RESEARCH PAPER

The Arabidopsis TRM61/TRM6 complex is a bona fide tRNA N1-methyladenosine methyltransferase

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Abstract
tRNA molecules, which contain the most abundant post-transcriptional modifications, are crucial for proper gene expression and protein biosynthesis. Methylation at N1 of adenosine 58 (A58) is critical for maintaining the stability of initiator methionyl-tRNA (tRNAi Met) in bacterial, archaeal, and eukaryotic tRNAs. However, although research has been conducted in yeast and mammals, it remains unclear how A58 in plant tRNAs is modified and involved in development. In this study, we identify the nucleus-localized complex AtTRM61/AtTRM6 in Arabidopsis as tRNA m1A58 methyltransferase. Deficiency or a lack of either AtTRM61 or AtTRM6 leads to embryo arrest and seed abortion. The tRNA m1A level decreases in conditionally complemented Attm61/LEC1pro::AtTRM61 plants and this is accompanied by reduced levels of tRNAi Met, indicating the importance of the tRNA m1A modification for tRNAi Met stability. Taken together, our results demonstrate that tRNA m1A58 modification is necessary for tRNAi Met stability and is required for embryo development in Arabidopsis.

Keywords: Arabidopsis, AtTRM6, AtTRM61, embryo, N1-methyladenosine, tRNA.

Introduction

Transfer RNA (tRNA) molecules, typically 76–90 nt in length, are responsible for delivering specified amino acids encoded by messenger RNAs (mRNAs) to the protein synthesis machinery in the cytoplasm and organelles. tRNAs therefore play an essential role in gene expression and protein biosynthesis. Pre-tRNAs undergo a variety of post-transcriptional modifications related to their maturation, stability, folding, and functioning (Helm et al., 1998; Hopper and Phizicky, 2003; Motorin and Helm, 2010; Machnicka et al., 2013). At least 96 chemical modifications have been identified on tRNAs (Machnicka et al., 2013). The functional roles of these modifications are dependent on their chemical properties and on the site on the clover-leaf structure of tRNAs (Burgess et al., 2016). In higher plants, as in bacteria and yeast, tRNAs are post-transcriptionally modified with numerous and diverse chemical moieties. A total of 26 RNA modifications have been identified by a combination of chromatography and mass spectroscopy techniques on purified tRNAs in Arabidopsis and hybrid aspen (Chen et al., 2010; Hienzsch et al., 2013). Among the tRNA nucleoside modifications, methylation is the most prevalent and abundant type (Hori, 2014). The existing data indicate that the modified tRNAs are not only essential for translation, but that they also...
functional as 'biosensors' for plants to adapt to their environmental and physical status (Björk and Neidhardt, 1975; Gustilo et al., 2008; Wang et al., 2017). In the model plants Arabidopsis and rice, the Am, Cm, m1A, and m1G methylated nucleosides of tRNAs are important for stress responses, while Gm, m1U, and m5C are involved in development (Chen et al., 2010; Wang et al., 2017). AtTRM5a (At3g56120) catalyses the formation of 1-methylguanosine (m1G) and 1-methylinosine (m1I) at position 37 on tRNAs and is necessary for vegetative and reproductive growth (Jin et al., 2019). TRM4B (At2g22400) acts as a tRNA 5-methylcytosine (m5C) methyltransferase and is required for root development (Burgess et al., 2015; David et al., 2017). AtTRM7 (At5g01230), a 2'-O-ribose methyltransferase, is functional in the efficient immune response to Pseudomonas syringae (Ramirez et al., 2015). AtTAD2 (At1g48175) and AtTAD3 (At5g24670) catalyse adenosine-to-inosine editing at position 34 of several cytosolic (cyt) tRNA species, and knockout of either gene leads to arrested embryo development at the globular stage (Zhou et al., 2014).

RNA N1-methyladenosine is an ancient modification that is conserved throughout archaea, bacteria, and eukaryotes. The free base, 1-methyladenosine (m1A), was first discovered by Dunn (1961) and was later found to be present in tRNAs (RajBhandary et al., 1966), rRNAs (Peifer et al., 2013; Sharma et al., 2013), and mRNAs (Dominissini et al., 2016; Li et al., 2016, 2017). tRNA is an indispensable participant in protein synthesis; however, research on the enzymes and regulation involved in the modification of tRNAs is at an early stage, especially in plants (Burgess et al., 2016). Most current knowledge about tRNA m1A is derived from archaea, bacteria, and eukaryotes other than plants. The m1A nucleoside bears a positive electrostatic charge under physiological conditions, suggesting that it is critical for the structural stability of tRNA through an electro-chemical interaction (Agris, 1996). In (cyt)tRNAs, the m1A modification occurs at positions 9, 14, 22, 57, and 58, and positions 9 and 58 have also been reported in mitochondrial (mt) tRNAs (Jühling et al., 2009; Suzuki et al., 2011; Oerum et al., 2017). The m1A modification at position 58 (m1A58) in the TΨC-arm is the most conserved and common in bacteria, archaea, and eukaryotes (Baker, 1971): m1A14 is rare and is only found in (cyt)tRNA of mammals, m1A22 occurs only in tRNAs from bacteria (Roovers et al., 2008), m1A57 just exists as an intermediate to 1-methylinosine (m1I) by hydrolytic deamination in archaea (Grosjean et al., 1996), and m1A9 has been found in (cyt)tRNA from archaea and mammalian (mt)tRNAs (Helm and Attardi, 2004).

S-adenosyl-L-methionine (AdoMet)-dependent tRNA methyltransferase is responsible for the m1A58 modification of tRNA and it has been identified in several organisms. In yeast, two essential proteins, namely GCD14 (TRM61) and GCD10 (TRM6), work in cooperation as tRNA m1A58 methyltransferase (Anderson et al., 2000). Sequence and architecture analyses have shown that TRM61 and TRM6 share a common ancestor and were produced by gene duplication and divergent evolution (Bujnicki, 2001; Wang et al., 2016). TRM61 contains a methyl donor (S-adenosyl-L-methionine, SAM) binding pocket and functions as the catalytic subunit, whilst TRM6 is short of the conserved motifs for SAM binding and is essential for tRNA binding. The TRM6 and TRM61 heterodimer forms an L-shaped tRNA-binding region (Wang et al., 2016), and two TRM6–TRM61 heterodimers assemble as a functional heterotetramer (Anderson et al., 2000). TRM1, which is the homolog of yeast TRM61, has been identified as the tRNA m1A38 methyltransferase of archaeal and bacterial, and forms a homotetramer to catalyse m1A58 modification (Droogmans, 2003; Roovers et al., 2004). Human hTRM61 (TRMT61A) and hTRM6 (TRMT6) also form a complex as (cyt)tRNA m1A58 methyltransferase (Ozanick et al., 2005). In the mitochondria of human cells, m1A58 modification has been found in tRNALeu(UUR), tRNAIle, and tRNASer(UCN). TRMT61B, a homolog of TRMT61A in humans, forms a homo-oligomer (presumably a homotetramer) and functions as the mitochondria-specific tRNA m1A58 methyltransferase (Chujo and Suzuki, 2012). In addition, m1A is a reversible modification, a trait it has in common with N1-methyladenosine (m1A) modification. The first reversible modification to be discovered, m1A can be erased by two ALKB family proteins, FTO (Fat mass and obesity-associated protein) and ALKBH5 (alkylation repair homologue protein 5) (Jia et al., 2011; Zheng et al., 2013), while m1A58 can be erased by ALKBH1 tRNA demethylase (Liu et al., 2016), indicating that tRNA m1A58 is a dynamic modification.

Functional studies have shown that the m1A58 modification of tRNA is important for cellular activity and development. Inactivation of TRM1 results in a thermosensitive phenotype in the bacterium Thermus thermophilus (Droogmans, 2003). In yeast, deficiency of either subunit of m1A58 methyltransferase results in growth arrest and cell death. Insufficiency of either TRM6 or TRM61 eliminates m1A58 modification in tRNAs, and leads to the formation of a non-tRNA-like structure for the initiator methionine tRNA (tRNAmet) and a reduction in levels of mature tRNAmet (Anderson et al., 1998; Anderson and Droogmans, 2005). The lack of m1A58 modification causes structural instability of tRNAmet by disrupting the unique A54–A58 interaction (Sigler, 1975; Schevitz et al., 1979; Basavappa and Sigler, 1991). In mammalian C6 cells, small interfering RNA (siRNA)-mediated depletion of either TRM6 and/or TRM61 causes significant effects on cell growth, cell death, and tRNAmet levels (Macari et al., 2016). In addition, loss of ALKBH1 causes embryonic lethality, defects in neural development, and distortion of the sex ratio in mice (Pan et al., 2008; Nordstrand et al., 2010; Ougland et al., 2012).

It has been determined that tRNA m1A58 plays important roles in yeast, mammals, and prokaryotes; however, no m1A58 methyltransferase has been yet functionally characterized in plants. Here, we demonstrate that the nuclear-localized AtTRM61/AtTRM6 complex in Arabidopsis acts as a bona fide tRNA m1A58 methyltransferase, and both AtTRM61 and AtTRM6 are essential for embryo development. Insufficient m1A58 modification of tRNA is consistent with low levels of tRNAmet in Attm61/lec1pro::AtTRM61 plants, indicating that tRNA m1A58 is vital for the accumulation of tRNAmet. Our findings shed light on the biological functions of tRNA m1A58 in plants.
Materials and methods

Plant material and growth conditions

Arabidopsis thaliana plants of ecotypes Columbia (Col-0) and Ts-1 were grown in an air-conditioned room at 22 °C under a 16:8-h light/dark cycle (90 μmol m⁻² s⁻¹). Seeds of T-DNA insertion mutants (SALK_024680, CS351262) from the Nottingham Arabidopsis Stock Centre (NASC) were in the Columbia background. Seeds were sterilized with 20% bleach for 10 min, then rinsed five times with sterile water, and germinated on Murashige and Skoog (MS) plates with or without antibiotics.

tRNA isolation and LC-MS analysis

Total RNA was extracted using TRIZol reagent (Invitrogen) according to the manufacturer’s instruction, with an additional DNase I treatment to eliminate DNA contamination. The RNA was then purified using a RNA Clean & Concentrator™-5 kit (R1016, Zymo Research).

tRNA was isolated from the total RNA by gel electrophoresis using 7.5% PAGE (29:1 acrylamide:bisacrylamide) containing 7 M urea. Bands of 60–90 nt tRNA were cut from the gel, extracted using 0.3 M NaHAc, and precipitated with glycogen and ethanol. The purified tRNA was then hydrolysed to single nucleosides and dephosphorylated in a 50-μl reaction containing 10 U benzonase (Sigma), 0.1 U phosphodiesterase 1 (US Biological), and 1 U alkaline phosphatase (NEB). The reaction was kept at 37 °C for 3 h, and then the solution of pre-treated nucleosides was de-proteinized using a Sarotous 10 (000-Da MWCO spin filter). Analysis of the nucleoside mixtures was performed using an Agilent 6460 QQQ mass spectrometer with an Agilent 1260 HPLC system. The multi-reaction monitoring (MRM) mode was used because of the high selectivity and sensitivity attained when working with parent-to-product ion transitions. The LC-MS data were acquired using the Agilent Qualitative Analysis software. The MRM peaks of each modified nucleoside were extracted and normalized to quantify the tRNA modifications (Yan et al., 2013; Su et al., 2015). Briefly, the total RNA solution with an equal activity of T7 polymerase (NEB), after which the tRNAiMet transcript was transcribed of tRNAiMet and northern blotting

Bioinformatics analysis of TRM61 and TRM6 homologues

The sequences of yeast TRM61 and TRM6 were used as queries to BLAST search homologs in the NCBI database (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and aligned sequences were edited with GeneDoc (https://www.softpedia.com/get/Science-CAD/GeneDoc.shtml). A non-rooted Neighbor-joining tree was constructed using the MEGA5 software (https://maldownload.informer.com/mega-5/).

Yeast complementation assays

The following yeast strains were obtained from EUROSCARF (www.euroscarf.de): BY4741 (S288C isogenic yeast strain: MATa; his3D1; leu2D0; met15D0; trm6D0, trm6-506 (BY4741; MATa; trm6D0); leu2D0; his3D1; met15D0; gpd10-506; kanMX), and trm61-4 (BY4741; MATa; trm6D0; his3D1; met15D0; gpd14-4; kanMX).

The coding sequences (CDs) of yeast TRM61 and TRM6, and Arabidopsis AtTRM6 and AtTRM61 were amplified and individually cloned with BamHI and SalI into pESC-Leu, a vector with the galactose-inducible GAL1-10 promoter active in yeast (Ozanick et al., 2005). To construct the vector for co-expression of AtTRM6 and AtTRM61, the AtTRM61 CDS was cloned with NotI and Sp4 into pESC-Leu-AtTRM6 to give pESC-Leu-AtTRM6-AtTRM61. The recombinant vectors were transformed into tnm6-506 and tnm14-4, and the control (empty) vector was transformed into tnm6-506, tnm14-4, and BY4741. The transformants were incubated on SD/–Leu plates (synthetic dextrose minimal medium without leucine). Serial dilutions of these incubations were dotted onto selective SC/gal/raf plates (synthetic complete media supplemented with galactose and raffinose) at permissive (28 °C) and non-permissive (37 °C) temperatures for 3 d.

Yeast two-hybrid and co-immunoprecipitation assays

For yeast two-hybrid (Y2H) assays, the CDS fragments of AtTRM6 and AtTRM61 were cloned into pGADT7 (AD) and pGBKKT7 (BD), respectively (Clontech). The resulting constructs were co-transformed in pairs to yeast strain AH109. The transformed cells were grown on SD/–Leu–Trp (SD/2) and SD/–Trp–Leu–His–Ade (SD/4) plates for 3–7 d at 30 °C.

For co-immunoprecipitation (Co-IP) assays in plant cells, the CDS fragments of AtTRM61 and AtTRM6 were cloned into pBSK-35S-C-3×Myc and pBSK-35S-C-3×HA, respectively. Leaf protoplasts were co-transfected with 100 μg plasmid DNA of these constructs and then incubated overnight at 22 °C with 45 rpm shaking. The protoplasts were harvested by soft spinning at 100 g, collected, and 60 μl was reserved as the control for total protein. The rest was incubated with anti-c-Myc agarose (Sigma) by rotating at 4 °C for 4 h. The samples were centrifuged at 12 000 g for 10 min at 4 °C, the supernatant was collected, and 60 μl was reserved as the control for total protein. The rest was incubated with anti-c-Myc agarose (Sigma) by rotating at 4 °C for 4 h. The samples were spun down at 400 g for 3 min and then rinsed five times with washing buffer (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES, pH 5.7). SDS loading buffer was added to samples before western blotting.

Protein purification and methyltransferase activity assays

The CDSs of AtTRM61 and AtTRM6 were amplified and cloned into pET28a⁺ and pGEX-4T-2 plasmids, respectively. For pull-down assays, the constructs pET28a⁺-AtTRM61 and pGEX-4T-2-AtTRM6 were co-transformed into E. coli, and induced by 0.5 mM IPTG at 20 °C overnight. Ni-NTA agarose was used to immunoprecipitate the AtTRM61-His/AtTRM6-GST complex. Methyltransferase activity assays of purified Arabidopsis enzyme were conducted as described previously (Ozanick et al., 2005). Briefly, activity assays were carried out at 30 °C for 30 min using 30 mM AdoMet, 150 μM RNA substrate, and 15 nM purified

In vitro transcription of tRNAiMet and northern blotting

The genomic tRNAiMet (At2g23020) fragment was amplified by PCR, and the purified product was used as a template for in vitro transcription with T7 polymerase (NEB), after which the tRNAiMet transcript was purified using a RNA Clean & Concentrator™-5 kit (Zymo Research). Northern blotting procedures were performed according to the DIG Application Manual (Roche) using DIG-tRNAiMet as the probe.
proteins. After the reaction, the RNA substrate was purified and the m1A modification level was detected by immuno-northern blotting.

Phenotypic analysis
To determine the embryo phenotype, siliques were dissected using syringe needles and mounted with Herr’s solution containing lactic acid:chloral hydrate:phenol:clove oil:xylene (2:2:2:1, w/w). Embryo development was observed using a Zeiss Axioskop II microscope equipped with differential interference contrast optics.

Vector construction
For genetic complementation and protein subcellular localization analyses, the cGFP tag was fused to the last exon of native AtTRM61 and AtTRM6. For the construction of p1300-AtTRM61pro::AtTRM61::eGFP and p1300-AtTRM6pro::AtTRM6::eGFP, a 2317-bp fragment upstream ATG was used as the AtTRM61 promoter, and a 414-bp fragment downstream TAG was used as the terminator. A 741-bp sequence upstream ATG and a 418-bp sequence downstream TGA were used as the AtTRM6 promoter and terminator, respectively. These constructs were transformed to AtTRM61 and AtTRM6, respectively.

To construct the CRISPR/Cas9 gene-editing vector, the first exon sequences of AtTRM61 and AtTRM6 were each used to search for high-score targets using the software on the CRISPRscan website (http://www.crisprscan.org/index.php?genre=sequence). Software on the CRISPR RGEN Tools website (http://www.rgenome.net/cas-offinder/) was used for off-target analysis. Two targets of AtTRM61 (target1 sequence, CCGTCTTTACTTACTTAACTGG; target2 sequence, TGTCGACAGCTCGGATTGG) and two of AtTRM6 (target1 sequence, TCGCAAGCAATTCTGGAGTTTG; target2 sequence, GTTCTGCTGACATCAACGAT) were chosen and cloned into the vector pHEE401E, and these constructs were transformed into Col-0 plants (Xing et al., 2014).

For gene expression analysis, the promoter sequence of AtTRM61 was cloned into p1300-GUS-3U, and introduced into Col-0.

To construct the conditional complementation vector, p1300-LEC1pro::AtTRM61, a 2.7-kb fragment upstream ATG was used as the LEC1 promoter to drive native AtTRM61 (from ATG to 414 bp downstream of TAG). p1300-LEC1pro::AtTRM61 was then transformed into AtTRM61.

Protein subcellular localization and gene expression analysis
For examination of protein subcellular localization, the roots of 6-d-old transgenic plants were stained using DAPI. Images of the epidermal cells were captured using a confocal laser scanning microscopy (Zeiss). p1300-LEC1pro::AtTRM61 and p1300-LEC1pro::AtTRM6 were stained using DAPI. Images of the epidermal cells were captured using a confocal laser scanning microscopy (Zeiss).

Results
Identification of putative genes for tRNA m1A58 modification in Arabidopsis
Previous studies using HPLC and LC-MS have indicated that modifications of m1A are present on tRNAs of Arabidopsis and rice (Chen et al., 2010; Hienzsch et al., 2013; Wang et al., 2017; Fig. 1A); however, to date no functional studies on plant tRNA m1A modification have been conducted. Here, we used LC-MS to confirm the presence of m1A modification on tRNAs from 7-d-old seedlings of Arabidopsis (Supplementary Table S1 at JXB online). To provide rapid identification of the modification, a method based on RNA m1A antibody immuno-northern blotting was adapted to detect and quantify the level of m1A. Using this technique, we confirmed that the m1A modification was indeed present in tRNAs from different tissues in Arabidopsis (Fig. 1B).

The m1A58 modification on tRNAs is catalysed by the TRM61/TRM6 complex in Saccharomyces cerevisiae (Anderson et al., 1998). Therefore, we used the yeast TRM61 and TRM6 protein sequences as queries to BLAST search m1A candidate genes in the Arabidopsis genome. At5g14600 was the only candidate for yeast TRM61, with a blastP E-value of 2×10−54 and 41% protein sequence similarity, and it was named as AtTRM61 in TAIR. TRM61 is a conserved protein in archaea, eubacteria, protists, fungi, plants, and animals (Supplementary Fig. S1), but in the phyllogenetic tree of the homologs, the plant TRM61s were clearly separated from the other groups (Fig. 1C). Similarly, At2g45730 was the best candidate for the yeast TRM6 homolog in Arabidopsis, with a PSI-BLAST E-value of 5×10−7 and 28% protein sequence similarity, and it was named as AtTRM6 in TAIR. TRM6 is a conserved protein in Eukarya (Supplementary Fig. S2), and in the phyllogenetic tree of eukaryotic TRM6 homologs, animal and yeast TRM6s were separated from those of plants (Fig. 1D).

AtTRM61 and AtTRM6 together rescue yeast m1A58-defective mutants
To investigate whether the candidate genes were true orthologs for TRM61 and TRM6, a yeast complementation assay was employed. The candidate AtTRM61 and AtTRM6 genes were introduced into corresponding yeast mutants, trm61-4 and trm6-506, respectively (Supplementary Figs S1, S2). The results showed that cells containing yeast TRM61, both AtTRM61 and AtTRM6, and the wild-type control all grew well at 37 °C (Fig. 2A); however, the trm61-4 mutant cells containing AtTRM61 alone did not survive. This indicated that AtTRM61 alone could not rescue the temperature-sensitive phenotype, whilst co-expression of AtTRM61 and AtTRM6 could. Similarly, only co-expression of AtTRM61 and AtTRM6, and not AtTRM6 alone, could rescue the temperature-sensitive phenotype of the trm6-506 mutant (Fig. 2C). Immuno-northern blotting was also used to further determine whether m1A levels in tRNAs were recovered in the transgenic yeast mutants. The m1A levels of mutant strains with co-expression of AtTRM61 and AtTRM6 were clearly increased, but this was not the case when AtTRM61 or AtTRM6 were expressed alone (Fig. 2B, 2D). Since TRM61 and TRM6 function as a heterodimer, it is most likely that AtTRM61 and AtTRM6 were not able to form functional heterodimers with their yeast counterparts, but they were able form a functional heterodimer together in the yeast cells. Similarly, human hTRM6 does not work sufficiently to complement yeast trm6-504, and the yeast mutant can only be rescued by co-expression of hTRM6 and hTRM61 (Ozanick et al., 2005). Overall, our data suggested that AtTRM6 and AtTRM61 function as a tRNA m1A58 methyltransferase heterodimer in yeast.
AtTRM61 and AtTRM6 form a complex in Arabidopsis

To investigate whether AtTRM61 and AtTRM6 interact in Arabidopsis in the same way that TRM61 and TRM6 function as a heterotetramer to synthesize m^1A in tRNA in yeast, Y2H, pull-down, and Co-IP assays were performed. The results indicated that yeast cells co-expressing BD-AtTRM61 and AD-AtTRM6 grew well on SD/-Trp–Leu–His–Ade selection medium but the control did not (Fig. 3A), which suggested that AtTRM61 interacted with AtTRM6 in yeast. To demonstrate their physical interaction, a pull-down experiment was carried out by co-expression of AtTRM61-His and AtTRM6-GST in E. coli, and this indeed showed that AtTRM61-His was able to pull-down AtTRM6-GST (Fig. 3B). These results indicated that AtTRM61 could interact with AtTRM6 in vitro. To further verify whether this interaction occurred in Arabidopsis cells, a protoplast Co-IP assay was performed in which AtTRM61-Myc and AtTRM6-HA were co-transformed into leaf protoplasts. The results showed that AtTRM6-HA could be co-immunoprecipitated by AtTRM61-Myc in an anti-c-Myc agarose pull-down assay (Fig. 3C). These data together demonstrated that AtTRM61 could interact with AtTRM6 in Arabidopsis.

The AtTRM61/AtTRM6 complex has tRNA m^1A methyltransferase activity in vitro

To examine the enzyme activity of the AtTRM61/AtTRM6 complex, AtTRM61-His and AtTRM6-GST were co-expressed in E. coli cells and the AtTRM61-His/AtTRM6-GST complex was purified using Ni-NTA agarose (Fig. 3D). The purified complex was then incubated with a methyl donor (SAM) together with a substrate tRNAs extracted from the yeast mutant trm6-506 cells, which lacked m^1A modification. This resulted in m^1A modification being detected by an anti-m^1A antibody (Fig. 3E), demonstrating that the AtTRM61/AtTRM6 complex did indeed possess methyltransferase activity. Furthermore, the AtTRM61/AtTRM6 complex was able to catalyse the formation of m^1A on an in vitro transcribed tRNA in Arabidopsis, initiator methionyl-tRNA (tRNA^Met^t, At2g23020) (Fig. 3F). These results confirmed that the AtTRM61/AtTRM6 complex functions as a tRNA m^1A methyltransferase in vitro.
The AtTRM61/AtTRM6 complex is a tRNA m1A methyltransferase

Fig. 2. Yeast complementation assays using AtTRM61 and AtTRM6. (A) BY4741 and trm61-4 cells were transformed with the control vector pESC-Leu, and trm61-4 cells were also transformed with pESC-Leu carrying TRM61, AtTRM61, or AtTRM61/AtTRM6. The transformants were incubated on SD/–Leu plates and then serial dilutions were dotted onto selective SCgus/raf plates at a permissive (28 °C) or non-permissive (37 °C) temperature for 3 d. Each column is a 10-fold dilution. (B) Detection of tRNA m1A levels by immuno-northern blotting in the trm61-4 complement strains. (C) BY4741 and trm6-506 cells transformed with the control vector pESC-Leu, and trm6-506 cells complemented with TRM6, AtTRM6, or AtTRM61/AtTRM6. (D) Detection of tRNA m1A levels by immuno-Northern blotting in the trm6-506 complement strains.

Fig. 3. AtTRM61 and AtTRM6 form a complex and show tRNA m1A methyltransferase activity in vitro. (A) AtTRM61 interacts with AtTRM6 in yeast two-hybrid assays. SD/2, SD/–Leu–Trp; SD/4, SD/–Trp–Leu–His–Ade. (B) AtTRM61 interacts with AtTRM6 in pull-down assays. (C) Co-immunoprecipitation assays indicate the interaction between AtTRM61 and AtTRM6 in Arabidopsis protoplasts. (D) SDS-PAGE of the purified AtTRM61-His/AtTRM6-GST complex. (E) tRNA of trm6-506 was used as the substrate in m1A methyltransferase activity assays. AdoMet, S-adenosyl-L-methionine. (F) In vitro transcribed tRNA$^{\text{Met}}$ of Arabidopsis was used as the substrate in m1A methyltransferase activity assays.
AtTRM61 and AtTRM6 are essential for embryo development

To study the function of tRNA m1A58 modification in Arabidopsis, the T-DNA insertion mutants of AtTRM61 (Salk_024680) and AtTRM6 (CS351262) were obtained from NASC and renamed as Attrm61 and Attrm6, respectively. Both mutants contain a T-DNA in the second intron of the gene (Fig. 4A, 4B); however, no homozygous plants could be recovered for either of the mutants (n=300 in each case). To investigate whether the insertion affected the targeted gene expression, we made use of the SNPs present in the first exon of AtTRM61 and in the tenth exon of AtTRM6 between the Col-0 and Ts-1 ecotypes, respectively (Fig. 4C, 4D). Heterozygous Attrm61 and Attrm6 (Col-0) were crossed with wild-type Ts-1 plants. The full-length CDSs of AtTRM61 and AtTRM6 were amplified by RT-PCR from RNAs extracted from F1 plants with or without the T-DNA insertion and sequenced. The sequencing signal of AtTRM61 mRNA in the Col-0 version (AtTRM61/Col-0) from plants harboring the T-DNA insert was lower than that of plants without T-DNA (Fig. 4E). The RT-PCR products were subsequently cloned individually to a T-vector and hundreds of colonies were sequenced. In F1 plants without T-DNA, the ratio of AtTRM61/Col-0 mRNA was 82.4% [C/(C+T), n=111]; however, in those plants with the T-DNA insertion, the ratio of AtTRM61/Col-0 mRNA was 35.7% (n=135). These results indicated that the T-DNA insertion led to a reduction of the level AtTRM61 mRNA to 43.3% of that of the control (Fig. 4F). This indicated that Attrm61 is a knockdown mutant. AtTRM6 mRNA (AtTRM6/Col-0) could not be detected in F1 plants with the mutation (Fig. 4G), which indicated that Attrm61 is an AtTRM6 knockout mutant.

Since no homozygous plants of the Attrm61 and Attrm6 mutants were obtained, it was indicative of embryo lethality. Full seed set was observed in wild-type siliques (Fig. 4H), whereas 25.32% (n=661) ovules were aborted in Attrm61 plants (Fig. 4H). Siliques from heterozygous Attrm61 plants were serially dissected and the ovules were carefully observed after whole-mount clearing with Herr's solution. In siliques at 4 d after pollination (DAP), the embryos of normal ovules had reached the heart stage of development; however, the embryos of the aborted ovules were arrested between the one- and four-cell stage (Fig. 4I). Similarly, the seed abortion in Attrm6 heterozygous plants was 27% (n=602) (Fig. 4H) and mutant embryos were arrested at the one-cell stage, in contrast to normal embryos that were at the globular stage in the same siliques at 3 DAP (Fig. 4K). These data suggested that early embryo development was impaired by knockdown of AtTRM61 and by knockout of AtTRM6.

Genetic complementation assays were performed by introducing constructs of AtTRM61-eGFP and AtTRM6-eGFP in native regulatory expression boxes to heterozygous Attrm61 and Attrm6 plants. Plants homozygous for the Attrm61 or Attrm6 mutations with full seed set were obtained (Fig. 4H), confirming that the observed embryo lethality was indeed caused by loss of function of AtTRM61 or AtTRM6. This was further confirmed by CRISPR-Cas9 mutation of either AtTRM61 or AtTRM6. Target sites were chosen on the first exons of AtTRM61 and AtTRM6 for construction of the knockout vectors and the constructs were introduced individually into Col-0. Transgenic plants were identified by PCR followed by sequencing, and we obtained eight heterozygous mutants of AtTRM61 and six of AtTRM6. To confirm the mutation type, the PCR products were cloned into a T-vector and sequenced. We found an AtTRM61 knockout heterozygous plant that contained an adenine insertion after +156 bp of the first exon and an early stop codon at +207 bp (Supplementary Fig. S3A, B). In the T$_3$ generation, PCR screening identified a heterozygous plant that was AtTRM61-knockout and CRISPR-Cas9 T-DNA-free (hereafter referred to as AtTRM61CR plants). The seed set of AtTRM61CR plants indicated that the rate of aborted seeds (23%, n=425) was consistent with that of the Attrm61 mutant (Fig. 4H), where embryos were also arrested at the one- to four-cell stage (Fig. 4J). Similarly, in AtTRM6CR plants, an adenosine was inserted after +104 bp in the first exon and caused a premature stop codon at position +114 bp (Supplementary Fig. S3C, D). AtTRM6CR plants displayed 24.3% (n=1025) seed abortion (Fig. 4H), with embryos arrested at the one-cell stage (Fig. 4L). Taken together, these results demonstrated that AtTRM61 and AtTRM6 are essential to embryo development in Arabidopsis.

AtTRM61 and AtTRM6 are nuclear proteins expressed in fast-growing tissues

Previous studies have shown that yeast TRM61 and TRM6 are both localized in the nucleus (Anderson et al., 1998), whilst human hTRM61 is found in the nucleus and cytoplasm, and hTRM6 is in the nucleus (Macari et al., 2016). To examine the subcellular localization of AtTRM61 and AtTRM6, transgenic plants expressing AtTRM61::GFP and AtTRM6::GFP were used, and the fluorescent signals were found to be co-localized with the DAPI signal in the nucleus for both constructs (Fig. 5). These results indicated that both AtTRM61 and AtTRM6 are nuclear proteins.

To investigate the expression patterns of AtTRM61 and AtTRM6, we used qRT-PCR and a GUS reporter system. qRT-PCR analysis indicated that AtTRM61 and AtTRM6 transcripts were expressed in vegetative organs such as roots, stems, cauline and rosette leaves, inflorescences, opening flowers, and siliques (Fig. 6A, B). The GUS reporter system indicated that the AtTRM61 promoter was very active in the fast-dividing cells of the root and shoot tips, inflorescences, flowers, siliques, ovules, and embryos (Fig. 6C). Thus, AtTRM61 and AtTRM6 shared similar expression patterns and encoded nuclear proteins, suggesting that they probably play a role in plant growth and development.

The level of tRNA$^{Met}$ is correlated with tRNA m1A modification

Given that AtTRM61 and AtTRM6 are essential for embryo development, it was not possible for us to obtain a homozygous mutant of either gene. To investigate whether the AtTRM61/AtTRM6 complex was a tRNA m1A methyltransferase in...
vivo, we employed a conditional complementation approach. The AtTRM61 coding region driven by the 2.7-kb promoter of the embryo-specific gene LEAFY COTYLEDON 1 (LEC1) (Lotan et al., 1998), (LEC1pro::AtTRM61), was constructed and transformed into Attrm61 heterozygous plants. A total of 18 transgenic lines were obtained and nine of them
Fig. 5. AtTRM61 and AtTRM6 are nuclear proteins. Subcellular localization of AtTRM61 and AtTRM6 in the root cells of 6-d-old seedlings as determined by DAPI staining and green fluorescent protein (GFP) analysis of transformed plants.

Fig. 6. AtTRM61 and AtTRM6 are highly expressed in fast-growing tissues of Arabidopsis. Relative expression of (A) AtTRM61 and (B) AtTRM6 in different tissues as determined by qRT-PCR. Rt, roots; St, stems; CL, cauline leaves; RL, rosette leaves; IF, inflorescences; OF, open flowers; Sil, siliques. Expression is relative to that of the ACTIN2 gene. Data are means (±SE) of three replicates. (C) GUS staining of AtTRM61pro::GUS plants. Arrows indicate regions where AtTRM61 was actively expressed. Scale bars are 1 mm in the top row of images and 20 μm in the bottom row.
The AtTRM61/AtTRM6 complex is a tRNA m1A methyltransferase.

The homozygous Attm61/LEC1pro::AtTRM61 plants bore short siliques (Fig. 7A–D), but they contained normally developed seeds (Fig. 7B). The expression level of AtTRM61 was restored in the siliques of Attm61/LEC1pro::AtTRM61 plants to 4.47-fold that of the wild-type (Fig. 7E), indicating that this transgene was expressed specifically under control of LEC1pro. As expected given that Attm61 is a knockdown mutant (Fig. 4E, F), the expression of AtTRM61 in organs other than the siliques was lower than that of the wild-type (42% and 52% compared with the wild-type for leaves and inflorescences, respectively). Correlated with the expression of AtTRM61, the level of tRNA m1A modification was similar to that of the wild-type in siliques but decreased dramatically in

Fig. 7. Decreased expression of AtTRM61 results in reduced tRNA m1A modification and decreased levels of tRNAiMet. (A) Phenotypes of Attm61/LEC1pro::AtTRM61 and the wild-type (WT). Arrows indicate siliques. (B, C) Close-up images of the short siliques of Attm61/LEC1pro::AtTRM61. (D) Seed numbers in Attm61/LEC1pro::AtTRM61 and the WT. Data are means (±SE), n=30. The significant difference was determined using Student's t-test: **P<0.01. (E) Relative expression of AtTRM61 in different tissues of the WT and Attm61/LEC1pro::AtTRM61, as determined by qRT-PCR. Expression is relative to that of the ACTIN2 gene. Data are means (±SE) of three replicates. (F) tRNA m1A modification in different tissues as determined by immuno-northern blotting and northern blotting of tRNAiMet. Lanes 1, 3, 5 are the WT; Lanes 2, 4, 6 are Attm61/LEC1pro::AtTRM61.
vegetative organs including leaves and in florescences (Fig. 7F).

Interestingly, the m\(^{1}\)A modification of tRNA was not increased even in silences that overexpressed \( \text{AtTRM61} \) compared to the wild-type, indicating that overexpression of one component of the \( \text{AtTRM61/AtTRM6} \) complex did not correspondingly increase the level of tRNA m\(^{1}\)A modification. This suggested that \( \text{AtTRM61} \) is indeed involved in tRNA m\(^{1}\)A modification in Arabidopsis cells, and that the \( \text{AtTRM61/AtTRM6} \) complex acts as a tRNA m\(^{1}\)A methyltransferase in vivo.

In yeast and mammals, deficiency of tRNA m\(^{1}\)A58 results in instability of tRNA\(^{\text{Met}}\) reduced levels of mature tRNA\(^{\text{Met}}\), and interference in the initiation of translation (Anderson et al., 1998; Liu et al., 2016; Macari et al., 2016). We therefore examined the correlation of the abundance of tRNA\(^{\text{Met}}\) and tRNA m\(^{1}\)A levels in different parts of \( \text{Attm61/LEC1prc::AtTRM61} \) using s northern blotting method. The levels of tRNA\(^{\text{Met}}\) decreased in tissues with low levels of tRNA m\(^{1}\)A, except for very similar levels of tRNA\(^{\text{Met}}\) compared to the wild-type in the silences where the \( \text{LEC1} \) promoter is specifically active, where a normal abundance of tRNA m\(^{1}\)A was observed (Fig. 7F).

These results indicated that the abundance of tRNA\(^{\text{Met}}\) was closely associated with the level of tRNA m\(^{1}\)A modification in Arabidopsis.

**Discussion**

In this study, we have shown that the heterodimeric complex \( \text{AtTRM61/AtTRM6} \) localized in the nucleus acts as a \textit{bona fide} tRNA \( m^{1}\)A58 methyltransferase in Arabidopsis. The genes of both proteins were ubiquitously expressed throughout the plant and were essential for embryo development, and knockdown or knockout of \( \text{AtTRM61 or AtTRM6} \) caused arrest of embryo development at an early stage. The level of tRNA\(^{\text{Met}}\) was correlated with tRNA m\(^{1}\)A modification, with low levels of tRNA m\(^{1}\)A resulting in reduced tRNA\(^{\text{Met}}\), with subsequent disruption of translation of proteins.

Quite a few tRNA modification enzymes are well characterized in \( E.\ coli \) and \( S.\ cerevisiae \), and some of them have been to be found fundamental for viability (Persson et al., 1992). In \( S.\ cerevisiae \), there are three enzymes for tRNA modification that are essential, TRM61/TRM6 for m\(^{1}\)A58 (Anderson et al., 1998), TAD2/TAD3 for adenosine-to-inosine editing at position 34 (Gerber and Keller, 1999), and THG1 for the attachment of a guanine nucleotide to the 5′-end of tRNA\(^{\text{His}}\) (Gu et al., 2003). In Arabidopsis, it has been shown that the AtTAD2/AtTAD3 complex that edits adenosine-to-inosine at position 34 of several (cyt)tRNA species is dispensable for embryo development (Zhou et al., 2014). I34 at the first position of the anticodon is believed to play a critical role in protein synthesis, because it can base-pair with A, C, or U, and this alternative pairing in the third position of codons allows a tRNA to decode multiple codons for the same amino acid (Crick, 1966; Jukes, 1973; Elias and Huang, 2005). However, in Arabidopsis chloroplasts (plastids), a nucleus-encoded tRNA adenosine deaminase arginine (TADA) that is responsible for deamination of the wobble nucleotide of chloroplast tRNA\(^{\text{Arg}}(\text{AGC})\) to tRNA\(^{\text{Arg}}(\text{ICG})\) is not necessary for plant survival (Delannoy et al., 2009). Our results showed that another tRNA methyltransferase, the tRNA m\(^{1}\)A58 methyltransferase complex \( \text{AtTRM61/AtTRM6} \), is essential in Arabidopsis, as it is also necessary in yeast. Knockout of either one of \( \text{AtTRM61/AtTRM6} \) caused early embryo arrest (Fig. 4), indicating the key role of the complex in embryogenesis. In yeast and mammals, tRNA m\(^{1}\)A58 is a major modification and is critical for the stability of tRNA\(^{\text{Met}}\), which is vital for the initiation of translation (Liu et al., 2016). Hypomodified tRNA\(^{\text{Met}}\) can be polyadenylated by the TRAMP complex and then degraded by exonuclease Rex1p and exosome in yeast (Ozanick et al., 2009). Plant embryo development is characterized by large and rapid amounts of protein synthesis, so the highly dynamic proteome at different stages of embryogenesis will depend on a plentiful supply of tRNAs. The embryo lethality of \( \text{Attm61 or Attm6} \) may have been caused by insufficient tRNA\(^{\text{Met}}\) (Fig. 7F), as suggested in yeast (Anderson et al., 2000), or by a general impact on all tRNAs.

tRNAs are highly modified. Cell biology studies have indicated that tRNA modifications are processed via a temporal-special program in the nucleus or cytoplasm. Among the numerous types of modifications, m\(^{1}\)A58 occurs first, as it takes place on the initial transcripts of tRNA in the nucleus (Hopper, 2013). Our results suggested that the m\(^{1}\)A58 modification in Arabidopsis was also processed in the nucleus because both \( \text{AtTRM61 and AtTRM6} \) were found to be nuclear proteins (Fig. 5). It is not known if the \( \text{AtTRM61/AtTRM6} \) complex acts on methylation at other positions of tRNA within the nucleus, but it is not likely that the complex is responsible for modifications generated in the cytoplasm. It is also not conceivable that the \( \text{AtTRM61/AtTRM6} \) complex acts in methylation at other positions of tRNA within the nucleus, but it is not likely that the complex is responsible for modifications generated in the cytoplasm. The mechanism that underlies this tRNA modification is unknown in these two organelles, although mammalian TRMT6B (a homolog of TRMT61A) has been identified as a mitochondria-specific tRNA m\(^{1}\)A58 methyltransferase (Chujo and Suzuki, 2012). We therefore implemented a BLASTp search in Arabidopsis for homologs of human TRMT61B, but no candidate other than \( \text{AtTRM61} \) showed an \( E \)-value below \( 1 \times 10^{-6} \). Previous phylogenetic analysis indicates that TRMT61B is of bacterial origin (Chujo and Suzuki, 2012), so we used the protein sequence of TRM1 of \( T.\ thermophilus \) as a query to search for homologs in Arabidopsis. Apart from \( \text{AtTRM61} \), the only other homolog with a BLASTp value below \( 1 \times 10^{-6} \) was \( \text{AtPIMT1} \) (protein L-isoaspartyl methyltransferase; \( E \)-value \( 4 \times 10^{-7} \)). The PIMT enzyme system functions in protein repair and can recognize abnormal L-isoaspartyl residues in polypeptides and convert them to the normal L-aspartyl form. PIMT has consistently been found to be crucial to resistance
to ageing, high temperatures, and oxidative stress in bacteria and animals (Mishra and Mahawar, 2019). There are two PIMT genes in rice and Arabidopsis, and they are involved in both seed longevity and germination vigor (Xu et al., 2004; Ogé et al., 2008; Wei et al., 2015). AtPIMT1 is a cytosol protein, while PIMT2 isoforms are localized to the nucleus (Xu et al., 2004). It would be interesting to determine whether PIMT can methylate tRNAs, and further, whether cytosolic PIMT targets to mitochondria or plastids in Arabidopsis. Recent studies have shown that the TRM61A/TRM6 complex is involved in some m1A modifications on mRNA in human cells (Li et al., 2017), but whether AtTRM61/AtTRM6 has similar functions in Arabidopsis needs further study.

In summary, we have identified the AtTRM61/AtTRM6 complex as the tRNA m1A methyltransferase. Both AtTRM61 and AtTRM6 are nuclear proteins and are required for embryogenesis and protein translation. Our work therefore highlights the importance of tRNA m1A modification for embryogenesis in Arabidopsis.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Chemical modifications detected in tRNAs of Arabidopsis as determined by LC-MS.

Table S2. Primers used in this study.

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