Identification of Enzymes for Adenosine-to-Inosine Editing and Discovery of Cytidine-to-Uridine Editing in Nucleus-Encoded Transfer RNAs of Arabidopsis[1][W]

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In all organisms, transfer RNAs (tRNAs) contain numerous modified nucleotides. For many base modifications in tRNAs, the functional significance is not well understood, and the enzymes performing the modification reactions are unknown. Here, we have studied members of a family of putative nucleotide deaminases in the model plant Arabidopsis (*Arabidopsis thaliana*). We show that two Arabidopsis genes encoding homologs of yeast (*Saccharomyces cerevisiae*) tRNA adenosine deaminases catalyze adenosine-to-inosine editing in position 34 of several cytosolic tRNA species. The encoded proteins (AtTAD2 and AtTAD3, for tRNA-specific adenosine deaminase) localize to the nucleus and interact with each other in planta in bimolecular fluorescence complementation and coimmunoprecipitation assays. Both AtTAD2 and AtTAD3 are encoded by essential genes whose knockout is lethal and leads to arrested embryo development at the globular stage. Knockdown mutants for *AtTAD2* and *AtTAD3* display reduced growth and inefficient editing from adenosine to inosine in six nucleus-encoded tRNA species. Moreover, upon comparison of DNA and complementary DNA sequences, we discovered cytidine-to-uridine RNA editing in position 32 of two nucleus-encoded serine tRNAs, tRNA-serine(AGA) and tRNA-serine(GCT). This adds a unique type of RNA editing to the modifications occurring in nuclear genome-encoded RNAs in plants.

tRNAs undergo extensive posttranscriptional base modifications in all organisms and genetic compartments that have been investigated (Sprinzl et al., 1998; Helm, 2006; Kellner et al., 2010; Phizicky and Alfonzo, 2010). These modifications are introduced at the posttranscriptional level by specific enzymes. The enzymes recognize polynucleotide substrates and modify individual nucleotide residues at highly specific sites (Martin and Hopper, 1994; Slany and Kersten, 1994; Alfonzo et al., 1999; Grosjean et al., 2010; Motorin et al., 2010). Modifications in the anticodon usually alter decoding specificity and/or improve decoding accuracy. For example, the hydrolytic deamination of adenosine to inosine in position 1 of the anticodon facilitates wobbling, as noted already by Francis Crick (Crick, 1966). Adenosine-to-inosine (A-to-I) conversion is also referred to as tRNA editing, because (1) A-to-I modifications also occur in nucleus-encoded mRNAs of various animals (Sommer et al., 1991; Hurst et al., 1995; Petschek et al., 1996; Patton et al., 1997) and (2) inosine differs in its base-pairing properties from adenosine in that it preferentially pairs with C rather than U. Consequently, A-to-I changes often alter the coding properties of edited mRNAs (Bass, 1997; O'Connell, 1997) or the binding specificity of edited microRNAs (Kawahara et al., 2007).

In *Escherichia coli* and yeast (*Saccharomyces cerevisiae*), A-to-I conversion in the wobble position (position 34) of the anticodon is an essential tRNA modification in that its loss is incompatible with cellular viability (Gerber and Keller, 1999; Wolf et al., 2002). In *E. coli*, inosine occurs in the wobble position (position 34) of the anticodon of tRNA-Arg(ACG). The specific adenosine deaminase conducting this conversion (termed tadA, for tRNA-specific adenosine deaminase) is an essential enzyme, and inactivation of the *tadA* gene is lethal, presumably because of the requirement for inosine in the wobble position to read the three Arg codons CGU, CGC, and CGA. A tadA homolog exists in chloroplasts (plastids) of plants and generates inosine-34 in the same tRNA species, tRNA-Arg(ACG) (Delannoy et al., 2009; Karcher and Bock, 2009). Interestingly, the gene encoding the plant tadA homolog (TADA) is not essential, even though plastid protein biosynthesis (Ahlert et al., 2003; Rogalski et al., 2006, 2008b; Fleischmann et al., 2011) and most plastid tRNA species (Rogalski et al., 2008a; Alkatib et al., 2012a, 2012b) are known to be essential for cell survival.

In yeast, seven cytosolic tRNAs undergo A-to-I editing in the wobble position of the anticodon. This modification is performed by a complex of two proteins (TAD2 and TAD3), both of which are encoded by essential genes (Gerber and Keller, 1999). Eukaryotic A-to-I tRNA deaminases are also referred to as ADATs (for adenosine deaminases acting on tRNA). Their deaminase domain comprises three highly conserved zinc-binding amino acid residues and a proton-shuttling Glu residue (Rubio et al., 2007; Fig. 1; Supplemental Figs. S1 and S2). Using this canonical deaminase motif and performing homology searches, we have identified putative A-to-I

[1] This work was supported by the Max Planck Society.

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Ralph Bock (rbock@mpimp-golm.mpg.de).

[W] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.114.250498

editing deaminases in plants (Karcher and Bock, 2009; Zhou et al., 2013; Fig. 1). We report here the functional analysis of three previously uncharacterized members of a gene family encoding putative nucleotide deaminases in the model plant Arabidopsis (*Arabidopsis thaliana*). Our work led to the identification of two proteins involved in generating the wobble inosine in cytosolic tRNAs and the discovery of a unique type of RNA editing in the plant nucleus.

RESULTS AND DISCUSSION

Identification of Putative Adenosine Deaminases in the Arabidopsis Nuclear Genome

In previous reports, we described a genome-wide search for candidate RNA-editing deaminases in the model plant Arabidopsis (Karcher and Bock, 2009; Zhou et al., 2013). The search employed the Patmatch tool (http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl)

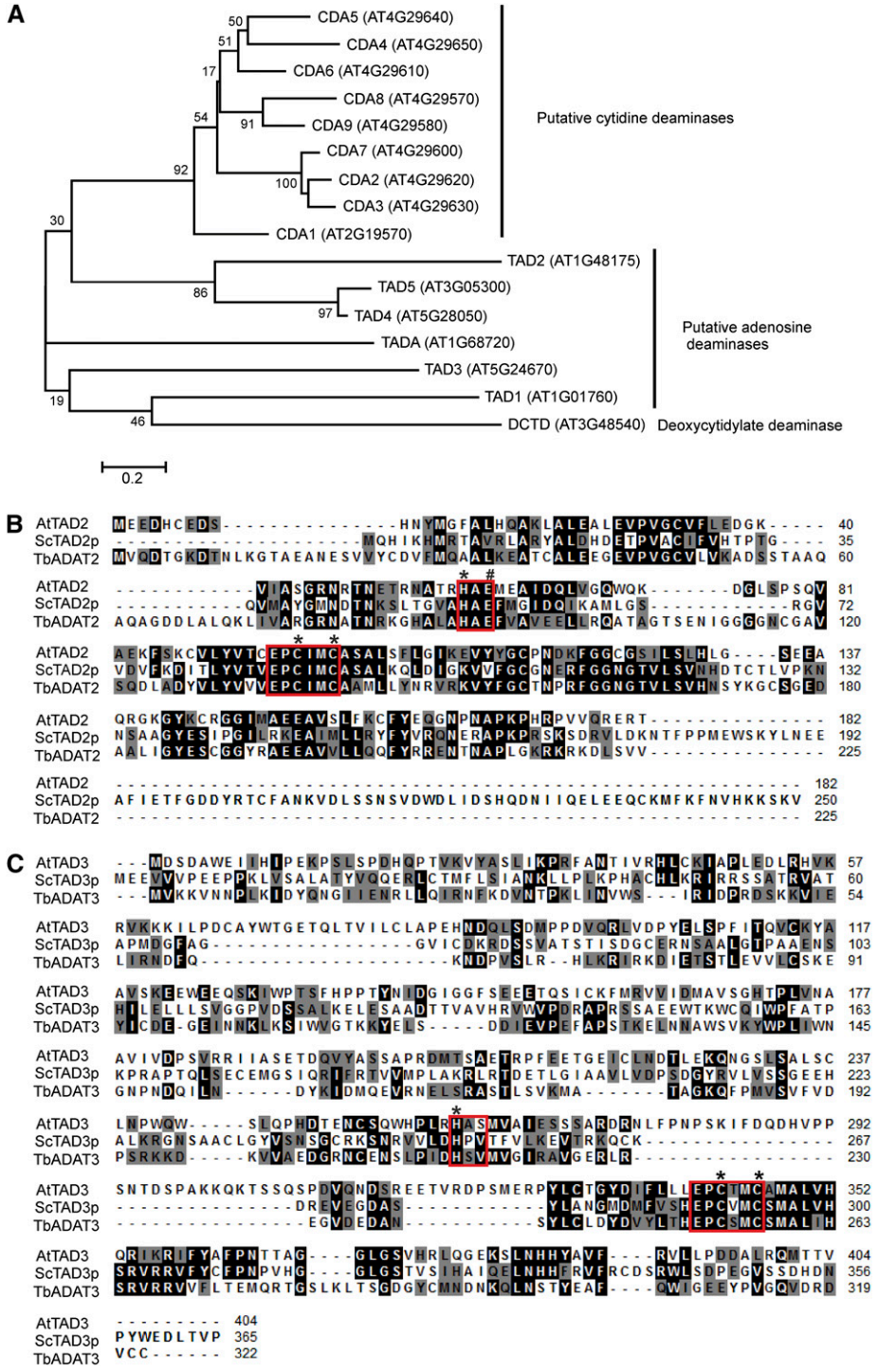

Figure 1. Identification of putative TAD genes in the genome of the model plant Arabidopsis. A, Phylogenetic tree analysis of putative purine and pyrimidine deaminases in Arabidopsis. Deaminases were initially aligned with ClustalW, and MEGA (version 5.05; http://www.megasoftware.net) was used to construct a phylogenetic tree based on the neighbor-joining method with 1,000 bootstrap replications. Bootstrap values are given as percentages of the 1,000 replications at each node. The scale bar indicates amino acid substitutions per site. B, Amino acid sequence alignment of the candidate AtTAD2 protein from Arabidopsis with the ScTAD2p protein from yeast (Gerber and Keller, 1999) and its homolog TbADAT2 from the protozoan parasite *Trypanosoma brucei* (Rubio et al., 2007). C, Amino acid sequence alignment of the candidate AtTAD3 protein from Arabidopsis with the ScTAD3p protein from yeast and its homolog TbADAT3 from *T. brucei*. Black and gray boxes indicate identical and similar amino acids, respectively. Dashes represent gaps in the sequences. The deaminase motifs are marked with red boxes. The His and two Cys residues involved in the coordination of a zinc ion are indicated by asterisks, and the essential Glu residue in the active site of the enzyme is denoted with a hash mark.

and was based on the consensus sequence motifs in the catalytic domain of all RNA-editing deaminases (Rubio et al., 2007). It uncovered several candidate proteins that were subsequently analyzed by BLAST searches to identify putative homologs in other species. This work led to the identification of the tRNA-editing deaminase that conducts the A-to-I modification in the anticodon of the chloroplast tRNA-Arg(ACG) (Karcher and Bock, 2009) and the enzyme that edits the position 3′ adjacent to the anticodon of the cytosolic tRNA-Ala (AGC) from adenosine to inosine (Zhou et al., 2013). It also prompted us to systematically analyze the entire gene family encoding putative A-to-I tRNA-editing enzymes in plants (Fig. 1). Construction of an evolutionary tree from all putative nucleoside deaminases encoded in the Arabidopsis nuclear genome (Arabidopsis Genome Initiative, 2000) revealed several additional proteins with similarity to the two functionally characterized A-to-I deaminases, TADA and TAD1 (Delannoy et al., 2009; Karcher and Bock, 2009; Zhou et al., 2013). We preliminarily designated these proteins AtTAD2, AtTAD3, AtTAD4, and AtTAD5 (for simplicity, subsequently referred to as TAD2, TAD3, TAD4, and TAD5; Fig. 1A). Analysis of published gene expression sets (https://www.genevestigator.com and https://arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp) and our attempts to amplify complementary DNA (cDNA) sequences from widely different tissues and developmental stages (including rosette and cauline leaves, stems, roots, siliques, seeds, flowers, and etiolated seedlings) revealed no evidence for TAD5 (encoded by locus AT3G05300) being expressed. We preliminarily concluded that *TAD5* may be a pseudogene, which is in line with the low degree of phylogenetic conservation, as also noted in a recently published study (Dahncke and Witte, 2013).

To identify new A-to-I editing deaminases, the remaining three genes, *TAD2*, *TAD3*, and *TAD4*, were subjected to functional analysis by reverse genetics. Previous work in yeast had shown that the inosine in the wobble position of tRNAs is generated by a heterodimeric complex of two proteins with similarity to nucleoside deaminases, Tad2p/ADAT2 and Tad3p/ADAT3 (Gerber and Keller, 1999). The protein encoded by Arabidopsis locus AT1G48175 (*TAD2*) displays significant amino acid sequence similarity to Tad2p/ADAT2 proteins (Fig. 1B), suggesting that it could be an A-to-I editing deaminase acting on tRNAs. Significantly, the sequence similarity is particularly high in the catalytic domain, including conservation of the three zinc-coordinating residues and the proton-shuttling Glu residue (Fig. 1B). The protein encoded by Arabidopsis locus AT5G24670 (*TAD3*) has very limited sequence similarity to Tad3p/ADAT3 (Fig. 1C). Putative homologs of the Arabidopsis TAD2 and TAD3 proteins are encoded in all sequenced genomes of vascular plants and in the genome of the unicellular green alga *Chlamydomonas reinhardtii* (Supplemental Figs. S1 and S2). However, while a TAD2-related protein was found to be encoded in the genome of the cyanobacterium *Synechocystis* sp. *PCC 6803*, no TAD3-like protein appears to be present in cyanobacteria (Supplemental Figs. S1 and S2). TAD4, encoded by Arabidopsis locus AT5G28050 and most similar to TAD2 (Fig. 1A), has very recently been suggested to act as a guanosine deaminase that generates xanthosine from guanosine in purine catabolism (Dahncke and Witte, 2013).

The TAD2, TAD3, and TAD4 Proteins Localize to the Nucleus and the Cytosol

If the TAD2, TAD3, and TAD4 proteins are indeed A-to-I editing enzymes acting on tRNA substrates, they would be expected to localize to the nucleocytosolic compartment. This is because the only inosine residue known to be present in an organellar RNA resides in the anticodon of the chloroplast tRNA-Arg(ACG), and the corresponding deaminase was identified as TADA (Delannoy et al., 2009; Karcher and Bock, 2009; Fig. 1A).

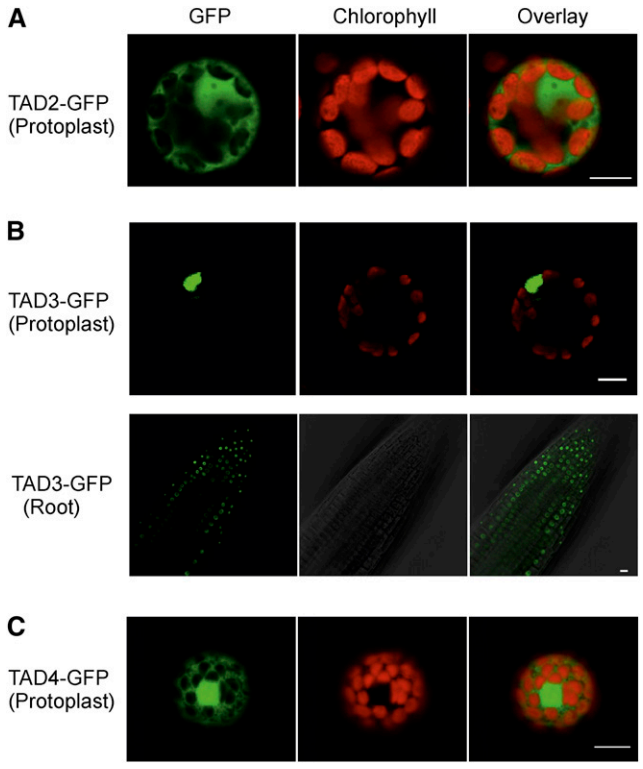

Figure 2. Subcellular localization of GFP fusion proteins of TAD2, TAD3, and TAD4. A, Localization of TAD2 in the nucleus and the cytosol. The TAD2-GFP fusion protein was transiently expressed in tobacco (*Nicotiana tabacum*) protoplasts. B, Localization of the TAD3 protein in the nucleus. In addition to localization in transiently transformed protoplasts, the TAD3-GFP fusion protein was also expressed from the cauliflower mosaic virus 35S promoter in stably transformed Arabidopsis plants. Shown here is the nuclear localization in root tip cells. C, Localization of the TAD4 protein. The TAD4-GFP fusion protein was transiently expressed in tobacco protoplasts. Images were obtained by confocal laser-scanning microscopy. GFP fluorescence (left), chlorophyll fluorescence (middle), and the overlay of GFP and chlorophyll fluorescence (right) are shown. Bars = 10 μm.

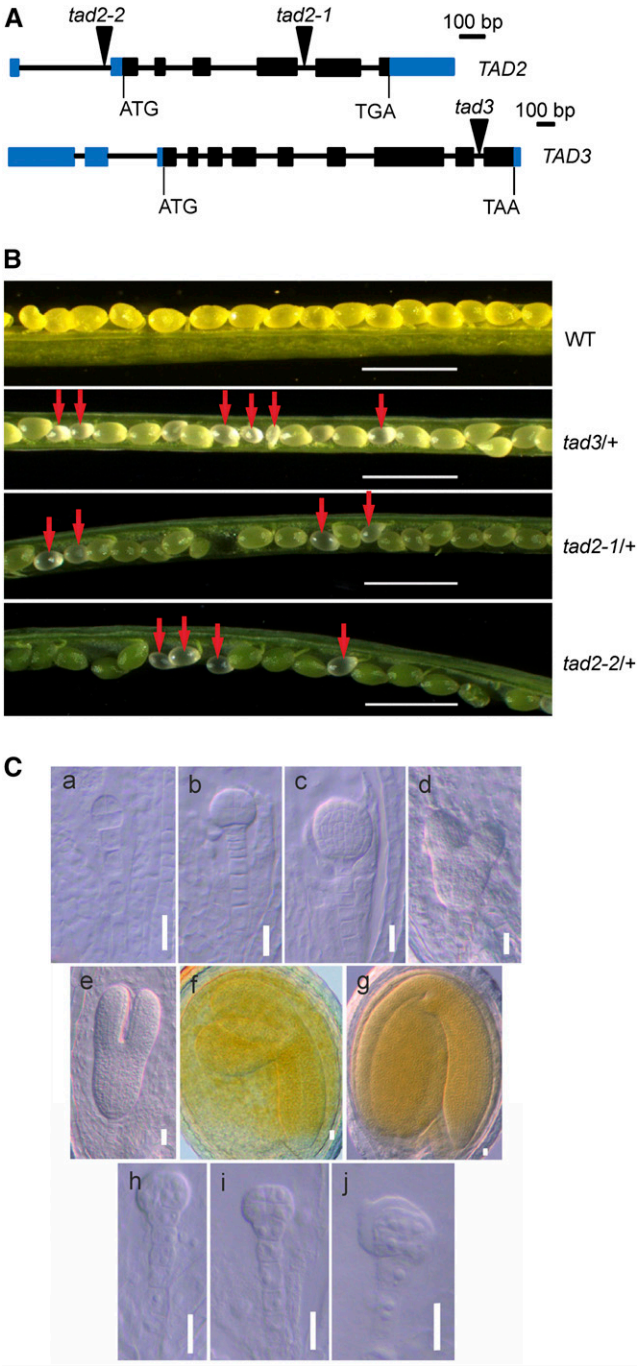

Figure 3. T-DNA insertions in the *TAD2* and *TAD3* genes cause embryo lethality in Arabidopsis. A, Schematic representation of the exon-intron structure of the *TAD2* and *TAD3* loci and location of the T-DNA insertions in *tad2* and *tad3* mutants (indicated by black triangles). Black boxes and blue boxes represent coding regions and untranslated regions, respectively. Introns are shown as black lines connecting the exon boxes. Translational start and stop codons are indicated by the respective triplets. B, Siliques from wild-type (WT) and heterozygous *tad2-1*/+, *tad2-2* /+, and *tad3* /+ plants observed with a binocular microscope. Aborted seeds (suggesting embryo lethality) are marked by red arrows. Bars = 1 mm. C, Embryo phenotypes of *tad2* and *tad3* mutants. Wild-type embryos at the eight-cell stage (a), early globular stage (b), late globular stage (c), heart stage (d), torpedo stage (e), cotyledon stage (f), and mature embryo stage (g) are shown. Mutant embryos in aborted seeds from *tad3* (h), *tad2-1* (i), and *tad2-2* (j) are arrested at the early globular stage. Bars = 20 μm.

To determine the subcellular localization of the TAD2, TAD3, and TAD4 proteins, in-frame fusions of all three genes with the gene for GFP were constructed. The fusion genes were inserted into a plant expression vector and introduced into plant cells by transient or stable transformation (Fig. 2). Analysis of GFP fluorescence in transformed cells and tissues by confocal laser-scanning microscopy revealed strong green fluorescence that localized predominantly to the nucleus and to a lesser extent to the cytosol (Fig. 2). This subcellular localization pattern would be compatible with a function of the proteins as tRNA-modifying enzymes.

TAD2 and *TAD3* Are Essential Genes

To test whether the proteins encoded by *TAD2* and *TAD3* are involved in A-to-I editing of nucleus-encoded tRNAs, we sought to address the gene functions by isolating null mutants. To this end, we searched the collection of publicly available Arabidopsis transfer DNA (T-DNA) insertion lines (http://arabidopsis.info/ and http://www.gabi-kat.de/). Two independent T-DNA insertion lines associated with the *TAD2* locus and one line associated with the *TAD3* locus could be identified (Fig. 3A). In one of the *tad2* mutant lines, subsequently referred to as the *tad2-1* allele, the T-DNA resides in the intron between exons 4 and 5 of the *TAD2* gene, whereas in the second line (*tad2-2* allele), the T-DNA insert is in the first intron upstream of the start codon of the *TAD2* reading frame. The T-DNA insertion in line *tad3* resides in the intron between exons 8 and 9 of the *TAD3* gene (Fig. 3A). To obtain homozygous *tad2* and *tad3* mutants, all mutant lines were selfed and the progeny were assayed for homozygosity of the T-DNA insertion by PCR. Despite genotyping of more than 30 progeny from each line, not a single homozygous plant could be identified, raising the possibility that the T-DNA insertions cause embryo lethality. To test this possibility, the phenotypes of immature seeds in developing siliques were analyzed. In all three mutants, approximately one-fourth of the seeds were smaller and less pigmented, suggesting that they contain aborted embryos (Fig. 3B). The ratios of aborted to normal seeds were 75:276 in *tad3*/+, 67:252 in *tad2-1*/+, and 89:290 in *tad2-2*/+.

To directly confirm embryo lethality, embryogenesis was investigated during seed development by microscopy. These analyses revealed an arrest of embryogenesis in the mutant seeds at the early globular stage (Fig. 3C) in the defective seeds of all three mutants. Taken together, the absence of homozygous mutant plants and the detection of mutant seeds displaying arrested embryogenesis strongly suggest that (1) all three T-DNA

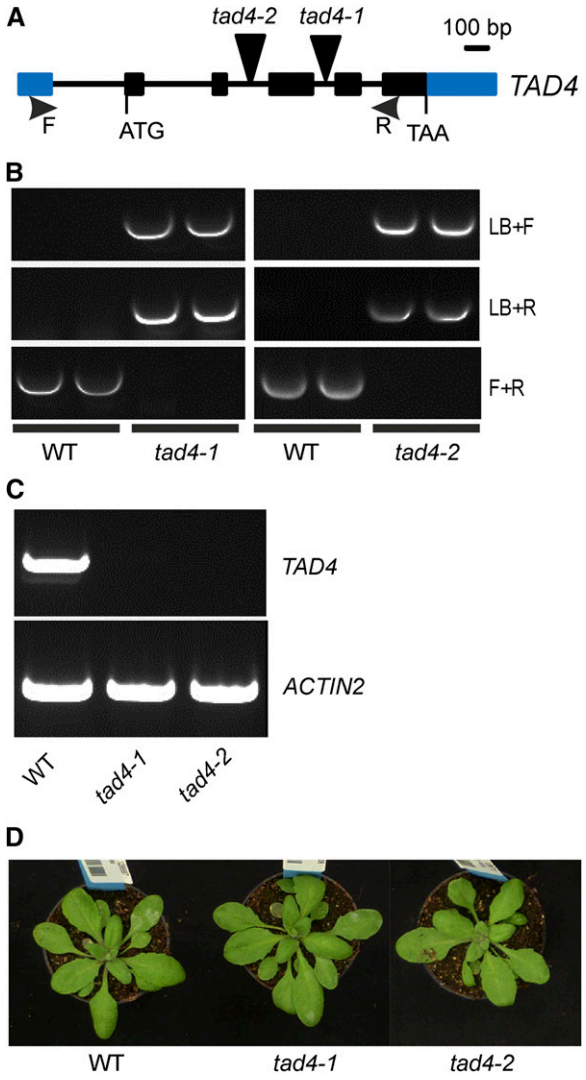

Figure 4. Identification and characterization of *tad4* mutants in Arabidopsis. A, Schematic representation of the structure of the *TAD4* gene and location of the T-DNA insertions in the *tad4-1* and *tad4-2* mutants (indicated by black triangles). Black and blue boxes represent coding regions and untranslated regions, respectively. Introns are shown as black lines connecting the exon boxes. Start and stop codons of the *TAD4* reading frame are indicated by the respective triplets. The gene-specific primer pair used for the identification of T-DNA insertions is indicated by arrowheads (F, forward primer; and R, reverse primer). B, Identification of homozygous T-DNA insertions in *tad4* mutants by PCR using genomic DNA as a template. Primer combinations are indicated at right. Two independent plant samples were analyzed for each line. C, Detection of *TAD4* mRNA in the wild type (WT) and two homozygous *tad4* mutants by semiquantitative RT-PCR. Absence of detectable transcripts from the *tad4* mutants suggests that the T-DNA insertions generate null alleles. The mRNA for an actin isoform (*ACTIN2*) was used as an internal control. D, Homozygous *tad4* mutant plants exhibit no obvious phenotype when compared with wild-type plants.

insertion lines represent loss-of-function alleles and (2) both *TAD2* and *TAD3* are essential genes.

In contrast to the *tad2* and *tad3* mutants, homozygous *tad4* mutants could be readily identified. From two independent T-DNA insertions (*tad4-1* and *tad4-2*; Fig. 4A), several homozygous mutant lines were isolated (Fig. 4B). Reverse transcription (RT)-PCR assays revealed the absence of detectable *TAD4* transcripts (Fig. 4C), suggesting that both T-DNA insertions generate functional null alleles. Consistent with a recent report (Dahncke and Witte, 2013), the homozygous knockout plants displayed no obvious phenotype under standard growth conditions (Fig. 4D).

Generation of RNA Interference Mutants for *TAD2* and *TAD3*

In the absence of homozygous T-DNA mutants for *TAD2* and *TAD3*, we sought to generate knockdown lines for both genes by RNA interference (RNAi). To this end, hairpin-type RNAi constructs were produced and stably introduced into Arabidopsis plants by *Agrobacterium tumefaciens*-mediated transformation. RNAi plants did not show a strong phenotype, but a mild growth retardation was noted in several lines

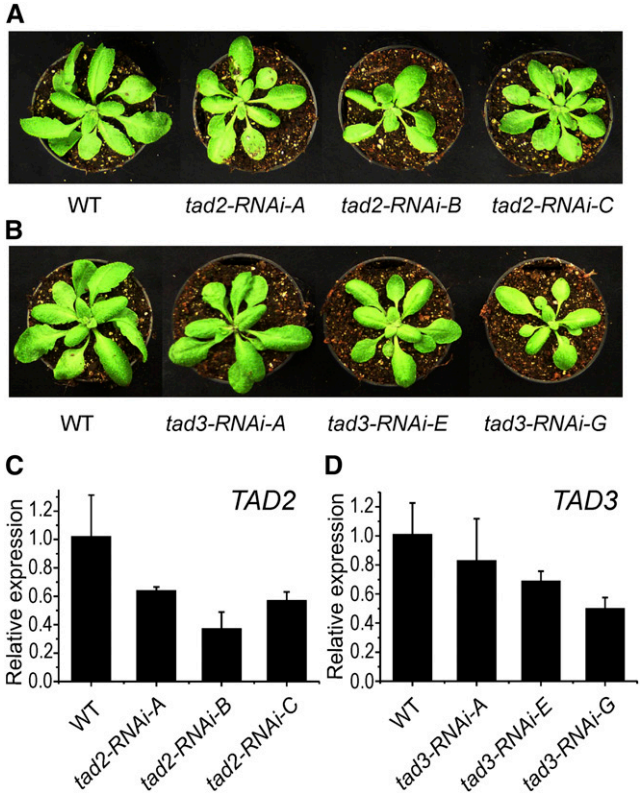

Figure 5. Phenotypic and molecular analyses of *TAD2* and *TAD3* RNAi mutants generated in Arabidopsis. A, Phenotypes of three independent *TAD2* RNAi lines after 16 d of growth under long-day conditions. B, Phenotypes of three independent *TAD3* RNAi lines after 16 d of growth under long-day conditions. C, Down-regulation of *TAD2* expression in the three independently generated RNAi lines as determined by quantitative RT-PCR. D, Down-regulation of *TAD3* expression in the three independently generated RNAi lines as determined by quantitative RT-PCR. Error bars indicate the SD ($n = 3$). WT, Wild type.

(e.g. *tad2-RNAi-B* and *tad3-RNAi-G*; Fig. 5, A and B). Quantitative RT-PCR was used to examine the expression of *TAD2* and *TAD3* in the RNAi lines. As expected, the level of down-regulation was variable between the transgenic lines of each set (Fig. 5, C and D), with the strongest lines showing a more than 50% down-regulation of *TAD2* and *TAD3*, respectively. Interestingly, the lines found to have the strongest level of down-regulation were the ones that displayed significantly delayed growth (*tad2-RNAi-B* and *tad3-RNAi-G*; Fig. 5, A and B), indicating that the *TAD2* and *TAD3* mRNA levels have fallen below a critical threshold in these lines.

Analysis of A-to-I Editing of Nucleus-Encoded tRNAs in *tad2*, *tad3*, and *tad4* Mutants

Having obtained mutants for all three putative deaminases, we next wanted to investigate the effect of the gene inactivation or knockdown on tRNA editing. Although the TAD4 protein was recently suggested to possess guanosine deaminase activity, we also included the *tad4* mutants in these analyses, because several RNA-editing enzymes have been demonstrated to display relaxed substrate specificity in that they, for example, can act on both ribonucleotides (in RNA) and deoxyribonucleotides (in single-stranded DNA molecules; Rubio et al., 2007). To exclude a similar moonlighting function of TAD4 in tRNA editing, both *tad4* knockout alleles were analyzed in addition to the *tad2* and *tad3* RNAi mutants.

Besides the two characterized A-to-I tRNA-editing events in position 37 of the cytosolic tRNA-Ala(AGC) and the chloroplast tRNA-Arg(ACG) (catalyzed by TAD1 and TADA, respectively; Delannoy et al., 2009; Karcher and Bock, 2009; Zhou et al., 2013), inosine occurs in the wobble position of the anticodon of eight nucleus-encoded tRNAs in Arabidopsis (Table I). We investigated the editing status of these potential substrate tRNAs by RT and sequencing. As inosine is read as guanosine by reverse transcriptases, the A-to-I editing events can be readily detected by sequencing of amplified cDNA obtained by RT of the tRNAs. cDNA synthesis and sequencing of mature tRNA molecules is challenging because of the small size of the tRNAs and their highly stable secondary structure. To alleviate these problems, an oligonucleotide linker was ligated to the extracted and purified tRNAs, which facilitated efficient RT and greatly improved sequencing quality.

Sequencing of reverse-transcribed tRNAs in the two *tad4* mutants revealed no evidence of reduced A-to-I conversion in any of the edited tRNA species (Fig. 6), supporting the view that the encoded enzyme acts exclusively as a guanosine deaminase in primary metabolism (Dahncke and Witte, 2013). By contrast, the strong RNAi lines for *TAD2* and *TAD3* showed reduced editing in several tRNAs. A-to-I conversion in position 34 of the anticodon of tRNA-Ala(AGC), tRNA-Ser(AGA), tRNA-Leu(AAG), tRNA-Ile(AAT), tRNA-Arg(ACG), and tRNA-Thr(AGT) was significantly lower than in the wild type (as evidenced by the presence of mixed A/G peaks in the sequence chromatograms; Fig. 6), suggesting that both TAD2 and TAD3 are involved in the editing of these tRNA species. Editing of two other tRNAs with inosine in the anticodon, tRNA-Val(AAC) and tRNA-Pro(AGG), was unaffected (Fig. 6; Table I), indicating either that these tRNAs are edited in a TAD2/TAD3-independent manner or, alternatively, that the knockdown of the two genes is not strong enough in our RNAi mutants to detect a significant effect on these two editing sites.

Table I. *Summary of tRNAs edited in their anticodon arm in Arabidopsis and the editing enzymes identified to date*

Edited bases were identified by cDNA sequencing. Data for tRNA-Ala(AGC) (Zhou et al., 2013) and chloroplast tRNA-Arg(ACG) (Delannoy et al., 2009; Karcher and Bock, 2009) are published; all other data are from this work.

tRNA Species	tRNA Origin (Genome)	Nucleotide Position and Type of Editing	Editing Enzymes
tRNA-Ala(AGC)	Nucleus	37 (A-to-I)	TAD1
tRNA-Ala(AGC)	Nucleus	34 (A-to-I)	TAD2, TAD3
tRNA-Ser(AGA)	Nucleus	34 (A-to-I)	TAD2, TAD3
tRNA-Leu(AAG)	Nucleus	34 (A-to-I)	TAD2, TAD3
tRNA-Ile(AAT)	Nucleus	34 (A-to-I)	TAD2, TAD3
tRNA-Arg(ACG)	Nucleus	34 (A-to-I)	TAD2, TAD3
tRNA-Thr(AGT)	Nucleus	34 (A-to-I)	TAD2, TAD3
tRNA-Val(AAC)	Nucleus	34 (A-to-I)	?
tRNA-Pro(AGG)	Nucleus	34 (A-to-I)	?
tRNA-Ser(AGA)	Nucleus	32 (C-to-U)	?
tRNA-Ser(GCT)	Nucleus	32 (C-to-U)	?
tRNA-Arg(ACG)	Chloroplast	34 (A-to-I)	TADA

Accumulation and Aminoacylation of Partially Edited tRNAs in *tad2* and *tad3* Mutants

Many nucleoside modifications in tRNAs enhance tRNA stability by promoting proper RNA folding and/or stabilizing the three-dimensional structure (Phizicky and Alfonzo, 2010). To test whether the adenosine-34 modification to inosine has a beneficial effect on tRNA stability in Arabidopsis plants, we examined tRNA accumulation in the wild type and the strong *tad2* and *tad3* RNAi mutants by northern-blot analyses. These experiments revealed that the only partially edited tRNAs, tRNA-Leu(AAG) and tRNA-Ser(AGA), in the mutants accumulate to similar levels to the fully modified tRNAs in wild-type plants (Fig. 7A), indicating that the A-to-I editing in position 34 of the anticodon has no significant impact on tRNA stability.

Finally, we investigated the possibility that the reduced tRNA editing in the *tad2* and *tad3* mutants affects the efficiency of tRNA charging with amino acids. When the level of tRNA aminoacylation was determined in the wild type and the mutants, no difference was seen in that the tRNA pools were fully aminoacylated in vivo in all plant lines analyzed (Fig. 7B). This result suggests that A-to-I editing in the anticodon is not required for efficient

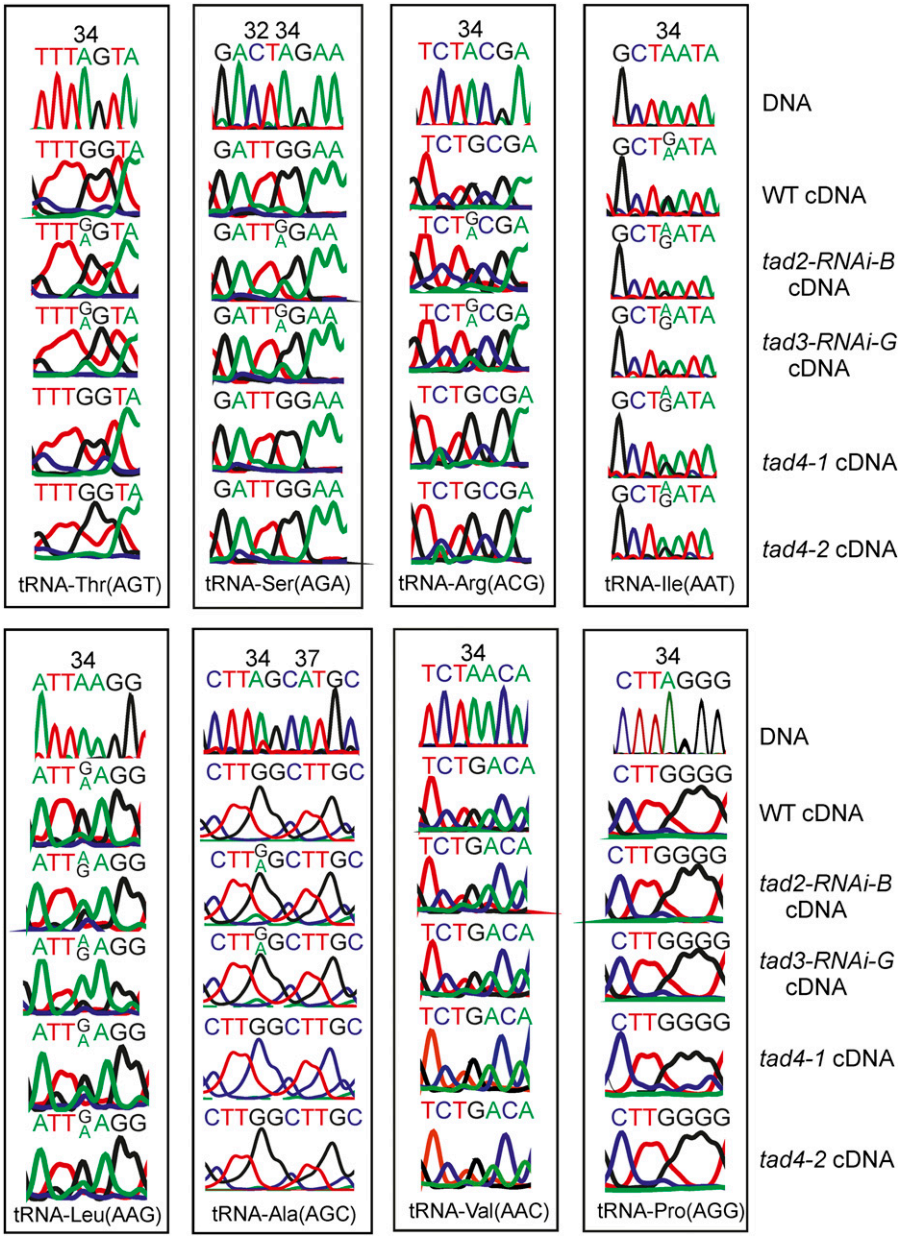

Figure 6. Analysis of A-to-I editing at position 34 of tRNAs in wild-type (WT) plants, *TAD2* and *TAD3* RNAi lines, and *tad4* knockout mutants. tRNAs were reverse transcribed, and the cDNA sequences were amplified by PCR and then directly sequenced. Reverse transcriptases read I as G. Adenosine-37 of tRNA-Ala (AGC) is deaminated by TAD1 to I, and inosine-37 then undergoes further modification by *S*-adenosyl-Met-dependent methylation to *N*1-methylinosine (m^1I_{37}). m^1I_{37} is read as T by reverse transcriptases (Zhou et al., 2013). Note that C is edited to U in tRNA-Ser(AGA) at position 32. Partial editing is indicated by two letters, one above the other (with the top letter denoting the dominating peak). The A peak sometimes seen together with the first C peak in tRNA-Arg(ACG) is likely a sequencing artifact (Supplemental Fig. S4).

tRNA aminoacylation. Therefore, we propose that the delayed growth of the RNAi lines is not due to inefficient charging but rather to less efficient use of the unedited tRNA by the ribosome.

TAD2 and TAD3 Form a Protein Complex in Vivo

In yeast, the Tad2p and Tad3p proteins form a heterodimeric protein complex that conducts A-to-I editing in tRNAs (Gerber and Keller, 1999). The essentiality of both *TAD2* and *TAD3* in Arabidopsis and the very similar editing phenotypes of the *tad2* and *tad3* mutants (Fig. 6; Table I) raise the possibility that the TAD2 and TAD3 proteins in plants also act in a protein complex. To test whether TAD2 and TAD3 engage in protein-protein interactions in planta, we used bimolecular fluorescence complementation (BiFC) assays (Walter et al., 2004; Waadt et al., 2008). These experiments revealed strong interaction between TAD2 and TAD3 (Fig. 8). The interaction was dependent upon the expression of both protein fusions in that coexpression of the C-terminal yellow fluorescent protein fragment (cYFP)-TAD3 fusion with an unfused N-terminal YFP fragment (nYFP) did not give rise to YFP fluorescence. As a positive control, TAD1, a tRNA adenosine deaminase known to act as a homodimer (Gerber et al., 1998; Zhou et al., 2013), was tested. As expected, when expressed as a fusion protein with both nYFP and cYFP, it yielded strong fluorescence complementation. No fluorescence complementation was seen when only one of the two YFP halves was

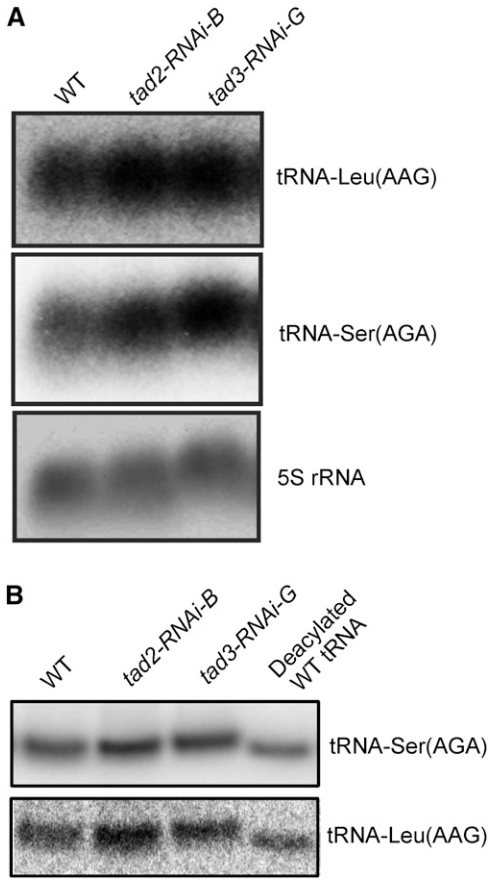

Figure 7. Accumulation and aminoacylation of tRNA-Leu(AAG) and tRNA-Ser(AGA) in wild-type plants (WT) and *TAD2* and *TAD3* RNAi lines. A, Northern-blot analysis to determine the accumulation of tRNA-Leu(AAG) and tRNA-Ser(AGA). Mitochondrial 5S rRNA was used as a loading control. B, In vivo aminoacylation assays for tRNA-Leu(AAG) and tRNA-Ser(AGA). On high-resolution polyacrylamide gels, the aminoacylated tRNAs migrate slower than the corresponding deacylated tRNAs.

fused to TAD1, supporting the specificity of the interaction observed (Fig. 8). The protein-protein interaction data support the existence of a TAD2/TAD3 protein complex in planta and provide further evidence for the two proteins being functional orthologs of the yeast adenosine deaminase subunits Tad2p and Tad3p.

To confirm the interaction between TAD2 and TAD3 by an independent method and, at the same time, obtain additional information about the functions of these proteins in vivo, we used stable transgenic plants expressing the TAD3-GFP fusion protein and performed coimmunoprecipitation experiments with anti-GFP antibodies. The composition of the purified complex was then determined by mass spectrometric protein identification (using a GFP-expressing line and wild-type plants as controls for contaminants; see "Materials and Methods"). The two most abundant proteins identified were the TAD2 protein, as expected, and a member of the DEA(D/H)-box RNA helicase protein family (Table II). These helicases unwind RNA and are involved in various aspects of RNA metabolism. It seems conceivable that the unwinding activity of such a helicase is needed to make the editing site accessible to modification by the TAD2/TAD3 proteins.

A New Type of RNA Editing in the Plant Nucleus

The tRNA-Ser(AGA) undergoes A-to-I editing in the wobble position (position 34) of the anticodon (Fig. 6; Table I). When sequencing amplified cDNAs derived from tRNA-Ser(AGA), we noticed, in addition to the A-to-I conversion at position 34, another nucleoside conversion at position 32. This editing event involves a cytidine-to-uridine (C-to-U) transition (Fig. 6; Table I), a type of editing that heretofore has not been found in the nucleus of plant cells. A cytidine in position 32 is present also in another Ser-isoaccepting tRNA species in the nucleus, the tRNA-Ser(GCT). This prompted us to investigate reverse-transcribed tRNA-Ser(GCT) for editing of position 32. Indeed, we found cytidine-32 in tRNA-Ser(GCT) to be edited as well (Fig. 9; Table I). The C in position 32 is conserved in all members of the two gene families encoding tRNA-Ser(AGA) and tRNA-Ser(GCT) in Arabidopsis (Supplemental Fig. S3), strongly suggesting that all tRNA transcripts undergo RNA editing.

At present, we can only speculate about the possible functional significance of this editing event. Base

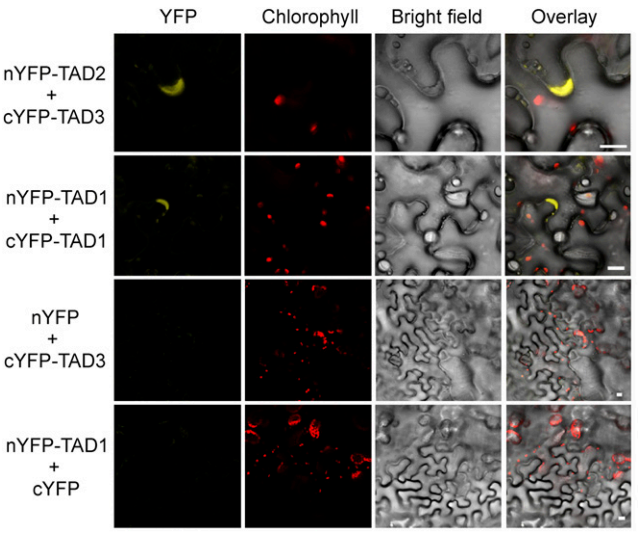

Figure 8. Interaction between the Arabidopsis TAD2 and TAD3 proteins in planta. BiFC assays were performed by transient transformation of *Nicotiana benthamiana* leaf epidermal cells. The nuclear YFP signal observed upon combination of TAD2 with TAD3 and TAD1 with TAD1 suggests that the corresponding proteins interact with each other. No YFP signal is detected in negative controls using unfused nYFP or cYFP in combination with the cYFP-TAD3 or nYFP-TAD1 construct. Bars = 10 μm.

Table II. *Mass spectrometry identification of proteins that coimmunoprecipitate with TAD3*

Arabidopsis Genome Initiative Code	Annotation	Mascot Score	Protein Sequence Coverage	No. of Peptides Matched
			%	
At1g72730	DEA(D/H)-box RNA helicase family protein	286	10.4	5
At1g48175	tRNA adenosine deaminase (TAD2)	208	22	4
At1g52040	Myrosinase-binding protein1 (MBP1)	137	7.1	2
At3g17390	*S*-Adenosyl-Met synthetase family protein (MAT4)	129	9.2	4
At3g16410	Nitrile specifier protein4 (NSP4)	45	4.4	2
At4g33070	Thiamine pyrophosphate-dependent pyruvate decarboxylase family protein (PDC1)	43	3	3
At4g27680	P-loop-containing nucleoside triphosphate hydrolase superfamily protein	38	5	2
At3g17690	Cyclic nucleotide-gated channel19 (CNGC19)	33	0.8	3
At5g35940	Man-binding lectin superfamily protein	33	6.3	2
At5g10550	Global transcription factor group E2 (GTE2)	28	1.2	3
At1g20200	PAM domain (PCI/PINT-associated module) protein	23	3.5	2

modifications in the vicinity of the anticodon have been shown to impact translational efficiency. For example, A-to-I editing in position 37 of the cytosolic tRNA-Ala(AGC) in Arabidopsis is required for full translational activity under conditions of environmental stress (Zhou et al., 2013). Whether the C-to-U editing in tRNA-Ser plays a similar role can only be addressed once the responsible enzyme is identified and the gene encoding it can be knocked out. Also, although the edited base is very likely to be uridine (Weigel and Nilsson, 1995; Alfonzo et al., 1999; Rubio et al., 2006), its chemical identity remains to be ultimately confirmed by tRNA sequencing. However, due to the difficulty with purifying large enough amounts of cytosolic tRNA species from plants, this has not yet been possible.

C-to-U editing occurs in mRNAs of higher plant mitochondria and plastids (for review, see Bock, 2000; Knoop, 2004; Schmitz-Linneweber and Small, 2008; Stern et al., 2010) and in the nucleus-encoded mRNA for apolipoprotein B in mammals (Powell et al., 1987). C-to-U editing in tRNAs was detected, for example, in plant mitochondria, where it usually corrects mismatches in stems of the cloverleaf secondary structure (Marechal-Drouard et al., 1996; Kunzmann et al., 1998; Schock et al., 1998), in the anticodon of the imported mitochondrial tRNA-Trp in *Leishmania tarentolae*, where it facilitates reading of the UGA stop codon (Alfonzo et al., 1999), and in the anticodon of a tRNA from marsupials, where partial editing leads to tRNA diversity and differential aminoacylation (Börner et al., 1996). Interestingly, in the nucleocytosolic compartment of plant cells, C-to-U RNA editing has not been found thus far.

C-to-U editing is catalyzed by cytidine deaminases, and at least the cytidine deaminase activities that have been biochemically characterized so far are evolutionarily related to the known A-to-I editing enzymes (Teng et al., 1993; Gerber and Keller, 1999; Rubio et al., 2007). However, C-to-U mRNA editing in plant cell organelles (mitochondria and plastids) has been suggested to be mediated by an evolutionarily unrelated protein family, the pentatricopeptide repeat proteins with a C-terminal DYW domain (Salone et al., 2007). The DYW domain has similarity to a cytidine deaminase domain (Iyer et al., 2011) and has been shown to bind zinc (Hayes et al., 2013) and to be essential for *ndhD* (encoding a subunit of the chloroplast NAD(P)H dehydrogenase) mRNA editing in the chloroplast (Boussardon et al., 2012). Whether the tRNA-editing events in the nucleus-encoded tRNA-Ser(AGA) and tRNA-Ser(GCT) discovered here are catalyzed by a cytidine deaminase that belongs to the cytidine deaminase superfamily (Gerber and Keller, 1999; Rubio et al., 2007), by DYW-type pentatricopeptide repeat

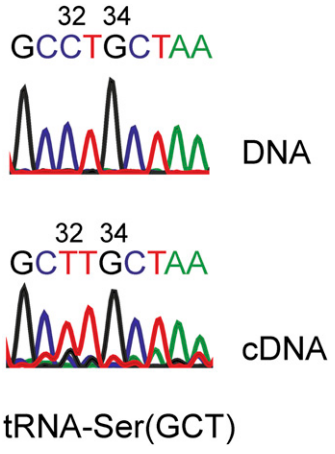

Figure 9. Detection of C-to-U RNA editing at position 32 of tRNA-Ser (GCT) in Arabidopsis wild-type plants. The genomic DNA sequence (DNA) is compared with the cDNA sequence (cDNA).

proteins, or by entirely different enzymes remains to be investigated. C-to-U editing in tRNA-Ser was unaffected in all our *tad2*, *tad3*, and *tad4* mutants (Fig. 6; Table I), suggesting that none of these enzymes is involved in C-to-U conversion. In general, much remains to be learned about the substrate recognition and substrate specificities of nucleoside deaminases acting on RNA. For example, there seems to be no strict nucleoside specificity of at least some deaminases in that an A-to-I tRNA-editing enzyme was shown to also perform C-to-U editing in single-stranded DNA, at least in vitro (Rubio et al., 2007). Likewise, the determinants of substrate specificity in both the deaminase enzyme and the substrate tRNAs are still largely obscure. Whether auxiliary factors influence the specificity of these enzymes in vivo is currently unknown. In this context, it certainly seems worthwhile to investigate some of the new proteins isolated as putative components of a TAD2/TAD3 multiprotein complex in our affinity purification approach (Table II).

In summary, our work reported here has identified two plant genes, *TAD2* and *TAD3* (or, according to the alternative nomenclature, *ADAT2* and *ADAT3*), that jointly are involved in site-specific A-to-I deamination at position 34 of several cytosolic tRNA species in the model plant Arabidopsis. Our data show that both genes are essential for plant viability and that the encoded proteins act as a protein complex in vivo that presumably contains additional constituents, including an RNA helicase. Moreover, we have discovered C-to-U conversional RNA editing in two Ser-isoaccepting tRNA species. To our knowledge, this is the first demonstration of C-to-U RNA editing in the nucleocytosolic compartment of plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) plant lines used in this study are in the Columbia-0 ecotype background. For growth under aseptic conditions, surface-sterilized seeds were stratified for 3 d and then sown onto one-half-strength Murashige and Skoog medium with 1% (w/v) Suc (Murashige and Skoog, 1962). All plant material was raised in controlled-environment chambers. T-DNA insertion mutants were identified by searching the collection cataloged on the SIGnAL T-DNA Express Web site (http://signal.salk.edu/cgi-bin/tdnaexpress). The genotype of the T-DNA lines was confirmed by genomic PCR using gene-specific primers in combination with T-DNA left border primers (Alonso et al., 2003). Exact T-DNA insertion sites were determined by sequencing of PCR products. The Arabidopsis T-DNA insertion lines CS16170 (*tad2-1*) and CS16171 (*tad2-2*) for the At1g48175 (*TAD2*) locus were obtained from the Nottingham Arabidopsis Stock Centre (http://www.arabidopsis.info/). In line CS16170, the T-DNA is inserted in an intron and accompanied by a deletion of 17 bp at the insertion site. In line CS16171, the T-DNA is also inserted in an intron and accompanied by a deletion of 300 bp at the insertion site (http://www.seedgenes.org). The T-DNA insertion line GABI_141G12 (*tad3*) for the At5g24670 (*TAD3*) locus was obtained from the GABI-Kat collection (http://www.gabi-kat.de/index.php). PCR with the T-DNA left border primer LB-GABI in combination with the At5g24670-specific primer F1 was used to confirm the position of the T-DNA insert. Sequencing showed that the T-DNA was inserted in the last intron (1,790 bp downstream of the start codon) in line GABI_141G12. Two independent T-DNA mutant alleles were characterized for the At5g28050 (*TAD4*) locus. In both lines SAIL_305B08 (*tad4-1*) and GABI_432D08 (*tad4-2*), the insertions were found to consist of an inverted T-DNA repeat situated in the fourth intron (852 bp downstream of the start codon in *tad4-1*) and the third intron (505 bp downstream of the start codon in *tad4-2*), respectively. Primers for the identification of T-DNA insertion sites are listed in Supplemental Table S1.

Generation of Knockdown Lines by RNAi

Constructs based on the pHANNIBAL vector (Wesley et al., 2001) were used for RNA-induced silencing of *TAD2* and *TAD3*. A cDNA fragment of 426 bp corresponding to the 3′ coding region of *TAD3* (nucleotide positions 700–1,125) was amplified by PCR using the primer pairs Fs1/Rs1 (introducing the restriction sites *Eco*RI and *Xho*I) and Fa1/Ra1 (introducing the restriction sites *Bam*HI and *Hin*dIII; Supplemental Table S1). The PCR product obtained with Fs1 and Rs1 was digested with *Eco*RI and *Xho*I and cloned (in the sense orientation) into multiple cloning site 1 of the similarly cut pHANNIBAL RNAi vector (Wesley et al., 2001). Subsequently, the PCR product generated with Fa1/Ra1 was digested with *Bam*HI and *Hin*dIII and inserted (in the antisense orientation) into multiple cloning site 2. The resulting vector contains a *TAD3*-specific hairpin construct under the control of the cauliflower mosaic virus 35S promoter and the *octopine synthase* gene terminator. The pHANNIBAL plasmid construct containing the sense and antisense fragments was then digested with *Sac*I and *Spe*I, and the excised RNAi fragment was ligated into the similarly cut binary plant vector pART27 carrying the *neomycin phosphotransferase II* selectable marker gene (Gleave, 1992).

For construction of a *TAD2*-specific RNAi vector, a 160-bp cDNA fragment from the *TAD2* coding region (corresponding to nucleotide positions 163–322) was amplified by PCR using the primer combinations Fs2/Rs2 and Fa2/Ra2 (Supplemental Table S1). Both PCR products were consecutively cloned into pHANNIBAL by digestion with *Xho*I/*Acc*65I and *Xba*I/*Hin*dIII, respectively. The RNAi construct was excised from this plasmid by digestion with *Not*I and ligated into the similarly digested and dephosphorylated pART27 vector.

The final constructs for RNAi suppression of *TAD2* and *TAD3* were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Plant transformation experiments were performed using the floral dip method (Clough and Bent, 1998). Transgenic Arabidopsis plants were selected for kanamycin resistance using standard protocols.

Analysis of the Subcellular Localization of TAD Proteins

GFP fusion constructs were assembled to study the subcellular localization of TAD proteins in vivo. To this end, the complete genomic sequence of *TAD2* was amplified by PCR using primer combination F3/R3 (Supplemental Table S1). The entire *TAD3* sequence was PCR amplified using primers F1 and R1, and the coding sequence of *TAD4* was amplified by PCR with primer pair F4/R4 (Supplemental Table S1) using cDNA as a template. Following digestion with the restriction enzymes *Xho*I and *Nco*I, the fragments were cloned into vector pA7-GFP (Voelker et al., 2006). Preparation and transient transformation of tobacco (*Nicotiana tabacum*) protoplasts was done according to published protocols (Huang et al., 2002). Subcellular localization of GFP fluorescence was determined by confocal laser-scanning microscopy (TCS SP5; Leica Microsystems; http://www.leica.com/). To construct a vector for stable transformation, the entire *TAD3* genomic sequence was cloned into the pENTR/SD/D-TOPO vector (Invitrogen). Following sequence verification, the *TAD3* fragment was transferred into a Gateway binary vector (pGWB5) by an LR (attL x attR) recombination reaction using the Gateway LR Clonase II enzyme mix (Life Technologies), producing a fusion gene that encodes a TAD3-GFP fusion protein. The construct was then transformed into *A. tumefaciens* strain GV3101 and introduced into Arabidopsis wild-type plants by the floral dip method (Clough and Bent, 1998). Transgenic plants were identified by selection for kanamycin resistance. At least 15 independent transgenic lines expressing TAD3-GFP were identified. Subcellular localization of GFP fluorescence was visualized using 8-d-old seedlings germinated on one-half-strength Murashige and Skoog medium with a confocal laser-scanning microscope. Excitation wavelengths and emission filters were 488 nm/band pass 505 to 530 nm for GFP and 488 nm/long pass 650 to 710 nm for chlorophyll fluorescence.

Analysis of Phenotypes of Mutant Plants and Embryos

To investigate the phenotypes of *tad2*, *tad3*, and *tad4* mutants, plants were transferred to soil 7 d after germination on synthetic medium and grown under long-day conditions (16-h-light/8-h-dark regime) at a light intensity of

120 μE m^{-2} s^{-1} at 22°C. To study the progression of embryogenesis in T-DNA insertion lines for *TAD2* and *TAD3*, developing seeds were isolated from siliques and cleared in a solution of 240 g of chloral hydrate and 30 g of glycerol in 90 mL of water for 30 min at room temperature (Goubet et al., 2003). Samples were analyzed with a light microscope (Olympus model BX-51) equipped with differential interference contrast optics.

Isolation of Nucleic Acids and cDNA Synthesis

Total plant DNA was extracted from fresh leaf tissue samples by a cetyltrimethyl-ammonium bromide-based method (Doyle and Doyle, 1990). Total RNA was isolated using a guanidine isothiocyanate/phenol-based method (peqGOLD TriFast; Peqlab) according to the manufacturer's protocol. RNA samples treated with TURBO DNase (Ambion) were reverse transcribed using SuperScript III reverse transcriptase (Invitrogen; http://www.invitrogen.com/) and oligo$(dT)_{18}$ primer according to the manufacturer's instructions. For DNA sequencing, amplification products were separated by electrophoresis on agarose gels and purified from excised gel slices using the NucleoSpin Extract II kit (Macherey-Nagel).

Quantitative RT-PCR

cDNAs synthesized from extracted total plant RNA samples were used as templates for quantitative real-time PCR with gene-specific primers (Supplemental Table S1). Real-time PCR was performed using the StepOnePlus real-time PCR system (Applied Biosystems) using Absolute SYBR Green ROX mix (Thermo Scientific) for quantitation. Three biological and three technical replicates were analyzed. The $2^{-\Delta\Delta CT}$ method was used to determine the relative transcript levels (Livak and Schmittgen, 2001). The *translation initiation factor1α* (At5g60390) transcript was used as a reference for cDNA quality.

RNA Gel-Blot Analyses

For northern-blot analysis, RNA samples (extracted total cellular RNA) were electrophoresed on formaldehyde-containing 2% (w/v) agarose gels and blotted onto Hybond XL nylon membranes (GE Healthcare). To detect tRNA-Leu(AAG) and tRNA-Ser(AGA) in hybridization experiments, antisense oligonucleotides were derived from the 5′ portion of the tRNAs (corresponding to the sequence upstream of the edited position 34; Supplemental Table S1) and 5′ end labeled using T4 polynucleotide kinase (New England Biolabs). For radioactive labeling, 20 pmol of the oligonucleotide was incubated with 10 units of T4 polynucleotide kinase and 30 μCi of [γ-^{32}P]ATP for 30 min at 37°C. Mitochondrial 5S ribosomal RNA (rRNA) was used as a loading control. A mitochondrial 5S rRNA-specific probe was generated by PCR amplification with specific primers (Supplemental Table S1). The probe was radiolabeled with [α-^{32}P]dCTP using the Multiprime DNA labeling system (GE Healthcare). Hybridizations were performed at 65°C in Rapid-Hyb buffer following the instructions of the manufacturer (GE Healthcare), and signals were analyzed using a Typhoon Trio+ variable mode imager (GE Healthcare).

Aminoacylation Assays

Aminoacylation of tRNA-Ser(AGA) and tRNA-Leu(AAG) was analyzed as described previously (Zhou et al., 2013). Specific probes for tRNA-Ser(AGA) and tRNA-Leu(AAG) were prepared as described above.

Sequence Analysis of Edited tRNAs

Editing sites in the anticodon arm of nucleus-encoded tRNAs were identified by cDNA sequencing. All tRNA species known to contain inosine in eukaryotes were included. tRNA purification and analysis of tRNA editing were performed as described previously (Karcher and Bock, 2009; Zhou et al., 2013). Purified tRNAs were ligated to oligonucleotide P3′Lig (5′-rCrCTGCAGCTACTGGCCGTCGTTTTACTCrAox-3′ [r, ribonucleotide; and ox, ribose oxidized to the dialdehyde]) using T4 RNA ligase (New England Biolabs). After RT of the tRNAs using oligonucleotide PM13 (5′-GTAAAACGACGGCCAGT-3′) as primer, tRNA-derived cDNAs were amplified by PCR with a forward primer specific to the tRNA species of interest (Supplemental Table S1) and the reverse primer PM13tRNA (5′-GGCCAGTAGCTGCAGGTGG-3′). Purified PCR products were directly sequenced, and A-to-I deamination was revealed by the presence of G in place of A. Genomic sequences of the tRNA genes were amplified with the specific primers listed in Supplemental Table S1. Gel-purified amplification products were cloned into vector pCR2.1-TOPO (Invitrogen) and subsequently PCR amplified using the M13 forward primer (Invitrogen) and a tRNA gene-specific primer as reverse primer. The PCR products were then sequenced with the M13 forward primer.

BiFC Assays

To test for protein-protein interactions in planta, BiFC assays were employed. For vector construction, the full-length reading frames of *TAD1*, *TAD2*, and *TAD3* were amplified using specific primer pairs (Supplemental Table S1) and cloned into vector pENTR/SD/D-TOPO (Invitrogen). The final expression vectors were produced by an LR recombination reaction between the entry vector and the pBiFP2 and pBiFP3 vectors (Azimzadeh et al., 2008), generating in-frame fusions between the coding sequence of interest and the N-terminal or C-terminal part of the *YFP* gene. Fusion proteins were expressed under the control of the cauliflower mosaic virus 35S promoter and contained the YFP sequence at the N terminus. The BiFC constructs were transformed into *A. tumefaciens* strain GV3101, and the transgenic *A. tumefaciens* strains were injected into *Nicotiana benthamiana* leaves. Transfected tobacco plants were grown at 25°C for 2 to 3 d prior to microscopic investigation. *N. benthamiana* epidermal cells were observed with a confocal laser-scanning microscope (TCS SP5; Leica Microsystems). For detection of YFP signals, an argon laser was used for excitation at 514 nm, and fluorescence emission was detected with a 520- to 550-nm filter.

Coimmunoprecipitation and Mass Spectrometric Protein Identification

Protein complexes were isolated from plants expressing the TAD3-GFP fusion protein using the μMACS GFP-tagged protein isolation kit (Miltenyi Biotec). Wild-type and GFP-overexpressing seedlings were used as negative controls to identify proteins that bind nonspecifically to the anti-GFP antibody beads. Twelve-day-old seedlings from all three plant lines (TAD3-GFP, the wild type, and 35S::GFP) were harvested, and proteins were extracted by grinding 10 g of fresh seedlings at 4°C in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% [v/v] Nonidet P-40, 5 μg mL^{-1} aprotinin, and protease inhibitor cocktail from Roche Diagnostics). Homogenates were centrifuged at 10,000*g* for 15 min at 4°C to remove cellular debris. The supernatants were mixed with 100 μL of anti-GFP microbeads (Miltenyi Biotec) and incubated on ice for 30 min with shaking. The mixtures were applied to μColumns (Miltenyi Biotec) in a magnetic field to capture the magnetic antigen-antibody complex. Lysis buffer was used to wash the samples on the column, and immunoprecipitates were eluted with 100 μL of elution buffer. After immunoprecipitation, the eluted proteins were separated on 12% (w/v) polyacrylamide gels followed by staining with Coomassie Colloidal Blue and destaining in distilled water. Subsequently, each lane was cut into slices and subjected to in-gel tryptic digestion. In-gel tryptic digestion and mass spectrometry were performed as described previously (Albus et al., 2010). Tandem mass spectrometry spectra were evaluated against The Arabidopsis Information Resource 10 database using Mascot (Matrix Science). Proteins identified from tandem mass spectrometry spectra were considered specific if (1) they were not pulled down with the negative controls, (2) they were represented by at least two peptides, and (3) their peptides were identified with a sufficiently high Mascot score greater than 20. Putative interaction partners of TAD3 identified by these stringent criteria are listed in Table II.

Bioinformatic Analyses

Using the conserved cytidine/deoxycytidylate deaminase motif ([H]XE-PCXXC; InterPro no. IPR002125) as query, the genome of the model plant Arabidopsis (http://www.arabidopsis.org/) was searched in order to identify candidate editing deaminases. Candidate proteins and the corresponding genes were further analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches to identify putative homologs from other species. Excluding the 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate deaminase (At4g20960), which was shown previously to be involved in riboflavin biosynthesis (Fischer et al., 2004), 16 proteins were selected: nine putative cytidine deaminases, six putative tRNA adenosine deaminases, and one putative deoxycytidylate deaminase.

tRNA-Ser(AGA) and tRNA-Ser(GCT) sequence alignments were produced using the MultAlin software (http://multalin.toulouse.inra.fr/multalin/). Genomic tRNA sequences were downloaded from The Arabidopsis Information Resource (http://www.arabidopsis.org/).

For phylogenetic analysis, the amino acid sequences of candidate deaminases were aligned with ClustalW (Thompson et al., 1997), and phylogenetic trees were constructed using the boot-strapped neighbor-joining algorithm in MEGA 5.05 with 1,000 trials (http://www.megasoftware.net/). Bootstrap values are indicated as percentages of the 1,000 trials at all nodes.

Supplemental Data

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

Supplemental Figure S1. Amino acid sequence alignment of the TAD2 protein from Arabidopsis and homologous proteins from other photosynthetic eukaryotes and the cyanobacterium *Synechocystis* sp. *PCC 6803*.

Supplemental Figure S2. Amino acid sequence alignment of the TAD3 protein from Arabidopsis and homologous proteins from other photosynthetic eukaryotes.

Supplemental Figure S3. Alignment of genomic sequences for the cytosolic tRNA-Ser(AGA) and tRNA-Ser(GCT) in Arabidopsis.

Supplemental Figure S4. Additional cDNA sequences of tRNA-Arg (ACG).

Supplemental Table S1. Oligonucleotides used in this study.

ACKNOWLEDGMENTS

We thank the Arabidopsis Biological Resource Center at Ohio State University and the GABI-Kat consortium for making available seeds of T-DNA insertion lines. We also thank Dr. Martine Pastuglia (Institut Jean-Pierre Bourgin, Institut National de la Recherche Agronomique) for providing the pBiFP2 and pBiFP3 plasmids for BiFC assays, Dr. Eugenia Maximova and Katrin Piepenburg (Max-Planck-Institut für Molekulare Pflanzenphysiologie [MPI-MP]) for help with confocal laser-scanning microscopy, Dr. Etienne Meyer (MPI-MP) for help with mass spectrometric protein analysis, and the MPI-MP GreenTeam for plant cultivation.

Received September 16, 2014; accepted October 11, 2014; published October 14, 2014.

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