-amiRNA* lines to less than 25% of the wild-type level (Fig. 6B; Supplemental Fig. S7). To determine the consequences of reduced *AtYbeY* expression for expression of the plastid genome, we used immunoblotting to analyze the levels of representative plastid-encoded protein components of thylakoidal protein complexes (Fig. 6C). In *atybeY-1*, the levels of D1 and D2, two reaction center proteins of PSII, were reduced to less than 25% of the wild-type levels, in line with the results of our chlorophyll fluorescence measurements (Fig. 4). Cytb6, a core subunit of the cytochrome b_6f complex, and AtpB, an essential subunit of the ATP synthase, were also significantly decreased in the *atybeY-1* mutant. Overall, the reduction in photosynthetic complex accumulation is in good agreement with the measured ETR values (Fig. 4B). In contrast to the plastid-encoded subunits of the photosystem cores, accumulation of Lhcb1, a nucleus-encoded antenna protein associated with PSII, was unaltered in the *atybeY-1* mutant. Collectively, the immunoblot data support the idea that the expression of chloroplast genes is impaired in *atybeY* mutant plants.

Chloroplast rRNA Processing Is Impaired in *atybeY* Mutants

The functions ascribed to the bacterial YbeY protein and the obtained evidence for defective plastid gene expression in *atybeY-1* mutants prompted us to investigate whether *AtYbeY* mutations have an impact on chloroplast rRNA processing. Northern-blot analysis using RNA samples extracted from 10-d-old seedlings showed that the level of mature 16S rRNA (1.5-kb band) was reduced in *atybeY-1* seedlings, while accumulation of the 16S rRNA precursor (1.7-kb band) was increased compared with the wild type (Fig. 7, A and B). Additional hybridizations using probes (a1 and a2; Fig. 7A)

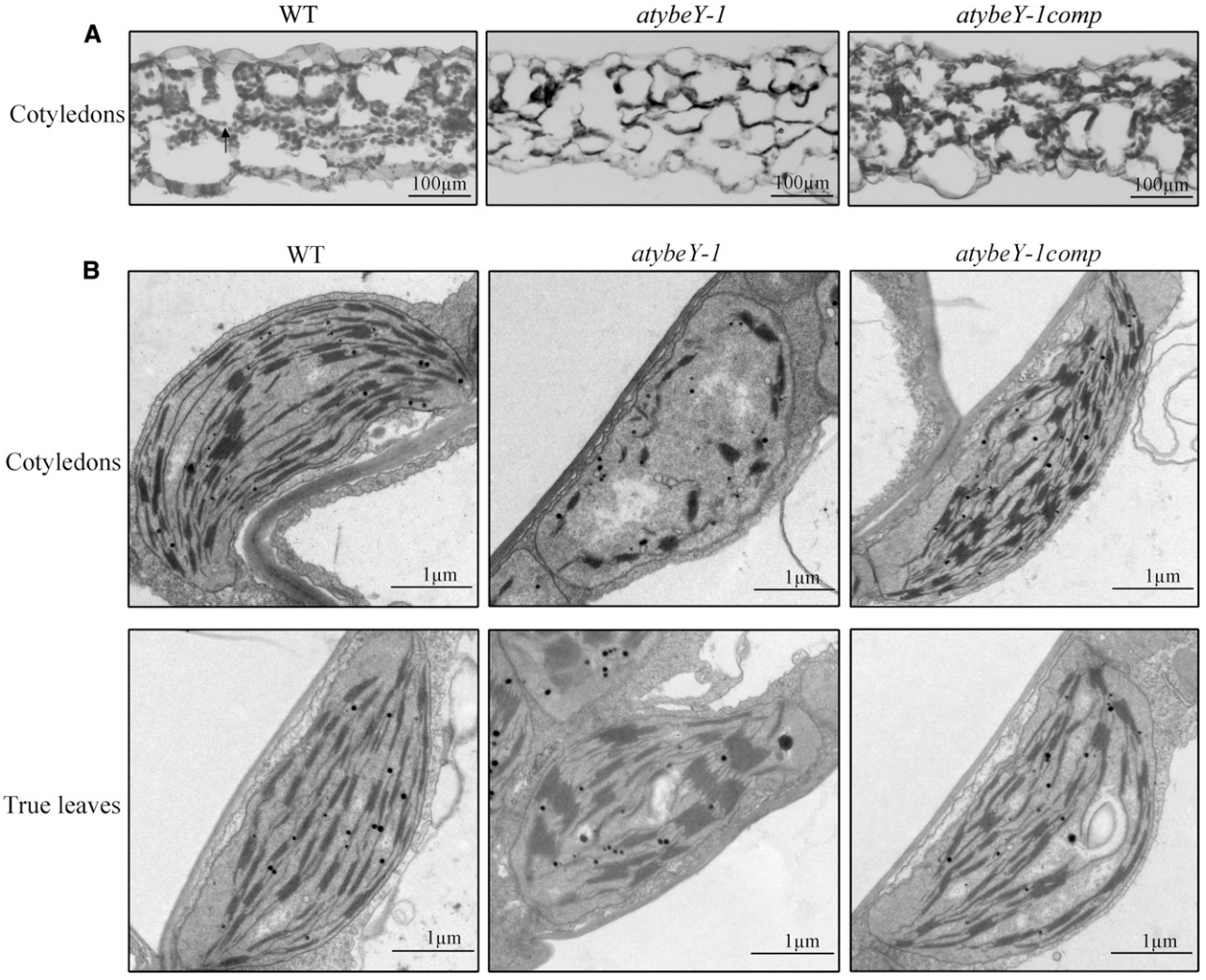


Figure 5. Loss of AtYebY expression affects chloroplast development. A, Transverse sections of cotyledons from 10-d-old wild-type (WT), *atybeY-1*, and complemented *atybeY-1* (*atybeY-1comp*) seedlings stained with toluidine blue. A chloroplast in the wild-type sample is indicated by a black arrow. B, Transmission electron microscopic images of chloroplasts from cotyledons and true leaves of 10-d-old wild-type, *atybeY-1*, and *atybeY-1comp* seedlings. Bars = 100 μm (A) and 1 μm (B).

that specifically recognize 5′ and 3′ unprocessed precursors revealed that both 5′-end and 3′-end maturation of the 16S rRNA were severely impaired in *atybeY-1* plants (Fig. 7B). Likewise, 23S rRNA precursors and processing intermediates (3.2-, 2.9-, 2.4-, and 1.7-kb bands) overaccumulated, while mature 23S transcripts were reduced in *atybeY-1* plants. The overaccumulation of the 23S-4.5S precursor (3.2-kb band) was confirmed by hybridization with specific probes (b1 and b2) that bind to 5′ and 3′ unprocessed precursor molecules (Fig. 7B). Also, abundance of the mature 4.5S rRNAs was slightly reduced in *atybeY-1* plants compared with the wild type, while pre-4.5S rRNAs overaccumulated in *atybeY-1* (Fig. 7B).

Because rRNAs are not stable if not assembled in ribosomal subunits, rRNA accumulation can serve as a proxy for ribosomal subunit accumulation. We, therefore, investigated the effect of *AtYbeY* mutations on ribosome biogenesis at the cellular level. In *atybeY-1* mutant plants, the accumulation level of 30S plastid ribosomal subunits was reduced to about 50% of the wild-type level (as evidenced by the ratio of cytosolic 18S rRNA to plastid 16S rRNA; Fig. 7C). The 23S rRNA is further processed into 0.5-, 1.2-, and 1.0-kb fragments (the hidden break of 23S rRNA [23HB] fragments; Nishimura et al., 2010). The ratio of 16S/23HB1 also decreased, whereas the ratio of 18S-23HB1 slightly increased in *atybeY-1* compared with the wild type (Fig. 7C). This result indicates that assembly of the 30S ribosomal subunit is affected more severely than that of the 50S subunit. Taken together, the ribosome accumulation data and the data from our rRNA processing analyses suggest that plastid ribosome biogenesis is specifically impaired upon AtYbeY deficiency.

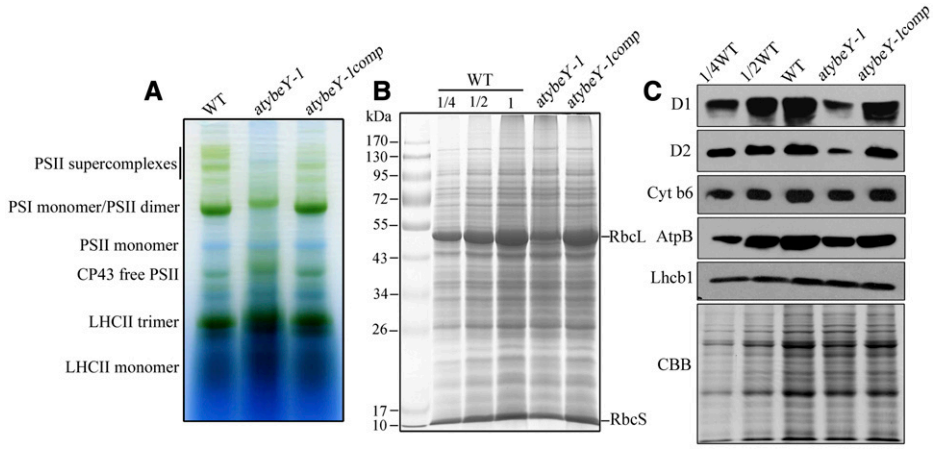


Figure 6. Analysis of photosynthetic complexes and of representative subunits. A, BN gel electrophoretic analysis of thylakoidal protein complexes from 10-d-old wild-type (WT), *atybeY-1*, and complemented *atybeY-1* (*atybeY-1comp*) seedlings. CP43, Subunit of the PSII reaction center. B, SDS-PAGE analysis of total proteins in wild-type, *atybeY-1*, and *atbybeY-1comp* seedlings. RbcL and RbcS indicate large and small subunits of Rubisco. C, Immunoblot analysis of diagnostic subunits of thylakoidal protein complexes. Isolated thylakoid samples were separated by SDS-PAGE and immunodecorated with specific antibodies against D1, D2, Cytb6, AtpB, and Lhcb1. For semiquantitative assessment, a dilution series of total protein from the wild type was loaded. A replicate Coomassie Brilliant Blue (CBB)-stained gel is shown to confirm equal loading.

The chloroplast rRNA operon also contains three tRNA genes: *trnI*, *trnA*, and *trnR*. We examined whether the processing of these tRNA transcripts was affected in the *atybeY-1* mutant. Compared with the wild type, the levels of mature *trnI*, *trnA*, and *trnR* transcripts were moderately reduced in *atybeY-1* plants, while the polycistronic rRNA operon precursor (7.4-kb band) overaccumulated (Fig. 7D). The *trnI* and *trnA* precursors were further analyzed using specific probes binding to their introns. No significant difference in the accumulation levels of the *trnI* and *trnA* precursors was detected between the wild type and the *atybeY-1* mutant, suggesting that splicing of these two tRNAs is not affected by the AtYbeY deficiency.

To further assess the role of AtYbeY in rRNA processing, 5′ and 3′ ends were mapped by circular reverse transcription (cRT)-PCR. As shown in Figure 8, among 24 sequenced 16S rRNA molecules from the *atybeY-1* mutant, 11 molecules were incorrectly processed at their 5′ end and 14 at their 3′ end. The smallest 23HB fragment was consistently found to be a 514-nucleotide RNA in wild-type plants. By contrast, five molecules with a 5′ extension of 69 nucleotides and 13 molecules with different 3′ extensions were detected in the *atybeY-1* mutant. Likewise, all 30 clones sequenced for the second-largest 23HB species represented the mature 1.0-kb fragment in wild-type plants, whereas 24 different 5′ extensions and 20 different 3′ extensions were observed in the *atybeY-1* mutant. Additionally, AtYbeY deficiency led to accumulation of abnormal 4.5S rRNA molecules carrying extensions at their 5′ and/or 3′ ends (Fig. 8). Thus, our findings indicate that AtYbeY is involved in processing of both the 5′ and the 3′ ends of chloroplast rRNAs.

mRNA Levels of Most Chloroplast Genes Are Not Altered in *atybeY* Mutants

To test whether AtYbeY has additional roles in the processing of other chloroplast RNAs, we examined the transcript levels of various chloroplast-encoded and nucleus-encoded genes using RNA gel-blot assay. No obvious differences between *atybeY-1* and wild-type plants were observed in transcript levels of plastid-encoded RNA polymerase-dependent genes (*rbcL*, *psaA*, *psbA*, *psbB*, *psbC*, or *atpB*), nucleus-encoded RNA polymerase-dependent genes (*accD*), and the examined nuclear genes for chloroplast proteins (*Lhcb1* and *rbcS*; Fig. 9A). Because protein accumulation of the plastid-encoded genes is reduced in the *atybeY-1* mutant (Fig. 6), these data provide further evidence for AtYbeY being required for chloroplast translation. Interestingly, overaccumulation of the plastid-encoded *petB* and *clpP1* transcripts was observed in the *atybeY-1* mutant (Fig. 9A), suggesting a possible relationship between AtYbeY and mRNA metabolism, as proposed previously (Caroca et al., 2013). RNA gel-blot analysis of a subset of plastid tRNA genes (*trnD*, *trnE*, and *trnQ*) revealed overaccumulation in the *atybeY-1* mutant (Fig. 9B). This could be a secondary consequence of the reduced usage of the tRNAs in translation, which may make them more stable. However, because the bacterial YbeY protein is an endoribonuclease (Jacob et al., 2013), it is also possible that AtYbeY has an additional role in mRNA and tRNA metabolism in chloroplasts.

Polysome Association of Chloroplast RNAs in *atybeY* Mutants

To further confirm that protein biosynthesis in chloroplasts is impaired in *atybeY* mutants, we analyzed the

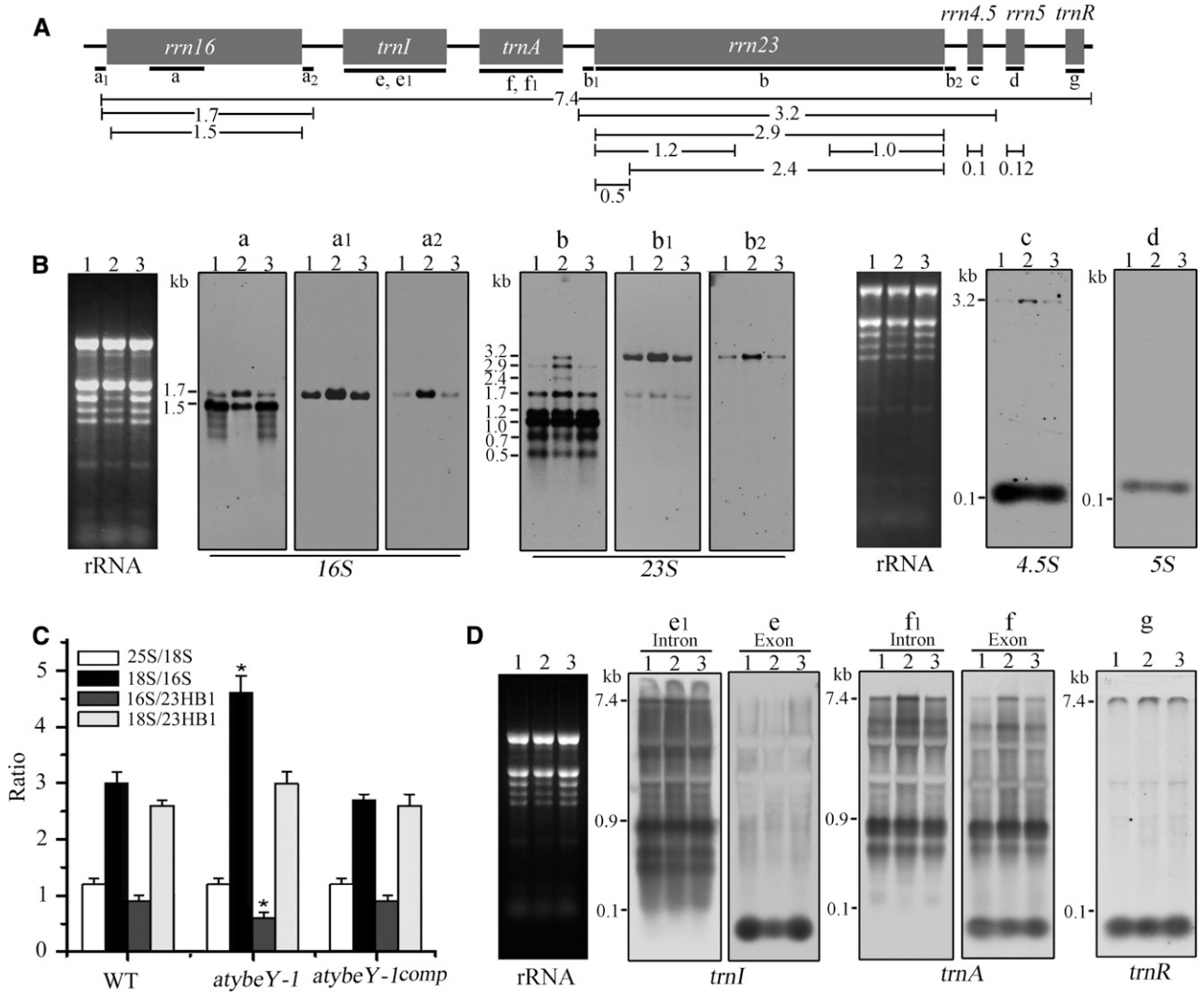


Figure 7. Expression and processing of chloroplast rRNAs in *AtYbeY* mutant plants. A, Structure of the chloroplast rRNA operon and location of probes used for RNA gel-blot analyses. Transcript sizes are indicated in kilobase pairs below the map. *rrn16*, rRNA 16S gene. B, Analysis of processing patterns of the four rRNA species encoded in the chloroplast rRNA operon. C, Accumulation of rRNAs as a proxy for the corresponding ribosomal subunits in wild-type (WT), *atybeY-1*, and complemented *atybeY-1* (*atybeY-1comp*) plants. The 25S and 18S rRNAs are components of the 60S subunit and the 40S subunit, respectively, of the cytosolic ribosome. 16S and 23HB1 (largest hidden-break product of the mature 23S rRNA) are components of the 30S subunit and the 50S subunit, respectively, of the chloroplast ribosome. Error bars indicate the SD ($n = 3$). D, Analysis of processing patterns of three tRNAs in the chloroplast rRNA operon. Total RNAs from 10-d-old wild-type (lane 1), *atybeY-1* (lane 2), and *atybeY-1comp* (lane 3) seedlings were separated in formaldehyde-containing agarose gels, and blots were hybridized to the probes indicated above. An ethidium bromide-stained rRNA gel is shown as a loading control.

association of chloroplast RNAs with ribosomes. To this end, polysomes (mRNAs loaded with translating ribosomes) were purified by Suc density gradient centrifugation. Northern-blot analyses of fractionated polysome gradients revealed that the 16S rRNA profile of the *atybeY-1* mutant was similar to that of the wild type. However, the mature 16S rRNA was underrepresented in the polysomal fractions (fractions 7–12) of the *atybeY-1* mutant (Fig. 10). Similarly, the mature hidden-break products of the 23S rRNA were reduced in polysomal fractions of the mutant, whereas the 23S-4.5S precursor overaccumulated in polysomes of *atybeY-1* (Fig. 10). We also examined the polysome association of six plastid mRNAs (Fig. 10). Compared with the wild type, the mRNAs of *petB*, *psaA*, *atpB*, and *rbcL* were underrepresented in polysomal fractions from the *atybeY-1* mutant (in that their distribution was shifted to lighter fractions of the gradient), indicating that these mRNAs are associated with fewer ribosomes and, hence, their translation is reduced. Taken together with defective processing of rRNAs in *atybeY-1*, these results indicate that AtYbeY deficiency leads to impaired plastid

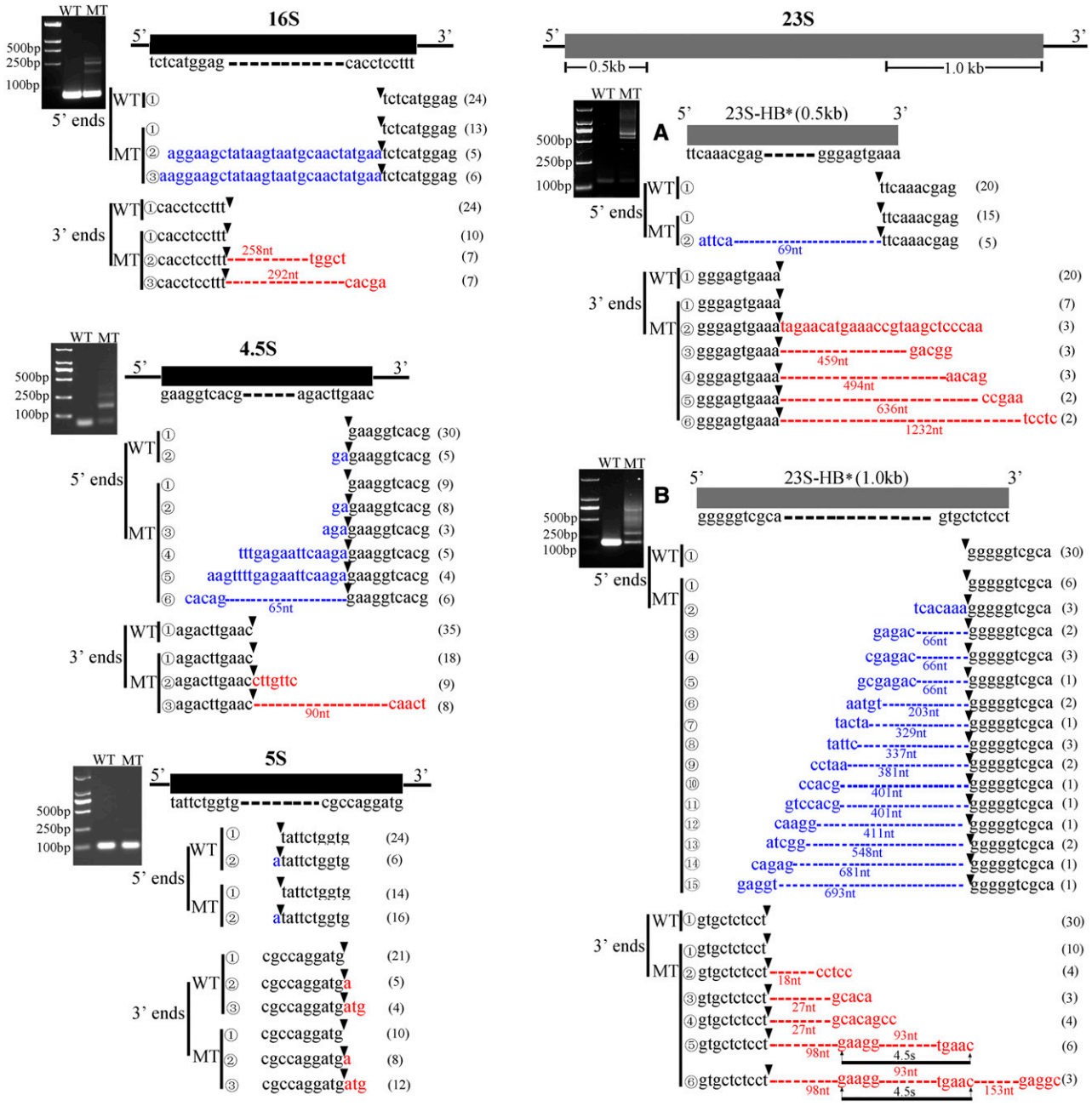


Figure 8. Precise 5′-end and 3′-end mapping of chloroplast rRNAs in wild-type (WT) and *atybeY-1* mutant (MT) plants by cRT-PCR. PCR products were separated on ethidium bromide-stained agarose gels. The 5′ and 3′ ends of the mature rRNAs are marked by black triangles. Mapped 5′ extensions and 3′ extensions of rRNAs are shown as blue and red nucleotide (nt) sequences, respectively. The numbers at the right of the sequences represent the number of sequenced clones that contained each of the sequence variants.

rRNA processing, which, in turn, causes inefficient ribosome biogenesis and reduced translational activity in chloroplasts.

AtYbeY Has Endoribonuclease Activity in Vitro

To test whether AtYbeY has RNase activity in vitro, we purified recombinant AtYbeY, mutated AtYbeY (R184A and H240A; without the putative plastid transit peptide), and GFP (as negative control) proteins from *E. coli* for activity assay (Supplemental Fig. S8). Recombinant AtYbeY protein efficiently degraded total rRNAs extracted from wild-type plants, while the mutated AtYbeY (R184A and H240A) protein degraded them weakly (Fig. 11A). Mutated *E. coli* YbeY carrying the same amino acid exchanges (R59A and H114A) was found to be ineffective in complementing

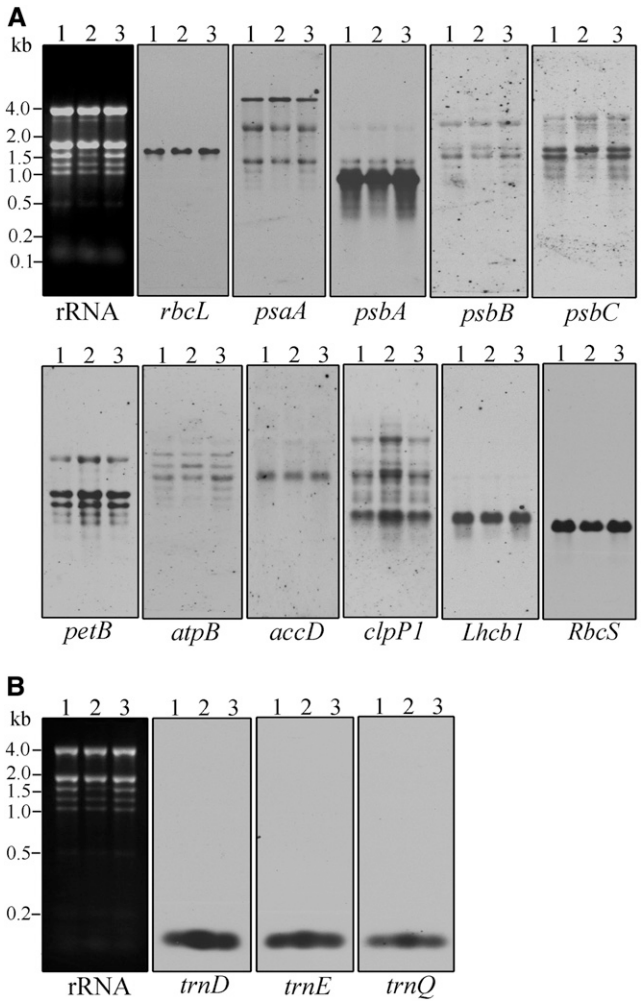


Figure 9. RNA gel-blot analysis of selected transcripts of chloroplast-encoded and nucleus-encoded genes. A, Analysis of chloroplast-encoded and nucleus-encoded mRNAs. B, Analysis of the transcripts of a subset of chloroplast-encoded *trn* genes. Total RNAs from 10-d-old wild-type (lane 1), *atybeY-1* (lane 2), and complemented *atybeY-1* (*atybeY-1comp*; lane 3) seedlings were separated in agarose-formaldehyde gels, and blots were hybridized to probes specific for the gene of interest. An ethidium bromine-stained gel is shown to confirm equal loading.

the growth phenotype of the *yeby* mutant (Davies et al., 2010). In addition, AtYbeY moderately degraded *rbcL* mRNAs synthesized by in vitro transcription (Fig. 11B) and had slight degrading activity toward chloroplast tRNA (Fig. 11C). As expected for a RNase, AtYbeY was unable to degrade either double- or single-stranded DNA (Fig. 11D). Consistent with its predicted metal-dependent hydrolase activity, AtYbeY endoribonuclease activity was inhibited by EDTA, a metal chelator (Fig. 11E). To further characterize the endoribonuclease activity of AtYbeY, we used synthetic 5′- or 3′-end biotin-labeled oligoribonucleotide substrates mimicking the 5′ and 3′ terminus of the 16S rRNA. Recombinant AtYbeY displayed an endoribonuclease activity toward the synthetic substrates, although it did not precisely cleave 5′ or 3′ terminal precursor sequences of the 16S RNA in vitro (Fig. 11, F and G). Taken together, these in vitro results strongly suggest that AtYbeY is an endoribonuclease.

DISCUSSION

In chloroplasts, transcript maturation and degradation are tightly controlled (Stern et al., 2010). rRNAs are first synthesized as precursor molecules bearing additional sequences at their 5′ and 3′ ends. Cleavage of the polycistronic rRNA operon transcript into monocistronic rRNAs and precise removal of 5′ and 3′ extensions are required to generate fully functional mature rRNA molecules. The work presented here reveals that a plant homolog of the highly conserved bacterial YbeY protein plays an important role in rRNA processing in chloroplasts of Arabidopsis.

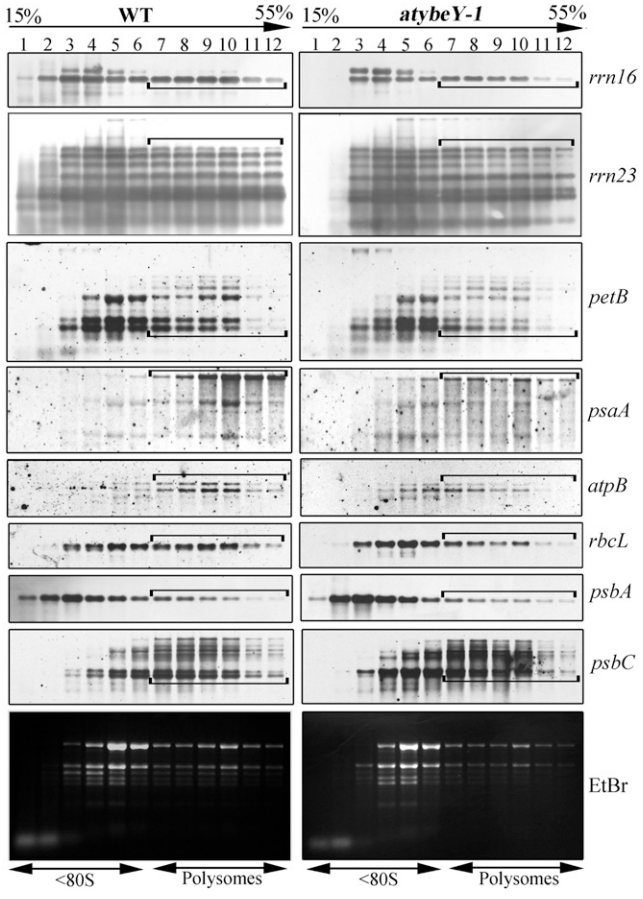
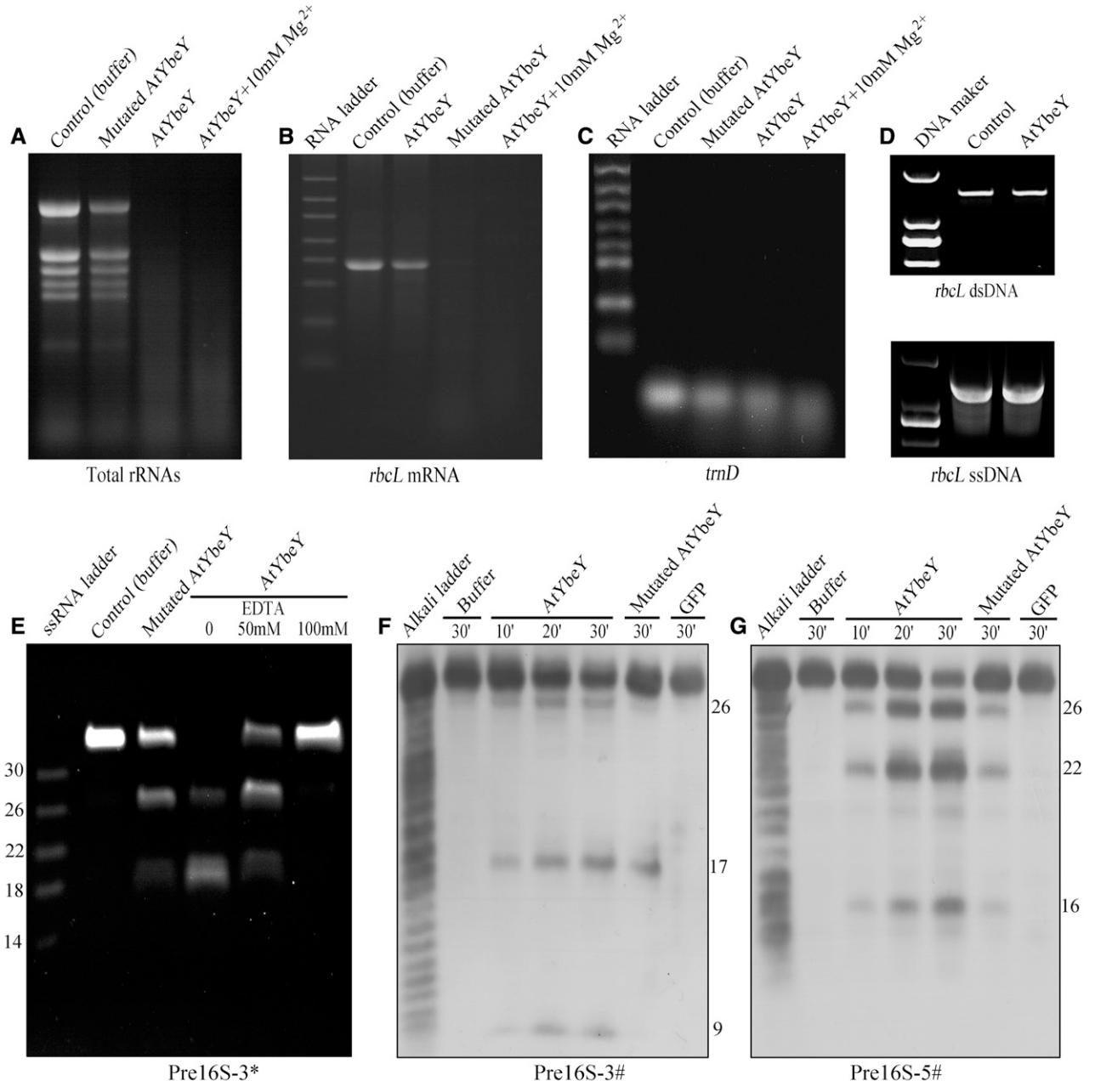


Figure 10. Polysome loading of chloroplast RNAs in wild-type (WT) and *atybeY-1* plants. Fractions from Suc density gradients were analyzed by RNA gel blots using gene-specific probes. Lanes 1 to 12 indicate the gradient fractions from top (15%) to bottom (55%). *rrn16*, rRNA 16S gene; EtBr, ethidium bromide.



Pre16S-3*: 5'-UGGAUCACC▼UCCUUU*UC▼AGGGAGAGC▼UAAUGCUUC-3'

Pre16S-3#: 5'(PO4)-U(biotin)GGAUCACC▼UCCUUU*UC▼AGGGAGAGC▼UA-3'

Pre16S-5#: 5'(PO4)-UGCAACUAUGAA*UCUC▼AUGGAG▼AGUU▼CG(biotin)-3'

Figure 11. Endoribonuclease activity assays of AtYbeY in vitro. A, Total RNA samples (3 μg) extracted from wild-type plants were treated with AtYbeY or mutated AtYbeY (R184A and H240A) for 30 min. B, Cleavage of *rbcL* mRNA (250 ng) by AtYbeY or mutated AtYbeY (R184A and H240A) for 30 min. C, Degradation of *trnD* transcripts (250 ng) by AtYbeY or mutated AtYbeY (R184A and H240A) for 30 min. Digestion products in A to C were analyzed in agarose-formaldehyde gels. The bands of RNA ladder from top to bottom are 6.0, 4.0, 3.0, 2.0, 1.5, 1.0, 0.5, and 0.2 kb. D, Lack of degradation of double-stranded DNA (dsDNA; 200 ng) and single-stranded DNA (ssDNA; 200 ng) derived from the *rbcL* gene by AtYbeY for 30 min. The bands of the DNA Maker from top to bottom are 2.0, 1.0, 0.75, and 0.5 kb. E, Inhibition of AtYbeY endoribonuclease activity by the metal chelator EDTA as demonstrated by using the short synthetic oligoribonucleotide Pre16S-3* (mimicking the 3′ terminus of 16S rRNA; 300 ng) as substrate. Digestion products stained with ethidium bromide were detected in polyacrylamide gels. F and G,

AtYbeY Is Essential for Chloroplast Development and Plant Viability

We have shown that knockout or knockdown of the *YbeY* gene in Arabidopsis leads to reduced chlorophyll content, impaired chloroplast development, and decreased photosynthetic efficiency (Fig. 3E; Supplemental Figs. S3, S4, and S7). At the molecular level, the inactivation of *AtYbeY* results in impaired chloroplast rRNA processing and a concomitant reduction in the efficiency of protein synthesis by chloroplast ribosomes. As expected, plastid genome-encoded proteins were decreased in their abundance in the mutants, while the nucleus-encoded proteins of the light-harvesting complex accumulated normally (Fig. 6). Chlorophyll deficiency and a reduced chlorophyll *a*/*b* ratio have previously been reported for a class of mutants referred to as grana-rich mutants (Nielsen et al., 1979). Also in chloroplasts of *atybeY* mutants, grana thylakoids are highly prominent, and in cotyledons, stroma thylakoids are barely observed (Fig. 5). Grana thylakoids are comprised of tightly appressed thylakoid membranes and play an important role in efficient light capture. PSI is located in unstacked thylakoids and at the margins of the thylakoid stacks, while PSII is located only in stacked grana. The grana-rich phenotype of *atybeY* mutants suggests that the relative level of PSII was higher than that of PSI. Previous reports provided evidence for trimeric LHCII playing a central role in grana stacking (McDonnel and Staehelin, 1980; Day et al., 1984; Allen and Forsberg, 2001). Consistent with this proposed role, the trimeric LHCII accumulated to higher relative levels in the mutants than in the wild type (Fig. 6A). While the YbeY endoribonuclease is unlikely to be directly involved in LHCII trimerization during grana formation, the changes in photosystem stoichiometry and antenna abundance suggest that the coordinated expression of plastid- and nucleus-encoded photosynthetic genes is impaired. Specifically, the reduced translation of plastid mRNAs in *atybeY* mutants is likely to cause a deficiency in the reaction centers of the photosystems (the subunits of which are encoded in the plastid genome), whereas synthesis of the nucleus-encoded LHC proteins is unaffected (and their accumulation is, to some extent, independent of the presence of photosystem cores).

Most RNases that were proposed to be involved in rRNA maturation in chloroplasts are not essential in that knockout mutants are viable. This is the case for both the exoribonucleases (RNase R and PNPase) and the endoribonucleases (RNase E and CSP41; Walter et al., 2002, 2010; Bollenbach et al., 2005; Germain et al., 2011; Qi et al., 2012). By contrast, the seedling-lethal phenotype of the *atybeY-2* null mutant (Fig. 3D) suggests that YbeY is essential for plant viability in Arabidopsis, at least under standard photoautotrophic growth conditions. Interestingly, YbeY is not essential in *E. coli* when cells are grown in normal conditions, but it becomes essential when cells are grown under stress conditions (Davies and Walker, 2008; Rasouly et al., 2009; Davies et al., 2010).

The Roles of AtYbeY RNase in rRNA Processing and Ribosome Biogenesis in Chloroplasts

In *E. coli*, endoribonuclease YbeY is directly involved in processing of 16S, 23S, and 5S rRNAs (Davies et al., 2010; Jacob et al., 2013). Similarly, our results show that YbeY in Arabidopsis chloroplasts plays an important role in processing both the 3′ termini and the 5′ termini of 16S, 23S, and 4.5S rRNAs (Figs. 7 and 8). The Arabidopsis exoribonucleases PNPase and RNase R have been shown previously to participate in processing the 3′ ends of chloroplast 16S and 23S rRNAs (Walter et al., 2002; Kishine et al., 2004; Bollenbach et al., 2005; Germain et al., 2011). Also, it has been reported that RNase J deficiency affects processing of the 5′ ends of 16S and 23S rRNAs in Arabidopsis (Sharwood et al., 2011). Interestingly, we observed defects in the processing of both the 3′ and the 5′ termini of chloroplast rRNAs in *atybeY* mutants. The *atybeY* mutants also show a much stronger overall effect on rRNA maturation than any of the RNases previously implicated in rRNA processing.

rRNA processing and ribosome assembly are interdependent. It has been reported that rRNA processing depends on ribosome maturation in *E. coli* (Srivastava and Schlessinger, 1988) and in plants (Barkan, 1993; Tiller et al., 2012). While correct rRNA processing is required for ribosome assembly, some steps in chloroplast rRNA processing occur in an assembly-assisted manner (Stoppel and Meurer, 2012). It is currently unclear whether the chloroplast AtYbeY, in addition to its function in rRNA processing, also has a direct function in ribosome assembly. It is possible that the loss of *AtYbeY*, through defective rRNA processing, also affects ribosome maturation, which, in turn, causes the reduced polysome loading of chloroplast RNAs observed in the *atybeY-1* mutant (Fig. 10). Alternatively, and similar to *E. coli*, the chloroplast AtYbeY protein together with other RNases may function in degradation of 70S ribosomes

Figure 11. (*Continued.*)
Analysis of in vitro endoribonuclease activity of AtYbeY using the short synthetic oligoribonucleotides Pre16S-3# (mimicking the 3′ terminus of 16S rRNA and carrying a 5′ biotin label; 250 ng) and Pre16S-5# (mimicking the 5′ terminus of 16S rRNA and carrying a 3′ biotin label; 250 ng) as substrates. Digestion products were analyzed by PAGE. The black triangles indicate cleavage sites of the synthetic oligoribonucleotide substrates (Pre16S-3*, Pre16S-3#, and Pre16S-5#) by AtYbeY in vitro, and in vivo cleavage sites of the 5′ and 3′ ends of 16S rRNA precursors are indicated by the asterisks. All assays were carried out in 50 mM HEPES-KOH (pH 7.5) buffer in a 20-μL volume at 37°C and used 2 μM of purified AtYbeY, mutated AtYbeY (R184A and H240A), or GFP.

with a defective 30S ribosomal subunit (Jacob et al., 2013). However, whether this is the case in chloroplasts needs to be further investigated.

In addition to rRNA processing, AtYbeY might be involved in other RNA metabolism in chloroplasts. For instance, the transcripts of *petB* and *clpP1* and some tRNA transcripts we detected here significantly overaccumulated in *atybeY* mutants compared with the wild type (Fig. 9). In the extreme thermophile *Thermus thermophilus* HB8, a gram-negative eubacterium, disruption of the YbeY gene had a greater impact on mRNA abundance (Ohyama et al., 2014). In Arabidopsis, RNase E- or PNPase-deficient mutants also exhibited defects in chloroplast mRNA or tRNA metabolism (Walter et al., 2010; Germain et al., 2012). It is unlikely that the higher steady-state levels of mRNAs and tRNAs result from an increased specific transcription, because the accumulation of mRNA of *accD* transcribed by nucleus-encoded RNA polymerase and mRNA of *rbcL* and *psbA* by plastid-encoded RNA polymerase in *atybeY* mutant was the same as in the wild type (Fig. 9). The PNPase has been proposed to act as a tRNA-degrading enzyme in chloroplasts (Walter et al., 2002). Our in vitro results (Fig. 11, B and C) suggest that AtYbeY may also participate in chloroplast mRNA and tRNA decay or metabolism.

Interplay between Chloroplast AtYbeY and Other RNA Processing Factors in Vivo

In *E. coli*, YbeY interacts genetically with RNase III, RNase R, and PNPase and plays important roles in rRNA maturation (Davies et al., 2010). In Arabidopsis, the absence of PNPase from chloroplasts caused defects in rRNA maturation and overaccumulation of tRNAs (Walter et al., 2002). Depletion of RNase R or RNase E in chloroplasts also leads to multiple abnormalities in RNA metabolism, including rRNA processing defects and overaccumulation of some precursor transcripts of mRNAs and tRNAs (Kishine et al., 2004; Walter et al., 2010; Germain et al., 2012). Whether the similar defects of chloroplast rRNA processing in *atybeY* mutants and other RNase mutants indicate partially overlapping functions is currently unclear. Also, it is not known whether chloroplast AtYbeY functions alone or acts together with other RNases. To explore the genetic and physical interactions of AtYbeY with other exoribonucleases (RNase R and PNPase) and endoribonucleases (RNase E and CSP41) will be an interesting future research direction.

Although different RNases act at different RNA target sites in vivo, most nucleases that attack RNA are thought to lack intrinsic cleavage specificity and show nonspecific activity in vitro (Stoppel and Meurer, 2012). Our results also show that recombinant AtYbeY acts as a nonspecific endoribonuclease in vitro (Fig. 11). Relative specificity and activity differences between in vivo and in vitro conditions were also observed for PNPase and RNR1 (Walter et al., 2002; Bollenbach et al., 2005). A possible cause of the observed differences could be the association of chloroplast transcripts with certain RNA-binding proteins (e.g. chloroplast-type ribonucleoproteins and pentatricopeptide repeat proteins) in vivo but not in vitro when synthetic transcripts are used. It was reported that pentatricopeptide repeat proteins can stabilize 3′ and 5′ termini of transcripts and consequently protect them from endo- or exoribonucleases. RNA-binding proteins like pentatricopeptide repeat proteins can exert a barrier function in vivo and impede ribonucleolytic degradation (Pfalz et al., 2009; Prikryl et al., 2011). An alternative possibility is that specificity is conferred onto AtYbeY enzyme activity by RNA secondary structures or interactions between AtYbeY and accessory factors present in vivo (Bollenbach et al., 2005; Germain et al., 2013). Finally, AtYbeY activity could also be fine-tuned by external stimuli (e.g. light) or the intracellular environment (e.g. metal ion concentrations).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*; ecotype Columbia) plants were grown in soil under long-day conditions (16 h of light/8 h of dark) at a light intensity of 120 μmol photons m^{-2} s^{-1} at 22°C. Seeds were surface sterilized and stratified for 3 d at 4°C, then sown on one-half-strength Murashige and Skoog medium with 1% (w/v) Suc. Two *atybeY* mutant lines, CS830701 (*atybeY-1*) and SALK_080307 (*atybeY-2*), were obtained from the Arabidopsis Biological Resource Center and identified by PCR using genomic DNA and T-DNA-specific primers in combination with gene-specific primers (Alonso et al., 2003). T-DNA insertion sites were determined by sequencing of PCR products. For the dark-to-light shift experiment, 3-d-old etiolated seedlings were exposed to normal growth conditions with a light intensity of 120 μmol photons m^{-2} s^{-1} for different times. After treatments, samples were harvested for total RNA extraction.

For mutant complementation and analysis of the subcellular localization of the AtYbeY protein, the entire coding sequence of *AtYbeY* was cloned into the Gateway binary vector pGWB2 and pGWB5 by an LR recombination reaction, generating an *AtYbeY* expression construct and an *AtYbeY-GFP* fusion construct, respectively. The constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and introduced into the *atybeY-1* mutants, heterozygous *atybeY-2*, and wild-type plants by the floral-dip method (Clough and Bent, 1998). To produce *AtYbeY* knockdown plants, an amiRNA (Schwab et al., 2006) construct for *AtYbeY* (*AtYbeY-amiRNA*) was generated. The chimeric amiRNA gene expressing the desired 21 mers was recombined into the pGWB2 vector, and the *AtYbeY-amiRNA* construct was introduced into Arabidopsis wild-type plants. Transgenic plants were selected for kanamycin resistance and assayed using PCR analysis.

Chlorophyll Content and Chlorophyll Fluorescence Analysis

Chlorophyll content was determined in 80% (v/v) acetone (Porra et al., 1989). Room temperature chlorophyll fluorescence of intact plants was recorded using a pulse amplitude modulated fluorimeter (Lohmann et al., 2006).

Microscopy Analysis

Chloroplast ultrastucture was analyzed by TEM as described by Zhou et al. (2009). For analysis of subcellular localization of AtYbeY, protoplasts were isolated from leaves of transgenic plants expressing AtYbeY-GFP, as described by Yoo et al. (2007). Fluorescence analysis of protoplasts was performed with a LSM500 confocal laser-scanning microscope. For cross-section analysis, cotyledons from 10-d-old seedlings were fixed in formaldehyde-acetic acid solution (50% [v/v] ethanol, 5% [v/v] acetic acid, and 3.7% [v/v] formaldehyde) and embedded in Paraplast Plus (Sigma). Eight-micrometer-thick sections

from embedded tissues were cut with a microtome, stained with toluidine blue, and observed by bright-field light microscopy.

RNA Preparation, RNA Gel-Blot Analyses, qRT-PCR, and cRT-PCR Analysis

Total RNA was extracted with the pBIOZOL Reagent (BioFlux), and complementary DNAs cDNAs were synthesized using the Prime Script RT reagent kit with gDNA Eraser (Takara). RNA gel blots were performed as described by Zhou et al. (2009). qRT-PCR was performed using the ABI 7500 real-time system with SYBR Green (Takara). The amplification of *Actin2* was used as an internal control for normalization. cRT-PCR was performed as described by Perrin et al. (2004). Total RNA was circularized using T4 RNA ligase, and cDNA spanning the junction of the 5′ and 3′ ligated ends was synthesized with reverse gene primers. The fragment was amplified, cloned into the pJET1.2/blunt cloning vector (Thermo Scientific) and sequenced. All primers used in this study are listed in Supplemental Table S3.

Protein Isolation, Immunoblotting, and BN-PAGE

Soluble total proteins were extracted as described by Bollenbach et al. (2005). Thylakoid membranes were isolated as described by Zhang et al. (1999), and complexes were separated by BN-PAGE (Schägger et al., 1994). For immunoblotting, proteins were resolved by SDS-PAGE, transferred to Hybond-ECL Nitrocellulose membranes (GE) and incubated with specific antibodies. The signals were detected using a chemiluminescence detection system (GE) according to the manufacturer's instructions.

Polysome Loading Assays and Quantification of rRNAs

Polysomes were fractionated by Suc density gradient centrifugation as described previously (Barkan, 1993). rRNA ratios were determined as described previously (Walter et al., 2010; Tiller et al., 2012). rRNAs were analyzed and quantified in leaf RNA preparations and polysome gradient fractions using the Agilent Bioanalyzer System (Agilent Technologies), the Agilent RNA 6000 Nano Kit, and the software provided by the supplier. The ratios of plastid 16S rRNA (component of the 30S subunit) to plastid 23S rRNA (component of the 50S subunit), cytosolic 18S rRNA to plastid rRNAs, and cytosolic 25S rRNA to cytosolic 18S rRNA were calculated for the wild type, *atybeY-1* mutant, and complemented *atybeY-1* (*atybeY-1comp*) plants.

Production of Recombinant AtYbeY and in Vitro RNA Degradation Assay

The cDNAs of GFP, AtYbeY, and mutated AtYbeY (R184A and H240A) without the sequence encoding the transit peptide were respectively cloned into the pET-29a vector with a C-terminal His tag. Site-directed mutagenesis of AtYbeY (R184A and H240A) was performed using a double-stranded plasmid mutagenesis procedure according to the manufacturer's protocol (TaKaRa MutanBEST Kit, Takara). AtYbeY, mutated AtYbeY (R184A and H240A), and GFP were expressed in bacterial strain BL21 (DE3) by the addition of 0.1 mM isopropylthio-β-galactoside for 5 h at 22°C and purified with Ni-NTA His-Bind Resin (Novagen) according to the manufacturer's protocol. The obtained AtYbeY, mutated AtYbeY (R184A and H240A), and GFP protein samples were then loaded onto a Superdex 75 column, and the fractions containing AtYbeY and mutated AtYbeY (R184A and H240A) were collected and passed through a Mono-Q column to remove any other potential contaminants. Thereafter, the fractions containing AtYbeY, mutated AtYbeY (R184A and H240A), and GFP were respectively collected and concentrated in a buffer of 10 mM Tris (pH 7.4), 200 mM potassium acetate, and 10% (v/v) glycerol and stored at –80°C. The purified proteins were run on a denaturing SDS polyacrylamide gel and subjected to mass spectrometry to ascertain their purities.

In vitro RNA degradation assays were carried out in 50 mM HEPES-KOH (pH 7.5) buffer at 37°C. The reaction products of the in vitro assays for total rRNAs and *rbcL* and *trnD* transcripts were detected by electrophoretic separation in agarose/formaldehyde gels stained with ethidium bromide. *rbcL* and *trnD* transcripts were synthesized in vitro using the TranscriptAid T7 High-Yield Transcription Kit (Thermo Scientific). Single-stranded DNAs corresponding to the *rbcL* gene sequence were synthesized in vitro using the Prime Script II First-Strand cDNA Synthesis Kit (Takara). The 5′- or 3′-end biotin-labeled oligoribonucleotide substrates (Pre16S-3# and Pre16S-5#) that mimic the 3′ and 5′ terminus of 16S rRNA were synthesized commercially (Takara) and used for in vitro endoribonuclease activity analysis of AtYbeY. The sequences of the synthetic substrates Pre16S-3# and Pre16S-5# were 5′(PO_4)-U(biotin)GGAUCACCUCCUUUUCAGGGAGAGCUA-3′ and 5′(PO_4)-UGCAACUAUGAAUCUCAUGGAGAGUUCG(biotin)-3′. The degradation products of synthetic substrates by AtYbeY were separated on 15% (w/v) denaturing polyacrylamide-urea gels, transferred electrophoretically to positively charged nylon membranes (Roche), and fixed by UV crosslinking. The blot was hybridized using anti-biotin-alkaline phosphatase antibody, and hybridization signals were detected with the CDP-Star reagent (Roche).

For database searches and sequence analysis, see Supplemental Methods S1. Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT2G25870 (AtYbeY), ATCG00490 (RBCL), ATCG00020 (PSBA), ATCG00350 (PSAA), ATCG00280 (PSBC), ATCG00680 (PSBB), ATCG00720 (PETB), ATCG00480 (ATPB), ATCG00500 (ACCD), ATCG00670 (CLPP1), AT1G29920 (LHCB1), AT1G67090 (RBCS), and AT3G18780 (ACTIN2).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phylogenetic analysis of YbeY proteins in *Escherichia coli*, thallophytes, and land plants.

Supplemental Figure S2. Effects of heat shock on *AtYbeY* transcript levels determined by qRT-PCR in Arabidopsis seedlings.

Supplemental Figure S3. Chlorophyll contents of wild-type and *AtYbeY-amiRNA* lines under long-day conditions.

Supplemental Figure S4. Chlorophyll *a/b* ratio of wild-type, *atybeY-1*, and *AtYbeY-amiRNA* lines.

Supplemental Figure S5. Complementation of the *atybeY-2* mutant.

Supplemental Figure S6. Siliques from wild-type, *atybeY-1*, and heterozygous *atybeY-2*+/− plants observed with a binocular microscope.

Supplemental Figure S7. SDS-PAGE analysis of total proteins extracted from 10-d-old wild-type and *AtYbeY-amiRNA* lines.

Supplemental Figure S8. SDS-PAGE analysis of the purified recombinant AtYbeY, mutated AtYbeY (R184A and H240A), and GFP proteins.

Supplemental Table S1. Amino acid sequences of 52 putative homologs of the *E. coli* YbeY protein.

Supplemental Table S2. Similarity analysis of UPF0054 domain sequences between *E. coli* YbeY and its homologs of photosynthetic eukaryotes and cyanobacteria.

Supplemental Table S3. List of synthetic oligonucleotides used as primers (forward or reverse primers) in this study.

Supplemental Methods S1. Database searches and sequence analysis.

ACKNOWLEDGMENTS

We thank the Arabidopsis Biological Resource Center (Ohio State University) for providing Arabidopsis mutant seeds.

Received December 4, 2014; accepted March 23, 2015; published March 25, 2015.

LITERATURE CITED

Allen JF, Forsberg J (2001) Molecular recognition in thylakoid structure and function. Trends Plant Sci **6:** 317–326

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 insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653–657

Bang WY, Chen J, Jeong IS, Kim SW, Kim CW, Jung HS, Lee KH, Kweon HS, Yoko I, Shiina T, et al (2012) Functional characterization of ObgC in ribosome biogenesis during chloroplast development. Plant J **71:** 122–134

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

Bayer RG, Stael S, Csaszar E, Teige M (2011) Mining the soluble chloroplast proteome by affinity chromatography. Proteomics **11:** 1287–1299

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

Bellaoui 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

Bisanz C, Bégot L, Carol P, Perez P, Bligny M, Pesey H, Gallois JL, Lerbs-Mache S, Mache R (2003) The Arabidopsis nuclear DAL 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 (2005) 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

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

Caroca R, Howell KA, Hasse C, Ruf S, Bock R (2013) Design of chimeric expression elements that confer high-level gene activity in chromoplasts. Plant J **73:** 368–379

Chi W, He B, Mao J, Li Q, Ma J, Ji D, Zou M, Zhang L (2012) The function of RH22, a DEAD RNA helicase, in the biogenesis of the 50S ribosomal subunits of Arabidopsis chloroplasts. Plant Physiol **158:** 693–707

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Davies BW, Köhrer C, Jacob AI, Simmons LA, Zhu J, Aleman LM, Rajbhandary UL, Walker GC (2010) Role of *Escherichia coli* YbeY, a highly conserved protein, in rRNA processing. Mol Microbiol **78:** 506–518

Davies BW, Walker GC (2008) A highly conserved protein of unknown function is required by *Sinorhizobium meliloti* for symbiosis and environmental stress protection. J Bacteriol **190:** 1118–1123

Day DA, Ryrie IJ, Fuad N (1984) Investigations of the role of the main light-harvesting chlorophyll-protein complex in thylakoid membranes. Reconstitution of depleted membranes from intermittent-light-grown plants with the isolated complex. J Cell Biol **98:** 163–172

Dyall SD, Brown MT, Johnson PJ (2004) Ancient invasions: from endosymbionts to organelles. Science **304:** 253–257

Fristedt R, Scharff LB, Clarke CA, Wang Q, Lin C, Merchant SS, Bock R (2014) RBF1, a plant homolog of the bacterial ribosome-binding factor RbfA, acts in processing of the chloroplast 16S ribosomal RNA. Plant Physiol **164:** 201–215

Germain A, Herlich S, Larom S, Kim SH, Schuster G, Stern DB (2011) Mutational analysis of Arabidopsis chloroplast polynucleotide phosphorylase reveals roles for both RNase PH core domains in polyadenylation, RNA 3′-end maturation and intron degradation. Plant J **67:** 381–394

Germain A, Hotto AM, Barkan A, Stern DB (2013) RNA processing and decay in plastids. Wiley Interdiscip Rev RNA **4:** 295–316

Germain A, Kim SH, Gutierrez R, Stern DB (2012) Ribonuclease II preserves chloroplast RNA homeostasis by increasing mRNA decay rates, and cooperates with polynucleotide phosphorylase in 3′ end maturation. Plant J **72:** 960–971

Harris EH, Boynton JE, Gillham NW (1994) Chloroplast ribosomes and protein synthesis. Microbiol Rev **58:** 700–754

Huang J, Taylor JP, Chen JG, Uhrig JF, Schnell DJ, Nakagawa T, Korth KL, Jones AM (2006) The plastid protein THYLAKOID FORMATION1 and the plasma membrane G-protein GPA1 interact in a novel sugar-signaling mechanism in *Arabidopsis*. Plant Cell **18:** 1226–1238

Jacob AI, Köhrer C, Davies BW, RajBhandary UL, Walker GC (2013) Conserved bacterial RNase YbeY plays key roles in 70S ribosome quality control and 16S rRNA maturation. Mol Cell **49:** 427–438

Kaczanowska M, Rydén-Aulin M (2007) Ribosome biogenesis and the translation process in *Escherichia coli*. Microbiol Mol Biol Rev **71:** 477–494

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

Komatsu T, Kawaide H, Saito C, Yamagami A, Shimada S, Nakazawa M, Matsui M, Nakano A, Tsujimoto M, Natsume M, et al (2010) The chloroplast protein BPG2 functions in brassinosteroid-mediated post-transcriptional accumulation of chloroplast rRNA. Plant J **61:** 409–422

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

Lohmann A, Schöttler MA, Bréhélin C, Kessler F, Bock R, Cahoon EB, Dörmann P (2006) Deficiency in phylloquinone (vitamin K1) methylation affects prenyl quinone distribution, photosystem I abundance, and anthocyanin accumulation in the Arabidopsis AtmenG mutant. J Biol Chem **281:** 40461–40472

Lu Y, Li C, Wang H, Chen H, Berg H, Xia Y (2011) AtPPR2, an Arabidopsis pentatricopeptide repeat protein, binds to plastid 23S rRNA and plays an important role in the first mitotic division during gametogenesis and in cell proliferation during embryogenesis. Plant J **67:** 13–25

Luro S, Germain A, Sharwood RE, Stern DB (2013) RNase J participates in a pentatricopeptide repeat protein-mediated 5′ end maturation of chloroplast mRNAs. Nucleic Acids Res **41:** 9141–9151

Manuell AL, Quispe J, Mayfield SP (2007) Structure of the chloroplast ribosome: novel domains for translation regulation. PLoS Biol **5:** e209

McDonnel A, Staehelin LA (1980) Adhesion between liposomes mediated by the chlorophyll *a*/*b* light-harvesting complex isolated from chloroplast membranes. J Cell Biol **84:** 40–56

Nielsen NC, Smillie RM, Henningsen KW, Von Wettstein D (1979) Composition and function of thylakoid membranes from grana-rich and grana-deficient chloroplast mutants of barley. Plant Physiol **63:** 174–182

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

Ohyama H, Sakai T, Agari Y, Fukui K, Nakagawa N, Shinkai A, Masui R, Kuramitsu S (2014) The role of ribonucleases in regulating global mRNA levels in the model organism *Thermus thermophilus* HB8. BMC Genomics **15:** 386

Pandey SP, Minesinger BK, Kumar J, Walker GC (2011) A highly conserved protein of unknown function in *Sinorhizobium meliloti* affects sRNA regulation similar to Hfq. Nucleic Acids Res **39:** 4691–4708

Park YJ, Cho HK, Jung HJ, Ahn CS, Kang H, Pai HS (2011) PRBP plays a role in plastid ribosomal RNA maturation and chloroplast biogenesis in *Nicotiana benthamiana*. Planta **233:** 1073–1085

Perrin R, Lange H, Grienenberger JM, Gagliardi D (2004) AtmtPNPase is required for multiple aspects of the 18S rRNA metabolism in *Arabidopsis thaliana* mitochondria. Nucleic Acids Res **32:** 5174–5182

Pfalz J, Bayraktar OA, Prikryl J, Barkan A (2009) Site-specific binding of a PPR protein defines and stabilizes 5′ and 3′ mRNA termini in chloroplasts. EMBO J **28:** 2042–2052

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta **975:** 384–394

Prikryl J, Rojas M, Schuster G, Barkan A (2011) Mechanism of RNA stabilization and translational activation by a pentatricopeptide repeat protein. Proc Natl Acad Sci USA **108:** 415–420

Prikryl J, Watkins KP, Friso G, van Wijk KJ, Barkan A (2008) A member of the Whirly family is a multifunctional RNA- and DNA-binding protein that is essential for chloroplast biogenesis. Nucleic Acids Res **36:** 5152–5165

Qi Y, Armbruster U, Schmitz-Linneweber C, Delannoy E, de Longevialle AF, Rühle T, Small I, Jahns P, Leister D (2012) Arabidopsis CSP41 proteins form multimeric complexes that bind and stabilize distinct plastid transcripts. J Exp Bot **63:** 1251–1270

Rasouly A, Davidovich C, Ron EZ (2010) The heat shock protein YbeY is required for optimal activity of the 30S ribosomal subunit. J Bacteriol **192:** 4592–4596

Rasouly A, Schonbrun M, Shenhar Y, Ron EZ (2009) YbeY, a heat shock protein involved in translation in *Escherichia coli*. J Bacteriol **191:** 2649–2655

Schägger H, Cramer WA, von Jagow G (1994) Analysis of molecular masses and oligomeric states of protein complexes by blue native

electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. Anal Biochem **217:** 220–230

Schmitz-Linneweber 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-mRNA. Plant Cell **18:** 2650–2663

Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D (2006) Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. Plant Cell **18:** 1121–1133

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

Sharma MR, Wilson DN, Datta PP, Barat C, Schluenzen F, Fucini P, Agrawal RK (2007) Cryo-EM study of the spinach chloroplast ribosome reveals the structural and functional roles of plastid-specific ribosomal proteins. Proc Natl Acad Sci USA **104:** 19315–19320

Sharwood RE, Halpert M, Luro S, Schuster G, Stern DB (2011) Chloroplast RNase J compensates for inefficient transcription termination by removal of antisense RNA. RNA **17:** 2165–2176

Stern DB, Goldschmidt-Clermont M, Hanson MR (2010) Chloroplast RNA metabolism. Annu Rev Plant Biol **61:** 125–155

Stoppel R, Manavski N, Schein A, Schuster G, Teubner M, Schmitz-Linneweber C, Meurer J (2012) RHON1 is a novel ribonucleic acid-binding protein that supports RNase E function in the Arabidopsis chloroplast. Nucleic Acids Res **40:** 8593–8606

Stoppel R, Meurer J (2012) The cutting crew: ribonucleases are key players in the control of plastid gene expression. J Exp Bot **63:** 1663–1673

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

Tiller N, Weingartner M, Thiele W, Maximova E, Schöttler MA, Bock R (2012) The plastid-specific ribosomal proteins of *Arabidopsis thaliana* can be divided into non-essential proteins and genuine ribosomal proteins. Plant J **69:** 302–316

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

Walter M, Piepenburg K, Schöttler MA, Petersen K, Kahlau S, Tiller N, Drechsel O, Weingartner M, Kudla J, Bock R (2010) Knockout of the plastid RNase E leads to defective RNA processing and chloroplast ribosome deficiency. Plant J **64:** 851–863

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

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, 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

Yehudai-Resheff S, Hirsh M, Schuster G (2001) Polynucleotide phosphorylase functions as both an exonuclease and a poly(A) polymerase in spinach chloroplasts. Mol Cell Biol **21:** 5408–5416

Yoo SD, Cho YH, Sheen J (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc **2:** 1565–1572

Zhan C, Fedorov EV, Shi W, Ramagopal UA, Thirumuruhan R, Manjasetty BA, Almo SC, Fiser A, Chance MR, Fedorov AA (2005) The ybeY protein from *Escherichia coli* is a metalloprotein. Acta Crystallogr Sect F Struct Biol Cryst Commun **61:** 959–963

Zhang L, Paakkarinen V, van Wijk KJ, Aro EM (1999) Co-translational assembly of the D1 protein into photosystem II. J Biol Chem **274:** 16062–16067

Zhou W, Cheng Y, Yap A, Chateigner-Boutin AL, Delannoy E, Hammani K, Small I, Huang J (2009) The Arabidopsis gene *YS1* encoding a DYW protein is required for editing of *rpoB* transcripts and the rapid development of chloroplasts during early growth. Plant J **58:** 82–96