Arabidopsis tRNA Adenosine Deaminase Arginine Edits the Wobble Nucleotide of Chloroplast tRNA^{Arg}(ACG) and Is Essential for Efficient Chloroplast Translation

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INTRODUCTION

tRNAs are key components of gene expression in every living organism. They serve as adaptor molecules helping to convert nucleic acid–based information into chains of amino acids: each RNA codon is recognized by the anticodon harbored by the corresponding tRNA, according to the wobble rules proposed by Francis Crick (Crick, 1966). However, several genetic systems, such as organelles and some bacterial parasites, encode fewer tRNAs than theoretically required for the translation of all codons (Shinozaki et al., 1986; Osawa et al., 1992).

This lack can be compensated for by import of cytosolic tRNAs as demonstrated for mitochondria of plants, fungi, and protozoa (Salinas et al., 2008), but import is unlikely to explain all cases of incomplete tRNA sets. For example, no import of cytosolic tRNA has been demonstrated in chloroplasts or in human mitochondria (Lung et al., 2006), which according to wobble rules lack complete tRNA sets. This suggests that exceptions to the wobble rules must exist in some genetic systems.

Two mechanisms have been postulated to explain how translation occurs with a reduced tRNA set: two out of three and superwobble decoding. Two out of three decoding postulates that a tRNA pairing with only the first two codon bases (usually a G and a C) can be sufficient for translation and that any base can occur at the third (wobble) codon position (opposite position 34 of the tRNA; Figure 1). This mechanism is supported by several examples (Lagerkvist, 1986). It has also been suggested to occur in chloroplasts (Shinozaki et al., 1986) and supported by in vitro experiments using wheat (Triticum aestivum) germ extracts and bean (Phaseolus vulgaris) chloroplast tRNAs (Pfitzinger et al., 1990). Further analysis showed that two out of three critically depends on the stability of the second base pair formed between the codon and anticodon and that this stability is influenced by the structure of the tRNA anticodon loop (Lehmann and Libchaber, 2008).

The so-called superwobble, or extended wobble, is a mechanism in which an unmodified uridine at the first anticodon position (position 34 of the tRNA; Figure 1) can read all four nucleotides at the wobble codon position. This mechanism has also been suggested to occur in chloroplasts (Shinozaki et al., 1986; Pfitzinger et al., 1990) and was demonstrated in tobacco (Nicotiana tabacum) when one of the two chloroplast
In this work, we identified the Arabidopsis thaliana tRNA adenosine deaminase arginine (TADA) gene that codes for a deaminase responsible for the editing of the adenosine at the wobble position of cp-tRNA^{Apg}(ACG). A mutation in TADA leads to slower chloroplast translation, causing profound effects on chloroplast function and plant development. However, as opposed to the case in prokaryotes, it is not lethal, adding weight to the hypothesis that a two out of three mechanism can occur in chloroplasts.

## RESULTS

### TADA Is a Nuclear Gene Encoding a Protein Containing a Conserved Deaminase Domain

Adenosine-to-inosine (A-to-I) editing of the wobble anticodon position of several eukaryote and prokaryote tRNAs is catalyzed by enzymes of the TAD/ADAT protein family (Schaub and Keller, 2002), which contain the conserved cytidine/deoxycytidylate deaminase motif (InterPro: IPR002125). This motif comprises three His and Cys residues involved in the coordination of a zinc ion and an essential Glu residue that is required for the hydrolytic reaction. The single chloroplast tRNA known to be edited by TADA is cp-tRNA^{Apg}(ACG), which has been directly sequenced in the alga Codium fragile and in bean (Francis et al., 1989; Pfitzinger et al., 1990). While searching for candidate genes that might be implicated in chloroplast RNA editing, we searched the Arabidopsis predicted proteome for sequences containing the characteristic deaminase motif. Fifteen genes were selected (see Supplemental Figure 1 online). Among them eight correspond to the CDA family of cytidine deaminases and were discounted because they lack the requirements for an organellar RNA editing enzyme, namely, organellar targeting sequences, affinity for RNA, deaminase activity on RNA substrates, and conservation in other plant species (Faivre-Nitschke et al., 1999; S.E. Faivre-Nitschke and J.M. Gualberto, unpublished data). Of the remaining candidate genes, only two code for proteins with predicted organellar targeting sequences: At4g20960, which probably codes for the chloroplast riboflavin

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**Figure 1.** Representation of cp-tRNA^{Apg}(ICG) Binding to Its Corresponding Codon.

cp-tRNA^{Apg}(ICG) depicted as a typical cloverleaf structure. The numbering of the anticodon loop residues is according to the convention recommended by Sprinzl et al. (1996). The modifications shown are those identified in other cp-tRNA^{Apg}(ICG) that are expected to be conserved in Arabidopsis (Francis et al., 1989; Pfitzinger et al. 1990). I, inosine; N, A, C, U, or G; m^3A, N^6-methyl A; D, dehydro U; Gm, 2-O-methyl G; T, 5-methyl U; ¥, pseudo U.
deaminase; and At1g68720, which codes for a large protein predicted by PREDOTAR (Small et al., 2004) and TargetP (Emanuelsson et al., 2000) to be possibly plastid or mitochondrial respectively. By sequence homology, we identified At1g68720 as likely to code for TADA.

The Arabidopsis TADA gene contains only three introns and codes for a large protein of 1307 amino acids with a C-terminal deaminase domain (Figure 2A). The recent identification of a full-size cDNA (accession number AK117889) confirmed the gene model. The presence of an in-frame stop codon in the 5′-untranslated region unambiguously identified the initiation codon. Most of the large 5′-terminal exon codes a sequence (1080 amino acids) that has no identifiable motif or similarities to sequences outside the plant kingdom. A probable ortholog of TADA is found in rice (Oryza sativa; Os06g0489500), which also codes for a large protein (1590 amino acids). Surprisingly, the predicted N-terminal regions of the Arabidopsis and rice proteins are poorly conserved (15% identity), while the region similar to other deaminases is 65% identical (see Supplemental Figure 3 online). Additional plant homologs can be identified in the databases of genomic and EST sequences (see Supplemental Figure 3 online).

**TADA Is Targeted to the Chloroplast**

To determine the subcellular localization of the TADA protein, several green fluorescent protein (GFP) fusions were prepared. The corresponding plasmids were biolistically transformed into *Nicotiana benthamiana* leaves, and the localization of the fluorescent fusion proteins observed by confocal microscopy. A full-length TADA:GFP fusion showed no GFP fluorescence. However, the first 259 amino acids of the TADA protein efficiently targeted GFP into chloroplasts (Figure 2B). We observed no fluorescence in mitochondria, cytoplasm, or in the nucleus. Thus, the TADA protein contains an N-terminal targeting sequence that can unambiguously target the protein into chloroplasts.

![Figure 2](https://example.com/figure2.png)

**Figure 2. The Arabidopsis TADA Gene Codes for a Large Protein Targeted to Chloroplasts.**

(A) Structure of the Arabidopsis TADA gene. The coding sequences of the exons are indicated by thick gray bars, and the transcribed region is represented by the arrow. The T-DNA insertion sites in the mutants described in this study are shown. The gene sequence selected for expression of a hairpin RNA in the RNAi line Agri-140-69-2-CATMA1a58100 is represented by the black bar. Primers described in the text are indicated by orange arrowheads.

(B) Localization of a protein-GFP fusion in epidermal cells of *N. benthamiana*. 1, Visible image; 2, merged fluorescence and visible images; green, GFP fluorescence; red, natural fluorescence of chloroplasts; yellow, colocalization of green and yellow fluorescence.

(C) RT-PCR analysis of TADA transcripts using primer P5 and P6 in four independent tada-1 homozygous plants. C, negative control without reverse transcriptase.

**TADA Is Required for the Specific Editing of Chloroplast tRNA$^{A^\phi}$ACG**

We obtained several T-DNA insertions lines for TADA. We could only confirm T-DNA insertions for two lines. Line SALK_024767 has a T-DNA insertion whose left border is located just eight nucleotides upstream of the predicted transcription initiation site (Figure 2A). However, plants homozygous for the insertion showed no decrease in TADA transcript abundance compared with wild-type Columbia-0 (Col-0) plants and showed no visible phenotype differences either. It is likely that promoters internal to the T-DNA efficiently drive expression of the downstream gene (Ulker et al., 2008). Line GK-119G08 contains a T-DNA insertion that was mapped to the first exon of the gene, spanning the region containing the T-DNA insertion (Figure 2C). Thus, if a transcript is generated and translated from that locus, the resulting truncated protein would lack most of the N-terminal sequence, including the chloroplast targeting sequence. Line GK-119G08 was therefore identified as mutant *tada-1*.

We have also characterized several lines transformed with an RNA interference (RNAi) construct that we obtained from the AGRIKOLA collection (Hilson et al., 2004). Plants from line Agri-140-69-2-CATMA1a58100 accumulate no or very little amounts of TADA mRNA (see Supplemental Figure 4B online).

We tested if TADA is involved in cp-tRNA$^{A^\phi}$ACG editing. The tRNA was amplified by RT-PCR, and the cDNA was sequenced. Inosine base pairs with cytosine during reverse transcription, so A-to-I deamination leads to an apparent change in sequence from A to G. In wild-type plants, the sequence profiles suggest that there are almost equimolar amounts of edited and unedited tRNA, as evaluated by the height of the A and G peaks at the position corresponding to the anticodon wobble nucleotide (Figure 3). However, no trace of cp-tRNA$^{A^\phi}$ICG could be found in tada-1 plants. The same result was obtained with RNA extracted from five independent homozygous plants. In RNAi
plants, amplification and sequencing of cp-tRNA Arg(ACG) confirmed that silencing of TADA correlates with loss of tRNA editing: in a plant where no TADA mRNA could be detected, there was no evidence of tRNA editing, while in plants showing partial TADA suppression, a residual G is visible at the position corresponding to the tRNA wobble nucleotide. Unaffected plants had equivalent amounts of edited and unedited tRNA, as in wild-type plants (see Supplemental Figure 4C online).

Apart from chloroplast tRNA Arg(ACG), several cytosolic Arabidopsis tRNAs are also expected to be edited by A-to-I deamination (http://www.inra.fr/internet/Produits/TAARSAT/table.html). We therefore tested the hypothesis that TADA is also responsible for editing of cytosolic tRNAs. Primers were designed to amplify cytosolic tRNA Ala(AGC), tRNA Val(AAC), tRNA Thr(AGU), and tRNA Arg(ACG). Our primers were designed to amplify only a subpopulation of tRNA Arg(ACG), for which there are several variants encoded by eight different genes in Arabidopsis. Both in the wild type and in tada-1 we found evidence for efficient editing of cytosolic tRNA Ala(AGC), tRNA Val(AAC), and tRNA Thr(AGU) (Figure 3). Surprisingly, we found no evidence that cytosolic tRNA Arg(ACG) is targeted for editing, either in wild-type Col-0 or in tada-1.

TADA is also not involved in editing of nucleotide A37 of tRNA Ala(AGC) which, in all eukaryotic systems studied, is edited to I and further modified to m1I. In yeast and humans, TAD1 is the deaminase involved (Gerber et al., 1998). In the sequence of the RT-PCR products of tRNA Ala(AGC), we find a T at position 37, both in wild-type Col-0 and in tada-1, consistent with the presence of m1I.

Thus, TADA seems to be exclusively involved in editing of the prokaryote-type cp-tRNA Arg(ACG), and other deaminases must be responsible for editing of cytosolic tRNAs. No other chloroplast tRNA can be a substrate for TADA because no other chloroplast tRNA has an A at the wobble position. Regarding mitochondria, no tRNA adenosine deaminase is theoretically required by the mitochondrial translation system, as there are no mitochondrially encoded tRNAs with an A at the wobble position, and all tRNAs that should require editing for function are imported (presumably preedited) from the cytosol (Duchene et al., 2008).

We also considered the hypothesis that TADA is responsible for C-to-U editing of chloroplast and/or mitochondrial mRNAs that, by analogy to animal APOBEC and AID proteins involved in
C-to-U editing, might be catalyzed by enzymes containing the same characteristic cytidine/deoxyctydylate deaminase domain (Conticello, 2008). However, the plant TADA is apparently not involved in organellar C-to-U editing; regions of the ndhB, ndhD, rps12, and rps14 chloroplast transcripts containing 11 editing sites were analyzed by RT-PCR and sequence, but no differences were found between mutant and wild-type plants (see Supplemental Figure 5 online). We conclude that TADA is not required for C-to-U chloroplast mRNA editing. We have also tested if editing of mitochondrial transcripts is affected, but none of 82 editing sites in the transcripts of ccnC, ccnFc, cox3, rps12, nad3, and nad5 is impaired in editing in the tada-1 mutant (see Supplemental Figure 6 online).

The Deaminase Domain of TADA Is Structurally Similar to Other Prokaryotic TADA Enzymes

We constructed a homology model of the TADA deaminase domain based on the crystal structure of *Staphylococcus aureus* TadA (2B3J) in complex with RNA (Losey et al., 2006). This model shows that most of the residues implicated in interactions with the zinc ion and the ligand are conserved (Figure 4). In particular, the interactions with base 34 of the tRNA are preserved (Figure 4B); Ile-26 is replaced by Val-1131 (as in *Escherichia coli* and *Aquifex aeolicus*), while the three other amino acids involved are absolutely conserved (Asn-1148, His-1159, and Ala-1160).

This allowed us to predict that the N-terminal domain, although not built in our model, is not a hindrance to tRNA recognition, being rather distant from the binding site (Figure 4A). A major difference between bacterial TadAs and plant chloroplast TADAs is an insertion of 16 residues that is present in all plant sequences found in the databases (see Supplemental Figure 3 online), except for the atypical *Chlamydomonas reinhardtii* protein that also has no predicted N-terminal extension and targeting sequence. This insertion is also poorly conserved in sequence among the different plant TADAs. Finally, our model also proposes a dimer of the same type as in the three bacterial TadA structures known. The portions of the chain involved in contact between the monomers are well conserved, with no sequence insertions and little sequence variation, comparable to that observed between the three known structures.

The Deaminase Domain of TADA Is Sufficient for the Deamination of cp-tRNA Arg(ACG) to cp-tRNA Arg(ICG) Both in Vitro and in Planta

We tested in vitro the activity of the C-terminal part of TADA on the deamination of cp-tRNA Arg(ACG). The sequence coding the last 194 amino acids of TADA that comprises the deaminase domain (∆N-TADA) was expressed in *E. coli* fused to an N-terminal His-tag, and the activity of the purified soluble protein fraction was tested on in vitro–synthesized cp-tRNA Arg(ACG) labeled with [32P]ATP. The presence of inosine was analyzed by thin layer chromatography (TLC) as described (Grosjean et al., 2007). One-dimensional TLC showed a radioactive spot comigrating with inosine monophosphate (IMP), as a consequence of adenosine deaminase activity by ∆N-TADA (Figure 5A1, lane c). No activity was detected in extracts prepared in the same conditions using an unrelated construct cloned in the same expression vector, used as control for contamination by bacterial Tada (Figure 5A1, lane b). The identity of the IMP spot was further confirmed by two-dimensional TLC analysis using different solvent systems (Figure 5A2). To definitely confirm that the activity does not result from bacterial TadA contamination, we also tested the activity of ∆N-TADA purified to homogeneity in denaturing conditions and

![Figure 4. Model of the Arabidopsis TADA Protein Bound to tRNA Substrate.](image)
renatured in vitro. This protein fraction was less active than the protein purified in native conditions but confirmed the deaminase activity of ΔN-TADA on cp-tRNA^Arg^AG(ACG) (Figure 5A3). We also tested the activity of ΔN-TADA on other tRNA substrates, namely, nuclear encoded cytosolic tRNA^Arg^AG(ACG) and cytosolic tRNA^Arg^AG(ACG). Only cytosolic tRNA^Arg^AG(ACG) was also recognized as substrate by ΔN-TADA (Figure 5A1, lanes d and e, respectively).

We also tested the activity of ΔN-TADA in planta by fusing it to the signal peptide of the chloroplast protein RECA1 (At1g79050) and expressing it in tada-1 under the control of the 3SS promoter. ΔN-TADA restored the deamination of cp-tRNA^Arg^ICG to cp-tRNA^Arg^ICG (Figure 5B), confirming that TADA is responsible for this activity in Arabidopsis and that the large N-terminal extension is dispensable for function both in vitro and in planta.

A Lack of cp-tRNA^Arg^ICG Impairs Photosynthesis

Homozygous tada-1 plants show severe phenotypes, including delayed growth, pale-green leaves, and very poor fertility (Figure 6). Leaf cross sections do not show any histological differences compared with young wild-type plants of the same size, and there is no apparent difference in the number of chloroplasts per cell (see Supplemental Figure 7 online). The pale-green color of the mutant is therefore a consequence of reduced chlorophyll content as confirmed by spectrophotometric quantification (see Supplemental Table 1 online). Complementation of the tada-1 mutation by ΔN-TADA targeted to chloroplasts restored a wild-type phenotype (Figure 6), showing that it is the absence of cp-tRNA^Arg^ICG that impairs plant growth. RNAi plants showed similar phenotypes of slow growth rate and delay in flowering, which correlated with the accumulation of TADA mRNA (see Supplemental Figure 4A online).

Different photosynthetic parameters were derived from saturation pulse-induced fluorescence measurements to determine the effects of the tada-1 mutation on photosynthesis and photoprotection (Kramer et al., 2004). The maximum quantum efficiency of photosystem II (PSII) photochemistry (Fm/Fp) of the tada-1 mutant was 60% of that of Col-0 (0.45 ± 0.039 versus 0.77 ± 0.013, respectively). The tada-1 mutation also affected other photosynthetic parameters, such as photochemical and nonphotochemical quenching (NPQ) (Table 1). Measurement of electron transport rates and chlorophyll fluorescence quenching indicated that tada-1 plants dissipated more excitation energy by NPQ than Col-0, as shown by the significantly 2.5-fold higher light-dependent thermal dissipation component of NPQ in tada-1 (0.039 versus 0.035), while, by contrast, the values for tada-1 plants (0.0) suggest that PSII is closed in the mutant (i.e., reduced Qa pool).

A Lack of cp-tRNA^Arg^ICG Has Profound Effects on the Chloroplast Transcriprome and Translation

To understand the reasons for the sharp drop in photosynthesis and chlorophyll content, we analyzed the accumulation of several chloroplast proteins by protein gel blots. In tada-1 total protein extracts, we saw a marked decrease of all chloroplast-encoded proteins tested (cytochrome f, cytochrome b6f, and D1), whereas most nucleus-encoded proteins were much less affected (Figure 7). Coomassie blue staining of protein gels also showed a decrease in RbcL accumulation. Interestingly, nucleus-encoded PsAD and plastocyanin were also strongly decreased in tada-1. These proteins are associated with the electron transport chain, and their decrease could be a consequence of a deficiency in the assembly of functional photosystems, as shown by the fluorescence measurements. The decrease in plastid-encoded proteins could be related to a decrease in protein synthesis or stability. A pulse-labeling experiment showed that Met incorporation in RbcL is strongly
reduced in tada-1, more than fivefold as determined by phosphor-imager quantification (Figure 8). This result does not exclude that the stability of plastid-encoded proteins is also affected but clearly shows that protein synthesis is decreased in tada-1 chloroplasts.

The decrease in the amounts of all chloroplast-encoded proteins tested as well as the decrease in RbcL synthesis suggests a global slowdown of chloroplast protein synthesis. This could be due to a decrease in chloroplast mRNA accumulation or translation. To discriminate between the two possibilities, we analyzed the accumulation of every chloroplast mRNA by quantitative RT-PCR (qRT-PCR) (Figure 9). Surprisingly, the tada-1 mutant overaccumulates most chloroplast transcripts compared with the wild type. This is a consequence of the lack of cp-tRNA\textsubscript{Arg}(ICG), as expression of ΔN-TADA restores a transcription profile that does not significantly deviate from the wild type. In tada-1, transcripts of petA, petB, and psbA (respectively encoding cytochrome f, cytochrome b\textsubscript{6}, and protein D1) are 3.7, 2.5, and 1.7 times more abundant in tada-1 compared with the wild type respectively, while the rbcL transcript is unchanged. In all cases, there is no correlation between the decrease in protein products and the accumulation of the corresponding transcripts that remain at least at the same levels as in wild-type plants. This further supports the hypothesis that tada-1 plants are impaired in chloroplast translation.

Furthermore, RNAs transcribed by the nucleus-encoded RNA polymerase, such as rpo, accD, and rpl transcripts, accumulate more than those transcribed by the plastid-encoded RNA polymerase (PEP), such as rbcL, psa, and most psb transcripts (Figure 9). Although the quantitative variations are different, this pattern is similar to the chloroplast transcript profiles of Arabidopsis PEP-deficient mutants, such as clb19 and ptac2 (Chateignier-Boutin et al., 2008), suggesting a partial inhibition of PEP activity in the tada-1 mutant. This inhibition is consistent with a decrease in chloroplast translation, as all four of the PEP subunits are encoded in the plastid genome.

**tada-1 Can Still Properly Synthesize Chloroplast Proteins**

In the absence of cp-tRNA\textsubscript{Arg}(ICG), if CGC and CGA codons could not be recognized by unmodified cp-tRNA\textsubscript{Arg}(ACG), plant survival should depend on misreading of these codons by a noncognate tRNA. This would result in incorporation of amino acids other than Arg at positions coded by CGC or CGA. We checked this possibility by studying the tryptic digest profile of RbcL. Trypsin is a protease cleaving specifically after Arg or Lys residues. If CGC and CGA codons are translated incorrectly, peptides corresponding to cleavage at these particular sites should no longer be detected. After fractionation of total tada-1 proteins by SDS-PAGE and trypsin digestion of the RbcL band, we could unambiguously identify 31 peptides derived from plastid-encoded proteins, namely, RbcL, AtpA, and AtpB (Table 2). Among these peptides, two correspond to cleavage at two out of the eight potentially mistranslated Arg residues in RbcL, three correspond to cleavage at three out of the eight potentially mistranslated Arg residues in AtpB, and two correspond to cleavage at two out of the eight potentially mistranslated Arg residues in AtpA. Therefore, although we cannot exclude that mistranslation occurs in tada-1 chloroplasts, our results show that Arg residues are accurately incorporated at positions corresponding to CGC and CGA codons.

### Table 1. Photosynthetic Efficiency

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<th>( \Phi_{\text{PSII}} )</th>
<th>( \Phi_{\text{NPQ}} )</th>
<th>( \Phi_{\text{f,D}} )</th>
<th>( \Sigma )</th>
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<td>Col-0</td>
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<td>tada-1</td>
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The photochemical efficiency, \( \Phi_{\text{PSII}} \), light-dependent thermal dissipation component of nonphotochemical quenching, \( \Phi_{\text{NPQ}} \), the sum of fluorescence quenching and light-independent thermal dissipation, \( \Phi_{\text{f,D}} \), and their respective sums, \( \Sigma \), were determined at normal light. Four 3-week-old plants per line were measured.
DISCUSSION

TADA Is a tRNA<sup>Arg</sup> Adenosine Deaminase

The chloroplast translation system is derived from its cyanobacterial ancestor and retains predominant prokaryotic characteristics. The recruitment of proteins from the symbiont host has had limited influence on the evolution of the plastid translation system because a significant proportion of its components are still encoded by the plastid genome. These include all rRNAs and tRNAs. Chloroplasts have not acquired exogenous gene sequences during evolution and do not import external tRNAs like plant mitochondria, which have evolved a translation system able to cope with tRNAs that originate from three different genomic compartments (Maréchal-Drouard et al., 1993). cp-tRNAs retain all the typical characteristics of prokaryotic tRNA<sub>5</sub>s. Among these is the editing of cp-tRNA<sup>Arg</sup>(ACG) into cp-tRNA<sup>Arg</sup>(ICG), a deamination reaction that we show is catalyzed by the TADA protein.

The Arabidopsis TADA gene encodes a large protein of which only the C-terminal part resembles bacterial tRNA adenosine deaminases. We modeled this C-terminal domain because the high sequence similarity with bacterial TadA made three-dimensional structure modeling relatively straightforward. In the three bacterial structures that have been determined (2B3J, 1Z3A, and 1WWR), the active site pocket is formed by residues from two different chains. Therefore homodimerization is an absolute requirement for the assembly of the biologically active unit. We identified the positions putatively involved in dimer formation in plant TADAs by comparing the accessible surface of the 2B3J dimer and an isolated monomer (chain 2B3J:A). A third of the residues in those positions is conserved compared with the three known structures. Residues of chain B involved in tRNA binding in pocket A are also well conserved (see Supplemental Figures 3 online): Arg-1176 and Arg-1200 are kept, and at position 1247 an Arg is replaced by a Lys, preserving its basic nature. However, positions 1178 and 1245 are less conserved: Glu is replaced by Ala-1178 (although the E. coli protein has an Ile at this position), and Asn is replaced by His-1245 in all plant sequences. Taken together, despite these small differences, our model suggests that the active form of plant TADA is likely to be a dimer, as in 2B3J, 1Z3A, or 1WWR.

We have shown that the TADA deaminase domain (ΔN-TADA) is sufficient for the deamination of cp-tRNA<sup>Arg</sup>(ACG) to cp-tRNA<sup>Arg</sup>(ICG) both in vitro and in planta (Figure 5). Interestingly, ΔN-TADA was also able to deaminate the cytosolic tRNA<sup>Arg</sup>(ACG) but not tRNA<sup>Ala</sup>(AGC). Because chloroplast and cytosolic tRNA<sup>Arg</sup>(ACG) share little sequence identity, apart from the anticodon loop bases, this suggests that the anticodon loop nucleotides are necessary and sufficient for tRNA recognition by TADA. Mutagenesis studies and the determination of TAD A structures showed that each one of the anticodon nucleotides is required for the recognition of the tRNA by bacterial TadA (Wolf et al., 2002; Kuratani et al., 2005; Losey et al., 2006; Luo and Schramm, 2008). The bacterial TadA is probably the ancestor of the eukaryotic ADAT2/TAD2 that, in yeast, forms a heterodimer with ADAT3/TAD3 and catalyzes deamination of seven tRNA species (Gerber and Keller, 1999). However, the bacterial TadA is

![Figure 7. Reduced Accumulation of Plastid-Encoded Proteins in tada-1.](image)

Protein gel blots of total leaf proteins of wild-type Col-0 and tada-1 plants were analyzed by immunodetection with antibodies recognizing plastid-encoded and nucleus-encoded chloroplast proteins. Plants were grown in vitro in agar plates without sucrose. PsbA, D1 protein; PetA, cytochrome f; PetB, cytochrome b<sub>6</sub>; PC, plastocyanine; PsaA, photosystem I reaction center subunit A; PsaD, idem subunit D; PsbO, oxygen-evolving enhancer protein 1; LHCII, 26-kD protein from light-harvesting complex II; PCOR, protochlorophyllide oxidoreductase; RbcS, small subunit of Rubisco; RbcL, large subunit of Rubisco.

![Figure 8. Reduced Synthesis Rate of RbcL in tada-1.](image)

Leaf discs of tada-1 and Col-0 were labeled with [35S]Met and incubated for 10, 20, or 30 min in the presence of cycloheximide to inhibit cytosolic translation. Total proteins were then fractionated by SDS-PAGE and autoradiographed. The asterisk indicates the RbcL band.
unable to deaminate substrates of ADAT2/TAD2 in vitro, with the exception of yeast tRNA_\text{Arg}. It is therefore not surprising that the plant TADA, which is phylogenetically related to bacterial TadA, is not involved in the deamination of other tRNAs. In vivo, chloroplast targeting of TADA probably prevents it from deaminating the cytosolic tRNA_\text{Arg}(ACG). The functional homolog of ADAT2/TAD2 in plants remains to be identified.

All higher-plant TADA sequences display large N-terminal domains, whose possible function(s) remain mysterious. They comprise the chloroplast targeting peptide, which in Arabidopsis is contained in the first 259 amino acids (Figure 2B), but the remaining N-terminal sequences contain no identifiable motifs, have no similarities to non-plant proteins and are poorly conserved within the plant kingdom (see Supplemental Table 2 online). The Arabidopsis N-terminal domain is dispensable for the activity and specificity of TADA in vitro (Figure 5A). In vivo, the C-terminal domain alone is sufficient to restore the deamination of cp-tRNA_\text{Arg}(ACG) to levels comparable to the wild type (Figure 5B), reestablishing normal plant growth (Figure 6).

### Table 2. Tryptic Digest of RbcL from tada-1

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</tbody>
</table>

After mass spectrometry analysis of the RbcL band of tada-1, 31 peptides derived from RbcL, AtpB, and AtpA were unambiguously identified. Only peptides corresponding to cleavage at potentially mistranslated Arg residues (in bold) are listed in this table. Ions score is $-10^\log(P)$, where P is the probability that the observed match is a random event. Ions scores >29 indicate identity or extensive homology ($P < 0.05$). Mr Calc, relative mass calculated from the mass-to-charge (m/z) ratio. The full list of ions observed during this experiment is available in the Supplemental Data Set 1.

Figure 9. Accumulation of Chloroplast Transcripts in tada-1 and ΔN-TADA.

Transcript abundances of all protein-encoding and rRNA genes of the chloroplast genome from tada-1 (black bars) and tada-1 complemented with ΔN-TADA (gray bars) were compared with the wild type by qRT-PCR and normalized against a set of nuclear housekeeping genes. The genes are sorted according to their physical location on the chloroplast chromosome. Error bars are standard deviations based on three biological replicates for tada-1 and technical triplicates for tada-1 complemented with ΔN-TADA. Asterisks mark transcripts for which the corresponding protein has been analyzed by protein gel blots (Figure 7). Values are given in Supplemental Table 3 online.
The Absence of cp-tRNA^{Arg(ICG)} Slows Down Chloroplast Translation, Which Affects Photosynthesis

Contrary to the situation in bacteriawhere, TadaA is essential for growth, knockout of the Arabidopsis TADA gene is compatible with plant survival. Nevertheless, the mutation has profound effects on the efficiency of chloroplast translation (Figures 6 to 9). In particular, the levels of key components of both photosystems (protein D1 and Psaa) and of the thylakoidal electron transport chain (cytochrome b6f complex subunit VI and cytochrome f) are substantially lower. Although levels of LHClI, a PSII light-harvesting chlorophyll a/b binding protein of the antenna system (Jansson, 1999), are not as affected as chloroplast-encoded proteins, total chlorophyll content is only 40% of wild-type levels in tada-1 plants (see Supplemental Table 1 online). The combination of inefficient energy transfer from the antenna complexes, reduced Qa pool, and impaired electron transport chain performance could result in a dramatically lower ratio of photons absorbed by PSII being channeled through photochemical processes (Fv/Fm, Kramer et al., 2004) and the more than doubled amount of nonphotochemical quenching, Fv/FPQ, in the tada-1 plants (Table 1).

A priori, the reduced protein synthesis in tada-1 chloroplasts could be due to any of the following defects: (1) reduced levels of translatable mRNA, (2) reduced rates of translation initiation, (3) reduced rates of protein synthesis (chain elongation), and (4) increased rates of protein turnover. We excluded the first of these possible explanations as we found that tada-1 mRNA levels are at least equivalent to those found in wild-type plants. Given that the primary defect in tada-1 plants is the loss of cp-tRNA^{Arg(ICG)}, the logical assumption is that chloroplast translation is impaired at the elongation step.

When stalled at particular sites, ribosomes can dissociate and/or induce cleavage of the mRNA (reviewed in Dreyfus, 2009). However, slower elongation rates have been associated with the accumulation of heavier polysomes both in yeast and mammals (Hovland et al., 1999; Shenton et al., 2006; Sivan et al., 2007). As a result, mRNAs densely covered with ribosomes might be protected from degradation by nucleases, becoming more stable (Deana and Belasco, 2005; Dreyfus, 2009). This phenomenon, also proposed by Pfalz et al. (2009), could explain the accumulation of plastid transcripts in tada-1, despite the expected reduction in PEP synthesis. A positive effect of reduced chloroplast translation on chloroplast mRNA accumulation was also found for the svr1 Arabidopsis mutant (Yu et al., 2008) but, surprisingly, not in transplastomic tobacco plants impaired in translation by deletion of one of the two cp-tRNA^{Gly} genes (Rogalski et al., 2008). This could be because different compensatory mechanisms may be in place. However, in the latter study, only three plastid mRNAs were monitored, including the rbcL transcript whose steady state level is also not affected in tada-1.

Arg Codon Usage Is Biased in Arabidopsis Chloroplasts

The absence of cp-tRNA^{Arg(ICG)} in tada-1 plants theoretically prevents translation of two out of the six Arg codons (CGA and CGC) in Arabidopsis chloroplasts. However, the mutation is not lethal, and Arg is still properly inserted at protein positions coded by CGA and CGC codons (Table 2), implying that normal wobble pairing rules can be infringed in chloroplasts. Even in wild-type plants, neither cp-tRNA^{Arg(ACG)} nor cp-tRNA^{Arg(IGC)} can form a wobble pair with CGG codons, which must be read by a nonstandard decoding mechanism. This might give a clue as to why a lack of cp-tRNA^{Arg(ICG)} does not completely block plastid translation.

A detailed analysis of the codon usage of Arabidopsis chloroplast genes shows that the group formed by rbcL, psb, and psa transcripts contains a significantly smaller proportion of CGG codons than other chloroplast transcripts (see Supplemental Table 4 online; Z-test, P value < 0.01). rbcL and psbA transcripts do not contain any CGG codons at all, suggesting that this particular codon is not compatible with high translation rates.

cp-tRNA^{Arg(ACG)} and cp-tRNA^{Arg(IGC)} have overlapping repertoires according to the wobble rules (both should read CGU codons efficiently), which raises the question of why only ~50% of cp-tRNA^{Arg(ACG)} is apparently deaminated. The Arg codon usage shows a significant bias toward CGU codons compared with CGA and CGG codons in highly translated mRNAs, such as those encoding subunits of ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco), PSI, and PSII (Z-test, P value < 0.001). This might explain the retention of cp-tRNA^{Arg(ACG)}, as it is expected to be more efficient than cp-tRNA^{Arg(ICG)} at translating CGU codons. These data therefore suggest that CGN codon usage of chloroplast genes is under selection for optimal translation efficiency, given the presence of both cp-tRNA^{Arg(ACG)} and cp-tRNA^{Arg(ICG)}, and that the preferred codon of the CGN series for highly translated RNAs is CGU. This codon bias, which minimizes the necessity for cp-tRNA^{Arg(ICG)}, is undoubtedly a major reason for why the tada-1 mutation is not lethal.

Two Out of Three Decoding Probably Occurs in Chloroplasts

A complete impairment of chloroplast translation is embryolethal in Arabidopsis and tobacco, as demonstrated by knockout mutants either of chloroplast ribosomal proteins (Ahlert et al., 2003; Rogalski et al., 2006; http://www.seedgenes.org), of aminoacyl-tRNA synthetases (Berg et al., 2005), or of some tRNAs (Rogalski et al., 2008). Therefore, the lack of cp-tRNA^{Arg(ICG)} is partially compensated for by some other mechanism in tada-1 chloroplasts. According to the superwobble rules, an unmodified uridine at the first anticodon position (position 34 of the tRNA; Figure 1) can read all four nucleotides at the wobble codon position. The possibility for a uridine to accommodate any nucleotide in the codon is restricted to position 34. For this reason, the translation of any of the CGN Arg codons by cp-tRNA^{Arg(UCU)} is most unlikely because this would involve superwobbling to occur at both positions 34 and 36 of the tRNA.

Most probably, the translation of CGN codons in tada-1 chloroplasts occurs via two out of three decoding using cp-tRNA^{Arg(ACG)}. This tRNA lacks a uridine at position 34 required for superwobble but can form the strong Watson-Crick pairing at positions 35 and 36 (Figure 1) required to stabilize codon-anticodon pairing when there is a mismatch at the third position (Lehmann and Libchaber, 2008). However, tobacco cp-tRNA^{Arg(GCC)} is apparently unable to decode by two out of three, despite that positions 2 and 3 of the anticodon are cytidines
(Rogalski et al., 2008). This could be because, in cp-tRNA\(^{\text{Gly}}\)(GCC), U\(_{26}\) and A\(_{29}\) can form a strong Watson-Crick pairing, whereas C\(_{26}\) and A\(_{29}\) in cp-tRNA\(^{\text{Ala}}\)(AGC) can only form a single weak hydrogen bond (Auffinger and Westhof, 1999). To allow two out of three, the N\(_{32}\)-N\(_{38}\) pair has to be non-Watson-Crick to allow the flexibility of the anticodon loop required for proper stacking of the bases (Lehmann and Libchaber, 2008).

These arguments are in favor of two out of three decoding occurring in plastids, even in wild-type Arabidopsis plants, as the Arg CGG codon cannot form a canonical wobble pairing with either cp-tRNA\(^{\text{Ala}}\)(AGC) or cp-tRNA\(^{\text{Gly}}\)(GCC). However, the decrease of translation activity in tada-1 clearly shows that cp-tRNA\(^{\text{Arg}}\)(ACG) cannot read all CGN Arg codons efficiently, demonstrating that in this particular case, two out of three pairing is much less efficient than wobble pairing. From this perspective, the deamination of cp-tRNA\(^{\text{Arg}}\)(ACG) to cp-tRNA\(^{\text{Arg}}\)(IGC) enhances plastidial translation by replacing inefficient two out of three by canonical wobble codon-anticodon pairing.

METHODS

Plant Materials and Growth Conditions

All experiments used the Col-0 strain of Arabidopsis thaliana as genetic background. Plants were grown on soil under long days (16 h light/8 h dark) at 22°C. For in vitro growth experiments, seeds were surface sterilized and plated on Murashige and Skoog agar plates with or without 1% sucrose. Plates were incubated in the dark at 4°C, except during two out of three by canonical wobble codon-anticodon pairing. In this perspective, the deamination of cp-tRNA\(^{\text{Arg}}\)(ACG) to cp-tRNA\(^{\text{Arg}}\)(IGC) by two out of three, the N\(_{32}\)-N\(_{38}\) pair has to be non-Watson-Crick to allow the flexibility of the anticodon loop required for proper stacking of the bases (Lehmann and Libchaber, 2008).

The activity of the protein was tested on in vitro–synthesized tRNAs labeled with \(^{32}\)P\(^{\text{ATP}}\). The cp-tRNA\(^{\text{Arg}}\)(AGC), cytosolic tRNA\(^{\text{Arg}}\)(AGC), and cytosolic tRNA\(^{\text{Ala}}\)(AGC) sequences were amplified with primers P53+P54, P55+P56, and P57+P58 directly fused to a T7 RNA polymerase promoter including a BstNI site at the 3' terminus to correctly generate CCA 3'-ends. After BstNI digestion, PCR fragments were used as templates for transcription using the RibopMAX RNA production system (Promega) in the presence of [\(^{\text{32}}\)P]\(^{\text{ATP}}\). Incubation conditions were as described (Wolf et al., 2002). Afterwards, the tRNA substrates were digested with nuclelease P1 to generate 5'-monophosphate nucleosides that were analyzed by one- or two-dimensional TLC as described (Grosjean et al., 2007).

Complementation of the tada-1 Mutant

The genomic fragment coding for the last 280 amino acids of TADA (\(\Delta\)N-TADA) was amplified with Gateway primers P47+P48 and recombined into pDONR221 by BP cloning (Invitrogen). Similarly the signal peptide of chloroplast protein RECA1 (A11g79050) was amplified with primers P49 and P50 and recombined by BP cloning into pDONRP4P1R. They were fused into the binary vector pB7m24GW35S under the control of the 3SS promoter by LR cloning (Invitrogen). The segregation of the complemented tada-1 mutant was checked as mentioned above, and the presence of the \(\Delta\)N-TADA construct was confirmed by PCR with primers pairs P51+P52 and P53+P54 amplifying the 5' and 3' borders of the construct, respectively.

Protein Gel Blots

Samples of 15 μg of proteins extracted from leaves of 3-week-old plants were fractionated by SDS-PAGE and blotted onto Immobilon-P membranes (Millipore). Antibodies against protein D1, PCOR, PsAD, plastocyanin, PsAA, PsBo, and cytochrome b\(_6\) were purchased from Agrisera and used following the manufacturer’s recommendations. Antibodies against LHCl, Rubisco small subunit, and cytochrome f were a kind gift of Géraldine Bonnard (Institut de Biologie Moléculaire des Plantes). Proteins recognized by the antibodies were revealed by ECL (GE Healthcare) and autoradiography.

Pulse Labeling of Chloroplast-Encoded Proteins

Pulse labeling experiments were conducted essentially as described (Takahashi et al., 2007). Leaf discs of 19.6 mm\(^2\) from young leaves of 3-week-old plants were vacuum infiltrated for 30 s in 0.5 mL of 1 mM KH\(_2\)PO\(_4\) pH 6.3, 0.1% Tween 20, 100 μCi of \(^{35}\)S methionine (specific activity >1000 Ci/mmol). Cycloheximide (100 μg/mL) was added to the infiltration buffer to inhibit cytoplasmic translation. After infiltration, leaf discs were washed in 10 mL water and floated in water plus 100 μg/mL cycloheximide. Discs were exposed to light at 25°C and immediately frozen in nitrogen at the indicated times (10, 20 and 30 min, four leaf discs for each time point). Total proteins were extracted, fractionated by SDS-PAGE and blotted onto Immobilon-P membranes (Millipore) before autoradiography. Quantifications were performed with a Fuji BAS 1000 Imager and MacBAS software version 2.1.

Analysis of RbcL by Mass Spectrometry

Total proteins from leaves of tada-1 were fractionated on a 12% SDS-PAGE gel. After Coomassie Brilliant Blue staining, the band corresponding to RbcL was excised, destained in acetonitrile 50% 10 mM NH\(_4\)CO\(_3\) and trypsin digested overnight. Peptides were extracted from the gel with 50% acetonitrile and 0.5% TFA, dried by vacuum, and analyzed with an 1100 Series HPLC coupled with a 6510 Q-TOF mass spectrometer (Agilent).

Observation of GFP Fluorescence

For in vivo intracellular localization, the sequences encoding the full-length and the first 259 amino acids of TADA were amplified with primers...
P2+P10 and P2+P4 and cloned in plasmid pCK-GFP3A to express protein:GFP fusions under the control of a 35S promoter as described (Vermel et al., 2002). The resulting plasmids were Nicotiana benthamiana leaves by bombardment, and images were obtained 24 h after transfection with a Zeiss LSM510 confocal microscope with >60 objectives. The excitation wavelength for GFP detection was 488 nm. Chloroplasts were visualized by the natural fluorescence of chlorophyll. Organelle targeting predictions were determined with programs Predotar and TargetP (http://urgi.versailles.inra.fr/predotar/predotar.html and www.cbs.dtu.dk/services/TargetP/).

Analysis of Chloroplast Transcripts

tada-1 mutants were grown under continuous light in soil, and the analysis of the steady state levels of chloroplast transcripts by qRT-PCR was conducted on three independent biological replicates as described (Chateigner-Boutin et al., 2008). Total Arabidopsis RNA was extracted with the RNeasy plant mini kit (Qiagen), and genomic DNA was removed using RNase-free DNase (DNA-free; Ambion). The absence of DNA was confirmed by PCR prior to reverse transcription with random hexanucleotide primers and Superscript III reverse transcriptase (Invitrogen). Real-time PCR analysis was conducted in 384-well plates with a LightCycler 480 (Roche) using primer pairs described by Chateigner-Boutin et al. (2008) and the LightCycler 480 SYBR I Master mix (Roche). Primer pairs actin2.8F + actin2.8R, Q18SF + Q18SR, UBC-1 + UBC-2, and YLS8-1 + YLS8-2 were used to measure the accumulation of nucleus-encoded transcripts. The specificity of each primer pair was checked by sequencing the PCR product and subsequently by melting curve analysis. For each primer pair and each comparison between mutant and wild-type Col-0, a standard curve based on serial dilutions of the Col-0 cDNA was included. The accumulation of each transcript was analyzed in triplicate and, as the total set of primers was analyzed over more than one PCR plate, primer pairs QC16S, QC23S, and QC18S were included in each plate to correct for variations between plates. The raw data were analyzed using the LightCycler 480 software release 1.5 (Roche) and crossing plate to correct for variations between plates. The raw data were analyzed using the LightCycler 480 software release 1.5 (Roche) and crossing point determined by second derivative maximum analysis. The accuracy of chloroplast transcripts was normalized by setting the average accumulation of each transcript was analyzed in triplicate using the LightCycler 480 software release 1.5 (Roche) and crossing point determined by second derivative maximum analysis. The accuracy of chloroplast transcripts was normalized by setting the average ratio of nuclear transcripts to 1.

Analysis of tRNA and mRNA Editing

Supplemental Data

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

Supplemental Figure 1. Cytidine/Deoxyxycytidylate Deaminase Motif Found in Several Arabidopsis Predicted Proteins.

Supplemental Figure 2. Alignment of the Arabidopsis, Rice, and E. coli TADA Proteins Highlights the Poor Conservation of the Large N-Terminal Domains of the Plant TADA.

Supplemental Figure 3. Alignment of the C-Terminal Active Domains of Plant TADA Sequences Found in Genomic and EST Databases Compared with Bacterial TADAs.

Supplemental Figure 4. RNAi Plants Deficient in TADA Expression Are Also Affected in cp-tRNA Editing.

Supplemental Figure 5. TADA Is Not Involved in Chloroplast C-to-U Editing.

Supplemental Figure 6. TADA Is Not Involved in Mitochondrial C-to-U Editing.

Supplemental Figure 7. tada-1 Plants Have Normal Leaf Structure and Equivalent Number of Chloroplasts per Cell as Wild-Type Plants.

Supplemental Table 1. Chlorophyll Content of tada-1 Plants.

Supplemental Table 2. Similarities between Plant TADA N-Terminal and C-Terminal Domains.

Supplemental Table 3. Accumulation of Chloroplast Transcripts in tada-1 and tada-1 Complemented with ΔN-TADA Compared with Col-0.
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