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

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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 microscopy 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 (25S 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.

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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 RNAs (Walter et al., 2002; Bollenbach et al., 2005). Although PNPase is involved in mRNA and 23S...
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). RNRI, 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 RNA 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 metalloendonuclease 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 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
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_{6}/f 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. 7A and B). Additional hybridizations using probes (a1 and a2; Fig. 7A).
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 5. Loss of AtYbeY 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).
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-dependen genes (rbcL, psaA, psaB, 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 cllP1 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 rRNA 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

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**Figure 6.** Analysis of photosynthetic complexes and of representative subunits. A, BN gel electrophoretic analysis of thylakoid 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 atybeY-1comp seedlings. RbcL and RbcS indicate large and small subunits of Rubisco. C, Immunoblot analysis of diagnostic subunits of thylakoid 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.
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 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. 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.
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...
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 chlo-
roplast 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.

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**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.

**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%). rRNA, rRNA 16S gene; EtBr, ethidium bromide.
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,

Pre16S-3*: 5′-UGGAUCACCUCUUULUCAGGGAGAGC-UAUGCUUC-3′
Pre16S-3#: 5′(PO4)-U(biotin)GGAUCACCUCUUULUCAGGGAGAGC-UA-3′
Pre16S-5#: 5′(PO4)-UGCAACUAUGAUUCUCUAUGGAGAGUGUU-C(biotin)-3′
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 RNA 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 endoribonuclease 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 RNA maturation than any of the RNases previously implicated in RNA processing.

rRNA processing and ribosome assembly are interdependent. It has been reported that RNA 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 RNA processing, also has a direct function in ribosome assembly. It is possible that the loss of AtYbeY, through defective RNA 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 RNA and carrying a 5′ biotin label; 250 ng) and Pre16S-5′ (mimicking the 5′ terminus of 16S RNA 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 μg 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 over-accumulated 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 *rcl* 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 RNA 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_080007 (*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 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 ultrastructure 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 LSM510 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 RNeasy Plant Mini Kit and treated with RNase-free DNase I according to the manufacturer's instructions. The integrity of the RNA was checked by gel electrophoresis. Complementary DNA (cDNA) was synthesized using the Prime Script RT Reagent Kit with gDNA Eraser (Takara) according to the manufacturer's instructions. The cDNAs were subsequently amplified using the PCR system described previously (Walter et al., 2010). The amplified fragments were visualized on an agarose/formaldehyde gel stained with ethidium bromide.

Protein Isolation, Immunoblotting, and BN-PAGE
Soluble total proteins were extracted as described by Bollenbach et al. (2003). Thylakoid membranes were isolated as described by Zibar 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 sucrose 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 25S 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 AtYbeY and mutated AtYbeY (R184A and H240A) were synthesized commercially (Jena Bioscience) and used for in vitro endoribonuclease activity analysis of AtYbeY. The sequences of the synthetic substrates Pre16S-3′ and Pre16S-5′ were 5′-PO4(biotin)5GGAUCACCUCCUUUCAGGGAGACCAU-3′ and 5′-PO4-UCCAAACUAUUCAUUCAUCAUCAUGAGAGUCC(biotin)-3′. The degradation products of synthetic substrates by AtYbeY were separated on 15% (w/v) denaturing polyacrylamide-urea gels, transferred electrotopically to positively charged nylon membranes (Roche), and fixed by UV cross-linking. The blot was hybridized using anti-biotin-alkaline phosphatase antibody, and hybridization signals were detected with the CDP-star reagent (Roche).

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