Arabidopsis Encodes Four tRNase Z Enzymes[1][W][OA]

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Functional transfer RNA (tRNA) molecules are a prerequisite for protein biosynthesis. Several processing steps are required to generate the mature functional tRNA from precursor molecules. Two of the early processing steps involve cleavage at the tRNA 5′ end and the tRNA 3′ end. While processing at the tRNA 5′ end is performed by RNase P, cleavage at the 3′ end is catalyzed by the endonuclease tRNase Z. In eukaryotes, tRNase Z enzymes are found in two versions: a short form of about 250 to 300 amino acids and a long form of about 700 to 900 amino acids. All eukaryotic genomes analyzed to date encode at least one long tRNase Z protein. Of those, Arabidopsis (*Arabidopsis thaliana*) is the only organism that encodes four tRNase Z proteins, two short forms and two long forms. We show here that the four proteins are distributed to different subcellular compartments in the plant cell: the nucleus, the cytoplasm, the mitochondrion, and the chloroplast. One tRNase Z is present only in the cytoplasm, one protein is found exclusively in mitochondria, while the third one has dual locations: nucleus and mitochondria. None of these three tRNase Z proteins is essential. The fourth tRNase Z protein is present in chloroplasts, and deletion of its gene results in an embryo-lethal phenotype. In vitro analysis with the recombinant proteins showed that all four tRNase Z enzymes have tRNA 3′ processing activity. In addition, the mitochondrial tRNase Z proteins cleave tRNA-like elements that serve as processing signals in mitochondrial mRNA maturation.

The generation of functional tRNA molecules is essential not only for protein biosynthesis but also for other cellular processes in all organisms (Söll, 1993). Maturation of tRNAs involves the removal of additional sequences from tRNA precursors (Hopper and Phizicky, 2003). Processing at the tRNA 5′ end has been well studied and is catalyzed by RNase P (Evans et al., 2006), but generation of the tRNA 3′ end is not as well understood. The endonuclease involved in this process has recently been identified and is termed tRNase Z (Schiffer et al., 2002). tRNase Z has been found in organisms from all three domains, bacteria, archaea, and eukarya. It exists in two forms: a short version of about 250 to 300 amino acids and a long version of about 700 to 900 amino acids. In bacteria and archaea, only short tRNase Z proteins are present. Eukaryotic organisms generally contain a long tRNase Z, and in some cases they have both forms, long and short tRNase Z proteins (Vogel et al., 2005; Redko et al., 2007).

tRNase Z proteins belong to the superfamily of the metallo-β-lactamases (MBLs), which are characterized by the α-β/β-α fold. All MBLs contain five highly conserved motifs that are involved in the metal coordination in the active site (Aravind, 1999). In addition to the MBL domain, members of this superfamily contain other different domains, resulting in a wide substrate spectrum for proteins of this family (Aravind, 1999; Callebaut et al., 2002; Dominski, 2007). Several enzymes of the MBL family are involved in nucleic acid metabolism. These include the polyadenylation factor CPSF73, the mRNA 3′ endonuclease (Ryan et al., 2004), and the ribonuclease J1 (Mathy et al., 2007).

tRNase Z was isolated and identified by biochemical purification of the enzyme from wheat (*Triticum aestivum*) embryos (Schiffer et al., 2002). Database searches identified four homologs of the wheat tRNase Z in Arabidopsis (*Arabidopsis thaliana*): two short tRNase Z proteins of 280 and 374 amino acids (termed AthTrZ^{S1} and AthTrZ^{S2}) and two long tRNase Z proteins of 890 and 943 amino acids (termed AthTrZ^{L1} and AthTrZ^{L2}; Schiffer et al., 2002; Vogel et al., 2005). Bioinformatic analyses using sorting servers showed that AthTrZ^{S1} has no predicted signal peptide while AthTrz^{S2} has a potential signal sequence for chloroplasts of about 68 amino acids (Schiffer et al., 2002).

The detection of four different tRNase Z enzymes in Arabidopsis was surprising, since all other organisms

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contain only one or two tRNase Z proteins (Vogel et al., 2005). Thus, the question arose, why does Arabidopsis need four tRNase Z enzymes? One possible explanation is that the four tRNase Z homologs have different RNA substrates. tRNase Z enzymes from bacteria and archaea have been shown to be able to process other substrates besides tRNA precursors: mRNAs (Perwez and Kushner, 2006) and 5S rRNA (Hölzle et al., 2008). In addition, it is known that the MBL enzymes show a broad substrate spectrum (Daiyasu et al., 2001; Callebaut et al., 2002; Dominski, 2007). Therefore, the four tRNase Z proteins in Arabidopsis might process different RNA substrates. Only a few ribonucleases have been identified in Arabidopsis, and the tRNase Z proteins are good candidates for the missing ribonucleases.

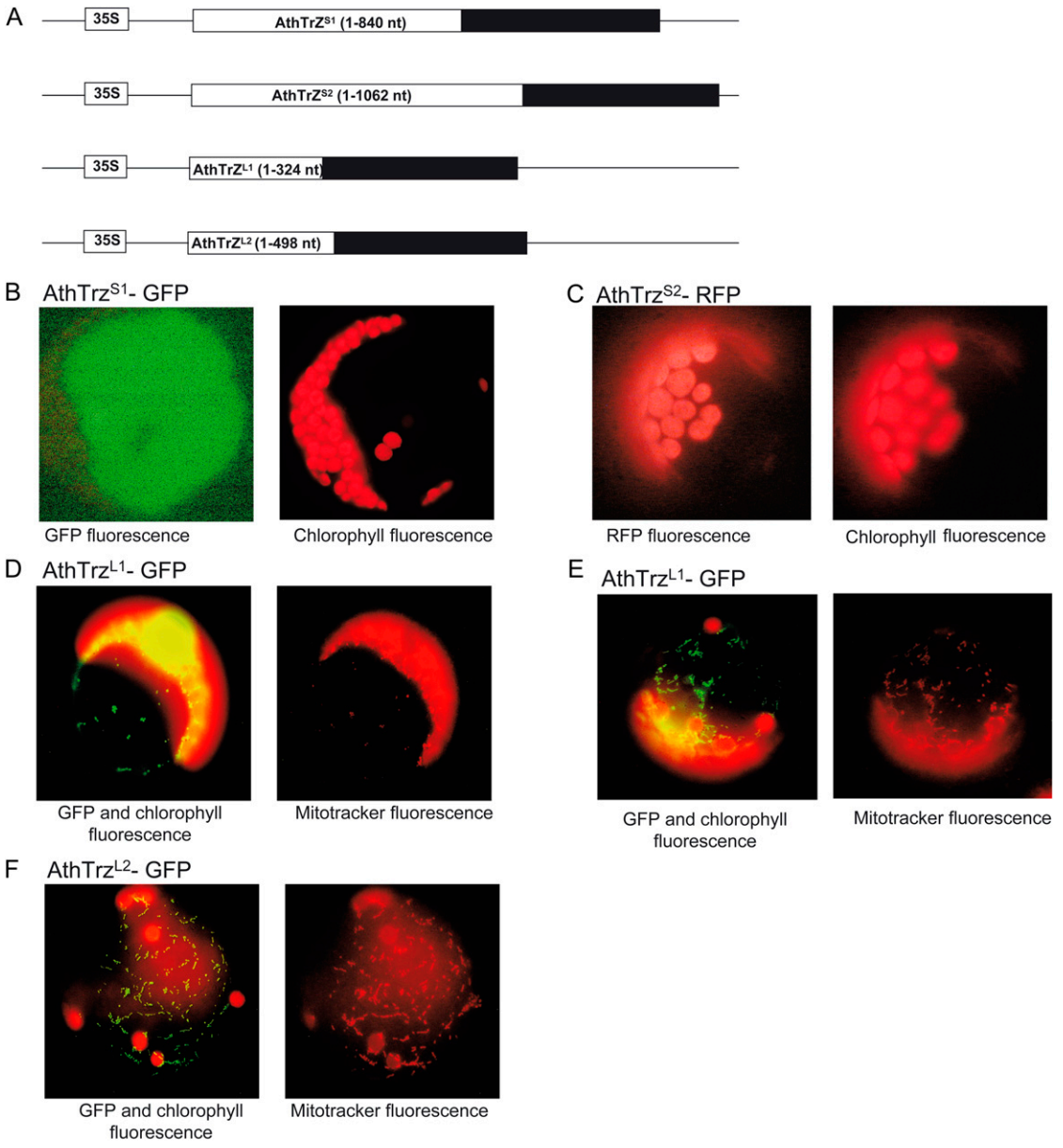

Figure 1. Subcellular localization of tRNase Z proteins in Arabidopsis. A, Schematic drawing of the GFP and RFP constructs used. B to F, The coding sequences of tRNase Z proteins were cloned in frame with the GFP and/or RFP, and the resulting fusion proteins were expressed in plants. The localization of AthTrz^{S1} and AthTrz^{S2} was analyzed in protoplasts of transformed Arabidopsis (B and C). The emitted fluorescence of AthTrz^{L1}-GFP and AthTrz^{L2}-GFP was investigated in transformed tobacco protoplast (D–F). Fluorescence emitted by GFP and chlorophyll was detected with a fluorescein isothiocyanate filter. A super-GFP filter was used to detect only the GFP fluorescence (excluding the chlorophyll fluorescence). A MitoTracker filter was used for the detection of the RFP protein fluorescence and the MitoTracker staining.

Another explanation for the occurrence of four tRNase Z proteins might be that they are active in different compartments or different tissues or are differentially expressed. The prediction by different sorting servers that three of the proteins are routed to organelles supports the hypothesis of ribonuclease activity in different compartments.

To reveal the specific function of each individual tRNase Z protein, we investigated their functions in detail using in vitro and in vivo approaches.

RESULTS

Arabidopsis is the only organism found to date that encodes four tRNase Z enzymes; all other organisms encode only one or two tRNase Z proteins (Vogel et al., 2005). Searches of the National Center for Biotechnology Information databases (http://www.ncbi.nlm.nih.gov/) revealed that full-length cDNAs corresponding to all four tRNase Z genes are available. Two full-length cDNAs have been reported for AthTrz^{S1} and AthTrz^{L1}, three for AthTrz^{L2} (the clone NM_112497 was wrongly assigned to AthTrz^{L1}), and five for AthTrz^{S2}. In general, all of the cDNA clones for one gene code for the same protein; thus, no alternative splicing seems to take place. The expression profile of the tRNase Z genes in Arabidopsis was monitored using the Genevestigator Web site (https://www.genevestigator.ethz.ch.at; Zimmermann et al., 2004, 2005). mRNAs corresponding to the tRNase Z proteins were found in all plant tissues tested. In particular, there was no significant variation between mRNA levels for AthTrz^{S1}, AthTrz^{L1}, and AthTrz^{L2}. Only in root tips was the expression significantly high for all three mRNAs. In contrast, AthTrz^{S2} was poorly expressed in nongreen tissue like flowers, pollen, and stamen and was abundant in young green tissue (rosette leaves) and in actively dividing tissue (i.e. shoot apex, pedicel, and root tips). All tRNase Z genes were expressed during all growth stages of Arabidopsis, in particular during the first stage of development (seedling) and at the start of flowering.

Four tRNase Z Proteins in Arabidopsis

The short tRNase Z proteins AthTrZ^{S1} and AthTrZ^{S2} have a sequence identity of 44%, and protein sequence alignments show that AthTrZ^{S2} has a longer N terminus, which suggests that this N terminus is a potential signal sequence (Supplemental Fig. S1). Sequence identity between the long tRNase Z enzymes is 69%. The short tRNase Z enzymes have sequence similarity to the C-terminal part of the long tRNase Z enzymes.

In silico analysis of subcellular targeting using different sorting servers (see "Materials and Methods") suggested that three of the four tRNase Z proteins are routed to organelles (Supplemental Table S1). AthTrZ^{S2} and AthTrZ^{L2} are predicted to be routed to chloroplasts, and AthTrZ^{L1} is predicted to be routed to mitochondria, chloroplasts, and the nucleus. AthTrZ^{S1} seems to contain no signal sequence.

Subcellular Localization of Arabidopsis tRNase Z Proteins

To investigate localization in vivo, the complete coding regions of AthTrZ^{S1} and AthTrZ^{S2} were cloned in frame upstream of the GFP (AthTrZ^{S1}) or the red fluorescent protein (RFP; AthTrZ^{S2}) genes (Fig. 1A). The fusion constructs were then stably transformed into Arabidopsis wild-type plants. Protoplasts were released from selected plants and analyzed by fluorescence microscopy (Fig. 1, B and C). Analysis of the AthTrZ^{S1}:GFP fusion protein showed a cytoplasmic location for this short tRNase Z (Fig. 1B). The red fluorescence emitted by the AthTrZ^{S2}:RFP fusion protein is identical to the pattern of chlorophyll fluorescence in the chloroplast, indicating that AthTrZ^{S2} is located in these organelles (Fig. 1C). To investigate the localization of AthTrZ^{L1} and AthTrZ^{L2}, the 5′ part of the corresponding cDNAs was cloned in frame upstream of the GFP gene (Fig. 1A). Constructs were transformed into tobacco (*Nicotiana tabacum*) protoplasts, and localization was investigated using fluorescence microscopy. This analysis revealed the AthTrZ^{L1}:GFP fusion proteins to be located in two different cell compartments: mitochondria and nu-

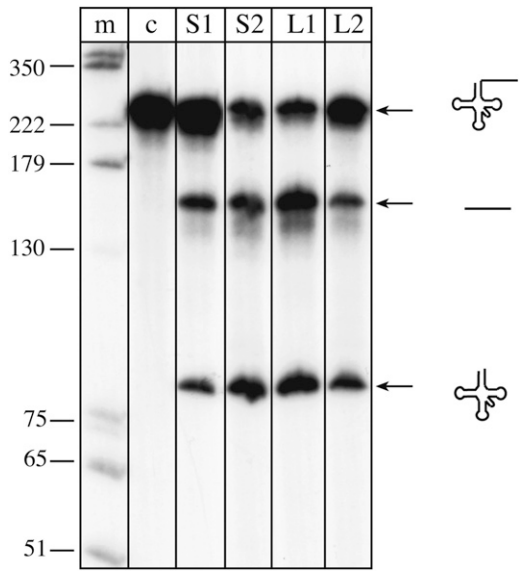

Figure 2. In vitro processing assay of recombinant tRNase Z proteins. Pre-tRNA^{Tyr} molecules were incubated with recombinant tRNase Z proteins, and reaction products were separated by denaturing PAGE. DNA size markers are shown on the left (lane m), and the control reaction without the addition of any proteins is shown in lane c. Lanes S1, S2, L1, and L2 indicate incubation with the tRNase Z proteins AthTrz^{S1}, AthTrz^{S2}, AthTrz^{L1}, and AthTrz^{L2}, respectively. Precursor and products are shown schematically on the right. All four tRNase Z proteins process the precursor.

cleus (Fig. 1, D and E). The same result was obtained by fluorescence analysis of AthTrZ^{L1}:RFP in tobacco protoplasts (data not shown). The analogous investigation of the AthTrZ^{L2}:GFP fusion protein showed this protein to be located in mitochondria, indicating that AthTrZ^{L2} is directed to this compartment (Fig. 1E). In contrast to the prediction by the TargetP server, neither AthTrZ^{L1} nor AthTrZ^{L2} is located in chloroplasts.

All Four Recombinant tRNase Z Proteins Process tRNA Precursors

The four tRNase Z proteins in Arabidopsis were identified solely by sequence similarity to the wheat tRNase Z. In vitro tRNA processing activity was only shown for the short tRNase Z AthTrZ^{S1} (Schiffer et al., 2002), confirming that this enzyme is a tRNA 3′ endonuclease. To determine whether the other three

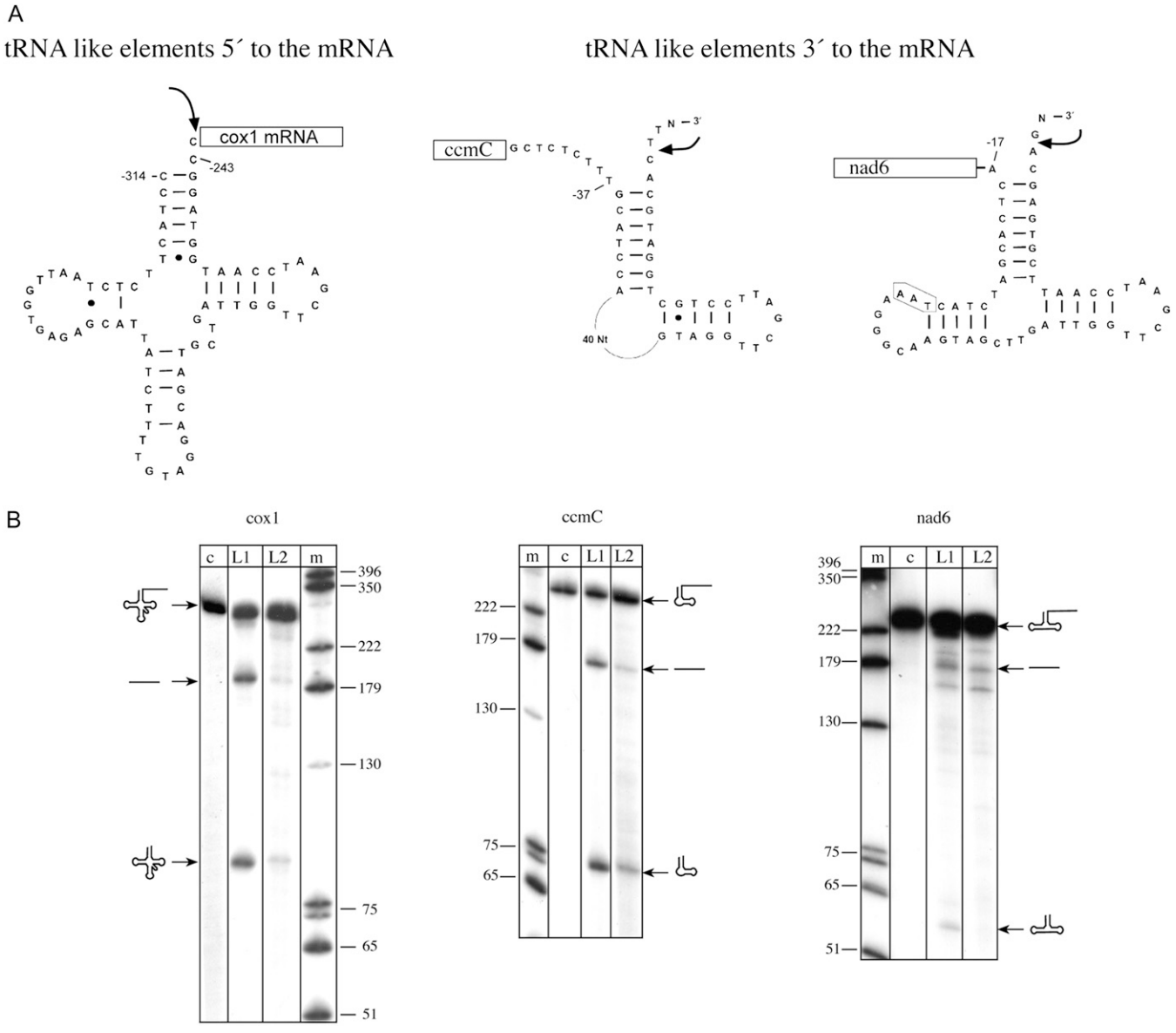

Figure 3. A, Schematic representation of the mitochondrial t-element substrates used. The secondary structures of the mitochondrial substrates used are shown. The sequence upstream of the *cox1* gene folds into an almost perfect cloverleaf tRNA structure, while the sequences downstream of the *ccmC* and *nad6* genes contain only parts of the tRNA (*ccmC*, only acceptor stem and T arm; *nad6*, only acceptor stem, D arm, and T arm). B, In vitro processing of mitochondrial transcripts by tRNase Z proteins. Purified tRNase Z enzymes from Arabidopsis were incubated with mitochondrial transcripts, and reaction products were separated by denaturing PAGE. The names of the transcripts used are indicated above the gels. At the sides, DNA size markers are indicated (lane m) as well as schematic drawings of precursors and products. Lane c, Control reactions without proteins; lane L1, addition of AthTrZ^{L1}; lane L2, addition of AthTrZ^{L2}.

homologs also have pre-tRNA processing activity in vitro, the corresponding cDNAs were cloned into pET expression vectors and expressed in *Escherichia coli*. All proteins were expressed in soluble form; proteins AthTrZ^{S1}, AthTrZ^{S2}, and AthTrZ^{L1} were obtained as pure recombinant protein fractions, while the purified fraction of recombinant AthTrZ^{L2} contained a few residual *E. coli* proteins (Supplemental Fig. S2). Incubation with precursor tRNA^{Tyr} (Fig. 2) showed that all tRNase Z homologs have tRNA 3′-processing activity. They cleave the precursor tRNA efficiently at the tRNA 3′ end, generating two products, the tRNA and the 3′ trailer (Fig. 2). The long tRNase Z AthTrz^{L2} processes the precursor less efficiently, which might be due to the fact that this enzyme was difficult to express and only low amounts of recombinant protein could be obtained. In addition, the recombinant protein fraction of AthTrz^{L2} was not as pure as those of the other tRNase Z proteins (Supplemental Fig. S2).

Processing of Mitochondrial tRNA-Like Structures

In plant mitochondria, tRNA-like structures, the so-called t-elements, are located adjacent to 5′ or 3′ mRNA termini (Hanic-Joyce and Gray, 1990; Bellaoui et al., 1997). In some of these transcripts, removal of the t-element by endonucleolytic cleavage was observed and a possible role of tRNase Z in this reaction was suggested (Forner et al., 2007).

Since AthTrZ^{L1} and AthTrZ^{L2} proteins are routed to mitochondria, we wondered whether recombinant AthTrZ^{L1} and AthTrZ^{L2} can process these mitochondrial tRNA-like structures in vitro. To analyze processing of RNAs containing a t-element, we first generated the *cox1* (for cytochrome *c* oxidase subunit 1) precursor RNA in vitro. The transcript contains the t-element structure and the 5′ part of the *cox1* mRNA (Fig. 3A). Both tRNase Z proteins cleaved the *cox1* substrate, although AthTrZ^{L2} cleaved the substrate less efficiently (Fig. 3B). As stated above, this may be due to the fact that expression of that protein was not as pure as that of the other proteins. tRNase Z cleavage resulted in two processing products: an 81-nucleotide-long RNA corresponding to the t-element and a 174-nucleotide-long RNA corresponding to the *cox1* mRNA (Fig. 3B). To investigate whether t-elements located 3′ to an mRNA can also be processed by tRNase Z proteins, substrates for the *ccmC* (for cytochrome *c* maturation subunit C) gene and for the *nad6* (for NADH dehydrogenase subunit 6) gene were prepared (Fig. 3A). These substrates contain the t-element downstream of the mRNA 3′ end. The *ccmC* substrate was processed by both tRNase Z enzymes tested, yielding two processing products of 73 and 159 nucleotides in length, which correspond in size to the t-element and the 3′ trailer (Fig. 3B). Again, processing by AthTrz^{L2} was less efficient. The *nad6* substrate was processed by AthTrZ^{L1}, although not very efficiently. Processing generates products of 58, 165, 175, and 180 nucleotides length. The two products of 58 and 175 nucleotides correspond to the t-element and the 3′ trailer. AthTrZ^{L2} also generates the mRNA fragment of the expected size (175 nucleotides), but the shorter t-element is not visible. Again, that could be due to the quality of the recombinant protein preparation.

Identification and Analysis of tRNase Z Knockout Mutants

The physiological function of a protein in Arabidopsis can be analyzed using T-DNA insertion mutants of the respective gene. T-DNA insertion mutants are available for all four tRNase Z genes in different Arabidopsis T-DNA collections (Supplemental Table S2). For all mutants, the presence and the localization of the T-DNA insertion were determined by PCR using genomic DNA and subsequent sequencing analysis. The absence of the corresponding tRNase Z mRNAs was confirmed by northern-blot analyses and reverse transcription-PCR (data not shown). In the T-DNA mutant for the AthTrz^{S1} protein gene, ΔAthZ1, the T-DNA is inserted between the second and fifth exons (the exact localization could not be determined because of a gene rearrangement probably caused by the

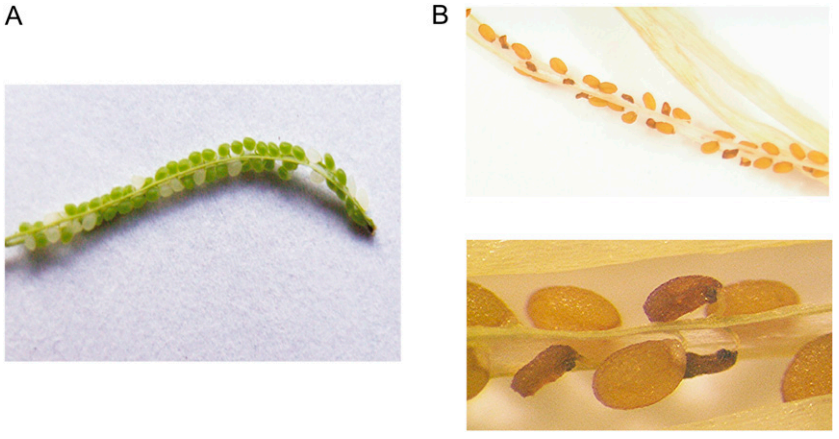

Figure 4. Seed phenotype of *ΔAthTRZ2*. Examples of an immature silique and a dry mature silique from a hygromycin-resistant plant heterozygous for the embryo-defective mutation. A, Immature silique containing green (42) and white (15) seeds. B, Mature silique after desiccation. The mutant seeds are severely shrunken.

insertion itself). In ΔAthZ2, the T-DNA mutant of the AthTrz^{S2} protein gene, the T-DNA is inserted one nucleotide upstream of the initiation codon. In the T-DNA mutants for the genes of the AthTrz^{L1} and AthTrz^{L2} proteins, ΔAthZ3 and ΔAthZ4, the T-DNA is localized in the second and the fifth exon, respectively.

Homozygous plants were isolated for three mutants: ΔAthZ1, ΔAthZ3, and ΔAthZ4. None showed a visible phenotype under standard conditions (see "Materials and Methods"). Since AthTrz^{S1} was shown to be involved in the processing of small nucleolar RNAs (snoRNAs; Kruszka et al., 2003; Barbezier et al., 2009), defects in snoRNA processing were analyzed in ΔAthZ1 by primer extension and RNase mapping (data not shown). No differences in snoRNA processing were detected between wild-type plants and ΔAthZ1. Northern-blot analyses were performed with RNA from ΔAthZ3 and ΔAthZ4 to investigate whether depletion of the respective tRNase Z protein results in processing defects of nuclear or mitochondrial tRNAs. Northern blots containing total RNA from mutant plants were hybridized with probes against nuclear tRNA^{Leu} and with probes against mitochondrial tRNA^{Ser}, tRNA^{Tyr}, tRNA^{Pro}, and tRNA^{Cys}. Again, no differences were detected between wild-type and mutant plants (data not shown).

Depletion of AthTrz^{S2} Results in an Embryo-Lethal Phenotype

It was impossible to obtain a homozygous plant for ΔAthZ2, since the absence of AthTrz^{S2} causes an embryo-lethal phenotype (Figs. 4 and 5). In immature siliques, embryo-defective seeds are readily distinguishable as white seeds compared with the green wild-type seeds (Fig. 4A). About 25% of the seeds are white. In mature siliques, the mutant seeds are severely shrunken (Fig. 4B). Mutant seeds are randomly distributed throughout the siliques. Analysis of these seeds with a microscope using Nomarski optics showed that the homozygous seeds did not develop beyond the globular stage of the embryo. In addition, the embryo in ΔAthZ2 was not detectable after the wild-type embryo reached the curled cotyledon stage of development (Fig. 5).

DISCUSSION

All four tRNase Z enzymes have in vitro tRNA processing activity and are able to generate tRNA 3′ ends ready for CCA addition, showing that they are true tRNase Z enzymes. Generally, the in vitro activity of AthTrz^{L2} is less efficient than that of the other three tRNase Z proteins, which is probably due to the fact that purification of the recombinant protein was not as efficient as for the other proteins (Fig. 2; Supplemental Fig. S2). Localization studies showed that each cell compartment is provided with at least one tRNase Z protein.

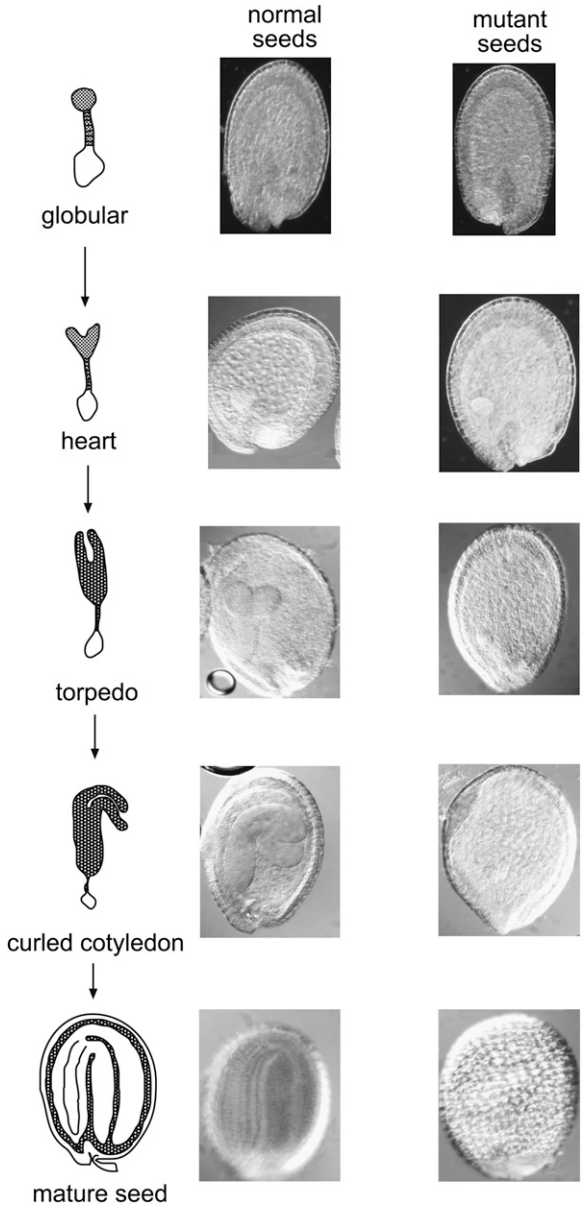

Figure 5. Embryo phenotype of *ΔAthTRZ2*. Seeds at different stages of development were collected from plants heterozygous for the T-DNA insertion in the *TRZ2* gene. Seeds were isolated from the siliques, cleared in Hoyer's solution, and analyzed with the microscope at 10× magnification with Nomarski optics. Seed developmental stages are shown schematically on the left.

Expression of the Chloroplast tRNase Z Protein in Arabidopsis Is Essential

Our in vivo analyses showed that the tRNase Z AthTrz^{S2}, which is located in chloroplasts, has a unique function that cannot be taken on by the other tRNase Z enzymes. AthTrz^{S2} is the only tRNase Z present in chloroplasts, and its function cannot be replaced by other nucleases. The localization of AthTrz^{S2} in chloroplasts is also indicated by the data on the Genevestigator site: the AthTrz^{S2} transcript is poorly

expressed in nongreen tissue and is highly expressed in green and actively dividing tissues, where high amounts of energy are required.

Analysis of heterozygous siliques showed about 25% aborted seeds: embryonic development is arrested at the preglobular/globular stage, which is the most common phase of arrest for embryo-lethal mutations (http://www.seedgenes.org/). Many embryo-defective lethal mutants are known to exhibit a nonrandom distribution of mutant seeds in siliques of heterozygous plants, suggesting that the corresponding genes are expressed prior to fertilization as well as having an essential function during embryogenesis (Meinke, 1991). In the case of the embryo-defective mutation described here, heterozygous mutant plants exhibited a random distribution of mutant seeds, suggesting that expression of *TRZ2* in haploid cells that give rise to pollen grains is not necessary for the development of pollen grains and growth of the pollen tube. Moreover, defective embryos that are homozygous for the mutant gene can develop at least to the globular stage, indicating that expression of the *TRZ2* gene is not necessary for seed development prior to the globular stage.

The role of the chloroplast in embryogenesis has not yet been clarified, but it is accepted that this organelle plays a significant role in plant embryogenesis (Tsugeki et al., 1996; Uwer et al., 1998; Budziszewski et al., 2001). Indeed, embryo-lethal phenotypes are often the result of a loss of function in chloroplast genes or in nuclear genes encoding proteins predicted to be directed to chloroplasts (Budziszewski et al., 2001). To date, it is not clear whether chloroplast proteins are required because of the need for energy at this developmental stage or whether they might have an additional function in embryogenesis.

The developmental stage of the chloroplast appears to regulate the expression of nuclear genes coding for proteins destined for the chloroplast. Inhibition of plastid protein synthesis, resulting from a lack of tRNAs caused by the missing tRNase Z, is expected to interfere with the complex signal-exchange program between plastids and the nucleus. A similar observation was made for mutants defective in the plastid glycyl-tRNA synthetase, since they also show an embryo-lethal phenotype (Ruppel and Hangarter, 2007). The failure to generate plastid proteins at that point of development seems to be signaled to the nucleus, and further development of the embryo ceases.

Two tRNase Z Proteins for Mitochondria

In contrast to plastids, mitochondria contain two different tRNase Z proteins: AthTrz^{L1} and AthTrz^{L2}. The observation that the T-DNA mutants of either of these proteins are viable suggests that they can functionally replace each other. Failure to obtain the double mutant AthTrz^{L1} × AthTrz^{L2} confirms this hypothesis (G. Canino and A. Marchfelder, unpublished data). In vitro processing analyses showed that recombinant AthTrz^{L1} and AthTrz^{L2} can process the mitochondrial t-element structures in precursor RNAs of *cox1* and *ccmC* and to some extent also *nad6*. Thus, these tRNase Z proteins might be responsible for cleavage at these sites in vivo.

One tRNase for the Nucleus

While AthTrz^{L2} is confined to mitochondria, AthTrz^{L1} is located in mitochondria and the nucleus. According to the fluorescence experiments, AthTrz^{L1} is the only tRNase Z protein located in the nucleus. Processing of tRNAs engaged in cytoplasmic protein biosynthesis occurs in the nucleus (Hopper and Phizicky, 2003). Thus, AthTrz^{L1} is the only tRNase Z responsible for the maturation of nuclear tRNAs. The knockout AthTrz^{L1} mutant is viable; therefore, a back-up system must exist for maturation of nuclear tRNAs.

Cytoplasmic Location for AthTrz^{S1}

The AthTrz^{S1}:GFP fusion protein showed diffuse fluorescence in the cell, indicating a cytoplasmic location, and the knockout mutant of AthTrz^{S1} showed no visible phenotype. Since the tRNAs required for cytoplasmic protein biosynthesis are processed in the nucleus, a possible function for a cytoplasmic tRNase Z would be repair of tRNA 3′ ends that have been loaded with only a partial CCA or a mutated CCA sequence, which would prevent interaction with the aminoacyl tRNA synthetases. The cytoplasmic tRNase Z could remove this incorrect CCA sequence to allow the addition of the correct terminal CCA sequence. This potential function of AthTrz^{S1} in the cytosol either is not essential or other proteins can take over its function, since the T-DNA mutant is viable.

Interestingly, it was not possible to obtain the double mutant AthTrz^{S1} × AthTrz^{L1} (G. Canino and A. Marchfelder, unpublished data). This suggests that AthTrz^{S1} is also localized in the nucleus and that the nucleus needs one of the tRNase Z enzymes: either AthTrz^{S1} or AthTrz^{L1}.

MATERIALS AND METHODS

In Vitro Processing Assay

Precursor of the mitochondrial tRNA^{Tyr} from *Oenothera berteriana* was prepared as described previously (Kunzmann et al., 1998). mRNA templates were prepared by PCR using total Arabidopsis (*Arabidopsis thaliana*) DNA. The template for the *cox1* substrate is 255 bp (from –319 to –64) in length. Templates for *ccmC* and *nad6* substrates are 232 bp (–37/195) and 233 bp (–17/+216) long. Primer sequences are available upon request. In vitro transcripts were prepared as described previously (Marchfelder et al., 1990). For each processing reaction, 200 ng of recombinant protein was used. All of the reactions were carried out in Cyto buffer (40 mM Tris, pH 8.4, 2 mM $MgCl_2$, 2 mM KCl, and 2 mM dithiothreitol) in a total volume of 100 μL at 37°C for 30 min. The reaction was stopped by phenol/chloroform extraction, and the products were separated on denaturant 8% polyacrylamide gels. Gels were analyzed by autoradiography.

Analysis of Signal Sequences

Target prediction was performed using the following prediction servers: WoLF PSORT (http://wolfpsort.org/; Horton et al., 2007), Predotar (urgi.versailles.inra.fr/ predotar/predotar.html; Small et al., 2004), and TargetP (www.cbs.dtu.dk/services/TargetP; Emanuelsson et al., 2007).

Expression of Arabidopsis tRNase Z Proteins in *Escherichia coli*

Cloning and purification of recombinant AthTrZ^{S1} (nuz) was carried out as described previously (Schiffer et al., 2002) with an additional purification step through the mini Q column (GE Healthcare; Späth et al., 2005). For the expression of recombinant AthTrZ^{S2}, the corresponding cDNA sequence without the coding sequence for the potential signal peptide was amplified from clone APD12e02 (Kazusa DNA Research Institute) and cloned into the pUC18 vector. The identity of the clone was verified by sequencing. The coding sequence for AthTrZ^{S2} was subcloned into the *Nco*I/*Xho*I sites of the expression vector pET32 (Novagen) and expressed in BL21-CodonPlus (DE3)-RIL as a fusion protein with both His and S tags. After purification using the S tag according to the manufacturer's instructions (Novagen), tags were removed by enterokinase digestion. To exclude the signal peptide, the cDNA from AthTrZ^{L1} was amplified without the first 153 bp (coding sequence for the first 51 amino acids) from the cDNA clone RAFL07-09-G16 (RIKEN Bioresource Center) and cloned into pBluescript KS II. After sequencing, the cDNA was subcloned into the *Not*I/*Xho*I sites of pET29 (Novagen) and expressed in BL21-AI (Stratagene) cells. The cDNA for AthTrZ^{L2} without the coding sequence for the first 66 amino acids representing the calculated target peptide was amplified from the cDNA clone RAFL16-86-L12, cloned in pBluescript KS II, and, after sequencing, subcloned into the pET29 expression vector (Novagen) previously digested with *Bam*HI/*Xho*I. AthTrz^{L2} was expressed in Rosetta cells (Novagen). Both AthTrZ^{L1} and AthTrZ^{L2} proteins were purified using the S tag according to the manufacturer's instructions (Novagen). The concentration of the recombinant proteins was evaluated with the Qubit (Invitrogen).

Cloning of tRNase Z Fusion Proteins with GFP and RFP and Plant Transformation

The complete coding sequences for AthTrZ^{S1} and AthTrZ^{S2} were cloned in frame upstream of the GFP and RFP genes, respectively, into the *Hin*dIII/*Eco*RI sites of the plant transformation vector pBI121 (BD Clontech). The vector was transformed in *Agrobacterium tumefaciens* GV2260 and introduced into Arabidopsis wild-type plants via floral dip (Clough and Bent, 1998). The transformed plants were selected on Murashige and Skoog medium containing kanamycin, and the presence of the construct was verified by PCR on genomic DNA with specific primers for the T-DNA insertion. For the localization of AthTrZ^{L1}, the coding sequence for the first 108 amino acids was cloned into the psmGFP4 vector (Arabidopsis Biological Resource Center) and used to transform tobacco (*Nicotiana tabacum* 'Petit Havana') protoplasts (Koop et al., 1996). For the localization of AthTrZ^{L2}, 497 bp of the coding sequence (corresponding to the first 166 amino acids) was cloned in frame with the GFP frame into the plant transformation vector pBI121 and used to stably transform tobacco by leaf disc transformation (Horsch et al., 1985). Arabidopsis protoplasts were prepared according to Koop et al. (1996). The expression of GFP and RFP fusion proteins in Arabidopsis and tobacco was investigated using a Zeiss Axioplan I microscope (Carl Zeiss). Mitochondria were stained with MitoTracker Red CM-H_2XROS (Molecular Probes).

Microscopy Analysis of Arabidopsis Seeds

Wild-type and mutant seeds were collected at different stages of development from a plant heterozygous for the insertion of the AthZ2 gene. The seeds were cleared in 1:5 Hoyer's solution (3.75 g of arabic gum, 2.5 mL of glycerin, and 50 g of chloral hydrate in 100 mL of water) for 4 h and analyzed under a Zeiss Axioplan I microscope (Carl Zeiss) provided with Nomarski optics and a 10× objective.

Plant Growth Conditions and Analysis of Arabidopsis T-DNA Insertion Lines

Wild-type and transformed plants were grown at 22°C in a 16/8-h light/dark cycle. T-DNA insertion lines were obtained from the Gabi Kat collection (http://www.gabi-kat.de/), the Signal collection of the Salk Institute (http://signal.salk.edu/), and the Cold Spring Harbor Laboratory collection (http://www.cshl.edu/). The presence of the T-DNA insertion in the gene coding for the tRNase Z proteins was determined using PCR on genomic DNA with primers specific for the insertion and the gene. Primer sequences are available upon request. Total DNA was extracted from 3-week-old leaves with the Phytopure DNA Kit (Amersham). Total RNA was extracted from 3-week-old leaves with the plant RNeasy kit (Qiagen).

Supplemental Data

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

Supplemental Figure S1. Conserved MBL motives of Arabidopsis tRNase Z proteins (A and B), and alignment of all four Arabidopsis tRNase Z proteins (C).

Supplemental Figure S2. Recombinant tRNase Z proteins of Arabidopsis.

Supplemental Table S1. Predicted localization of all four tRNase Z proteins.

Supplemental Table S2. Overview of tRNase Z genes, proteins, and T-DNA insertion mutants used in this study.

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