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The absence of A-to-I editing in the anticodon of plant cytoplasmic $tRNA^{Arg}_{ACG}$ demands a relaxation of the wobble decoding rules

Carolin A. Aldinger,[1] Anne-Katrin Leisinger,[1] Kirk W. Gaston,[2] Patrick A. Limbach[2] and Gabor L. Igloi[1,*]

[1]Institut für Biologie III; Universität Freiburg; Freiburg, Germany; [2]Rieveschl Laboratories for Mass Spectrometry; Department of Chemistry; University of Cincinnati; Cincinnati, OH USA

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Abbreviations: GFP, green fluorescent protein; TEA, triethylammonium acetate; HFIP, hexafluoroisopropanol; PAGE, polyacrylamide gel electrophoresis

It is a prevalent concept that, in line with the Wobble Hypothesis, those tRNAs having an adenosine in the first position of the anticodon become modified to an inosine at this position. Sequencing the cDNA derived from the gene coding for cytoplasmic $tRNA^{Arg}_{ACG}$ from several higher plants as well as mass spectrometric analysis of the isoacceptor has revealed that for this kingdom an unmodified A in the wobble position of the anticodon is the rule rather than the exception. In vitro translation shows that in the plant system the absence of inosine in the wobble position of $tRNA^{Arg}$ does not prevent decoding. This isoacceptor belongs to the class of tRNA that is imported from the cytoplasm into the mitochondria of higher plants. Previous studies on the mitochondrial tRNA pool have demonstrated the existence of $tRNA^{Arg}_{ICG}$ in this organelle. In moss the mitochondrial encoded distinct $tRNA^{Arg}_{ACG}$ isoacceptor possesses the I34 modification. The implication is that for mitochondrial protein biosynthesis A-to-I editing is necessary and occurs by a mitochondrion-specific deaminase after import of the unmodified nuclear encoded $tRNA^{Arg}_{ACG}$.

Introduction

Codon-anticodon recognition between triplets of an mRNA and a specific tRNA is the key element in the translation of the genetic code. In general, the precision of this process is dominated by a strict Watson-Crick base-pairing scheme. However, the degeneracy of the genetic code led Crick to propose the Wobble Hypothesis,[1] permitting a less restraining interaction with the third base of the codon and involving the participation of inosine for decoding C-ending codons. In formulating this concept, Crick predicted that, "…. inosine will be formed enzymically from an adenine in the nascent sRNA. This may mean that A in this position will be rare or absent, depending upon the exact specificity of the enzyme(s) involved."[1] The validity of this insight was subsequently confirmed, as evident from the collection of primary structures of numerous tRNAs[2] and elucidated in detail in a review[3] which pointed out a mere four exceptions to the rule.[4-7]

Recently, while characterizing an A-to-I editing system in Arabidopsis chloroplasts using the standard reverse transcriptase assay,[8] "surprising" data, which were not commented further, were presented showing that the mature cytoplasmic $tRNA^{Arg}_{ACG}$ counterpart showed no signs of an A-to-I conversion. This independent observation was in agreement with results that have emerged from our work on the plant arginine system[9] and confirmed our doubts on the accepted nature of the wobble base in this tRNA.

The RNA sequence of a wheat $tRNA^{Arg}$ isoacceptor whose gene encodes an ACG anticodon was reported in 1986.[10] Among other modified bases that were found, particular emphasis was placed on the presence of inosine at the wobble position of the anticodon. While its presence in a plant tRNA had not been reported previously, an inosine at this position of the anticodon was in line with the Wobble hypothesis and not inappropriate in view of its established localization in murine[11] and bacterial[12] $tRNA^{Arg}$, as well as subsequently in the chloroplast isoacceptor.[13] Glover et al.,[14] analyzed the group of tRNAs that are nuclear encoded and subsequently imported into wheat mitochondria. Here, using chemical sequencing, reverse transcriptase sequencing and 2D TLC, the presence of I34 in $tRNA^{Arg}_{2}$ confirmed the similar finding in imported potato mitochondrial $tRNA^{Arg}$.[15] Thus, plant $tRNA^{Arg}_{ICG}$ has been propagated in the literature[16-20] and has become an accepted member of the tRNA family. In view of the discrepancy between the accepted status quo and the published[8] data, we have performed a number of analyses from several plant species (including wheat) while using the corresponding isoacceptor from *E. coli* as a positive control. Whereas

*Correspondence to: Gabor L. Igloi; Email: igloi@biologie.uni-freiburg.de
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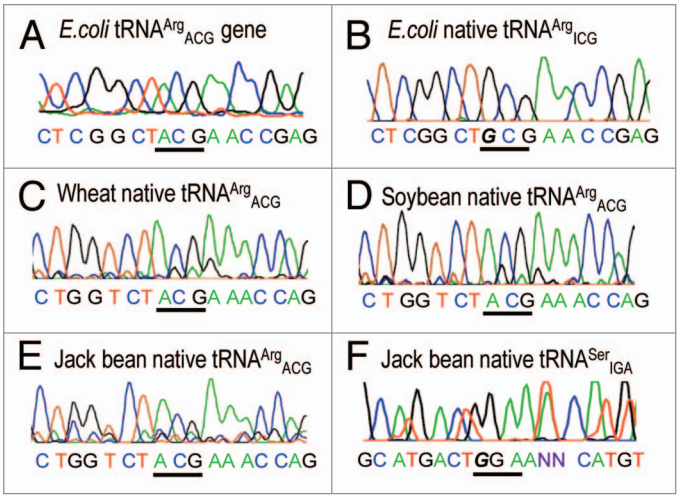

Figure 1. DNA sequence analysis of PCR products generated from genomic DNA (**A**) and reverse transcribed tRNAs (**B–F**). Anticodon stem/loop sequences are shown for the tRNAs indicated. For the purposes of this communication, native tRNA refers to tRNAs extracted directly from the organism, as opposed to transcript tRNA prepared by in vitro transcription. In each case, the anticodon is indicated by a bar and the A-to-I deaminated base 34 (read as G by the reverse transcriptase) in italics.

inosine is readily detected in the bacterial tRNA^{Arg}, we find no evidence for the presence of this modification in plant cytoplasmic tRNA^{Arg}.

Results

Reverse transcriptase analysis of plant tRNA$^{Arg}_{ACG}$. The in vivo gene product of the *E. coli* tRNA$^{Arg}_{ACG}$ is a classic example of a tRNA whose anticodon A34 is deaminated by tadA to inosine.[21] To confirm that the RT/PCR protocol[22] was able to detect authentic inosine in tRNA, partially purified, overexpressed *E. coli* tRNA$^{Arg}_{ICG}$ [23] was subjected to this analysis. Accordingly, **Figure 1B** reveals a G at this position as a result of I34 pairing with C during reverse transcription. In contrast, the unmodified genomic sequence tRNA (**Fig. 1A**) retains the A34 sequence. tRNAs extracted from three plants, including examples of both monocots and dicots, when examined by this method for the presence of inosine in tRNA^{Arg} are characterized by an unequivocal A34 sequence (**Fig. 1C–E**). As in the case of Arabidopsis,[8] we interpret this as evidence for the lack of deamination at this position.

Unlike prokaryote and chloroplast-encoded tRNAs, in eukaryotes tRNA^{Arg} is not the only isoacceptor in which inosine plays a role in codon-anticodon recognition.[24] As a representative of the other seven potential I34-bearing tRNAs, we analyzed cytoplasmic tRNA$^{Ser}_{IGA}$ which has been sequenced from two different plant species and by two independent groups (tobacco[25]; spinach[26]) and whose genomic sequence reveals an A34 (see e.g., NCBI trace archive gnl|ti|1697792733, for the Arabidopsis equivalent). Using primers derived from the soybean genomic sequence, the presence of inosine in the related jack bean tRNA$^{Ser}_{IGA}$ could be confirmed (**Fig. 1F**).

Mass spectrometry. The tRNA$^{Arg}_{ACG}$ isoacceptor was isolated from wheat leaves by oligonucleotide capture,[27] subjected to RNase T1 digestion, and analyzed by LC-MS/MS.[28,29] Digestion products of the tRNA containing inosine were not detected (**Fig. S1**); however, an unmodified version of the anticodon portion of the tRNA containing A34 was found. This RNase T1 digestion product was detected as a doubly charged species (*m/z* 955.9) (**Fig. 2A**). The doubly charged ion was selected for sequencing by tandem mass spectrometry (MS/MS), and the fragment ions (c-, y-, and w-ions) are consistent with an RNase T1 digestion product sequence of UCUACGp (**Fig. 2B**). This sequence is found in only one location of this tRNA, from position 31 to 37 (**Fig. 2C**). Further sequencing of additional RNase T1 digestion products (**Figs. S2–9**) were used to obtain a tRNA sequence that was in full agreement with the gene sequence and a modification pattern comparable to published data.[10,14]

In vitro translation. Cell free wheat germ extracts have been used for decades[30] for the efficient translation of mRNA in vitro. Total tRNA from a commercial wheat germ extract was isolated and the tRNA$^{Arg}_{ACG}$ isoacceptor sequenced by the RT/PCR procedure. As in the case of tRNA isolated from wheat leaves, this sequence was characterized by an unmodified A34 (**Fig. 3A**). Although the success of this extract in translating a host of different mRNAs over the years favors the conclusion that the arginine CGC codon (assumed to be read by ICG) does not hinder translation despite the lack of inosine, we tested its efficacy using a GFP gene in which all six arginine codons had been converted to CGC (pEGFP-N3; Accession number U57609). **Figure 3B** shows that a translation product of the correct size is obtained. Fluorescence measurements with the GFP translation product gave an emission spectrum typical (E_{max} 507 nm) for this protein (**Fig. 3C**) compared with the negative control (translation vector without GFP insert or luciferase gene insert). Similarly, the manufacturer's control luciferase gene product whose arginines at positions 437 and 513 are coded by CGC, is efficiently translated to a product of the expected size. As only full-length luciferase is active (Promega, Technical Notes), the positive luminescence measurements obtained with this product (data not shown) confirmed the translation of the two CGC codons.

Mitochondrial decoding requires A-to-I editing. In contrast to angiosperms, which rely on the import of cytoplasmic tRNA$^{Arg}_{ACG}$ into the mitochondria,[31] the mitochondrial genome (AB251495) of the bryophyte *P. patens* encodes a distinct tRNA$^{Arg}_{ACG}$ isoacceptor.[32] Ligation of an RNA tail to the 3'end of moss total RNA followed by a tail specific reverse transcription provides a cDNA population from which the mitochondrial tDNA$^{Arg}_{ACG}$ may be specifically amplified using the corresponding 5' targeted primer. Sequence analysis of this amplicon clearly shows the effect of ACG to ICG conversion with the tell-tale evidence of GCG in the DNA sequence trace (**Fig. 4**).

Discussion

The concept that A34 of tRNA$^{Arg}_{ACG}$ in all eukaryotes, eubacteria and plant chloroplasts is converted to I34 is firmly anchored in the literature. Auxilien, et al.,[33] pointed out that in archaea, in the

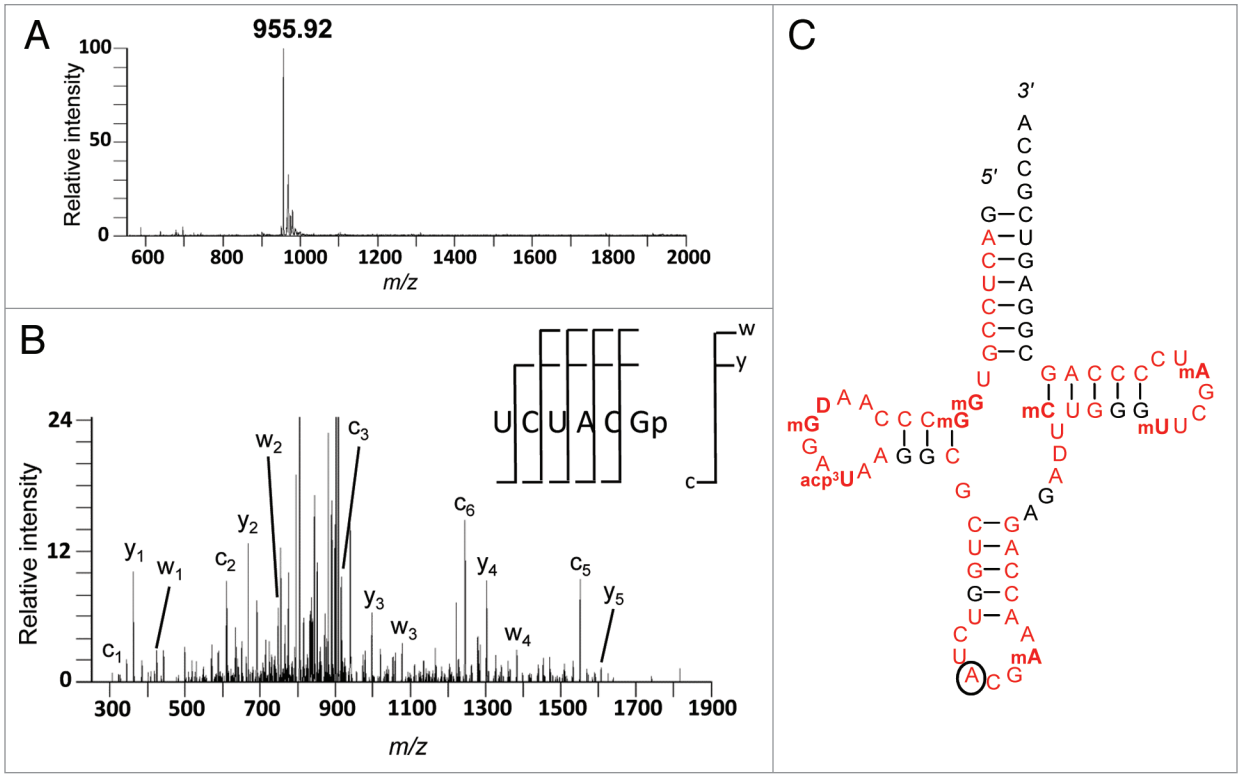

Figure 2. Mass spectrum (**A**) depicting a doubly charged ion (*m/z* 955.92) and fragmentation pattern after collision induced dissociation (**B**) of the RNase T1-derived oligonucleotide covering the anticodon region of $tRNA^{Arg}_{ACG}$ from wheat with the sequence UCUACG. All c- and y-type fragment ions and 4 of 5 possible w-type ions were detected and used to confirm the sequence. The position of A_{34} in the compiled sequence of the entire tRNA is circled (**C**). RNase T1 digestion products whose fragmentation pattern (Figs. S1–9) was used to establish the sequence are red. Modified bases are shown in bold.

mitochondria of single-cell organisms and in animal mitochondria no tRNA has been found to contain inosine 34. Following the revelation that the Arabidopsis cytoplasmic $tRNA^{Arg}_{ACG}$ showed a complete lack of A-to-I conversion,[8] we have performed a series of experiments that substantiate our claim that higher plant cytoplasmic $tRNA^{Arg}_{ACG}$ does not harbor an inosine in its anticodon.

RT PCR of tRNAs is the method of choice for detecting certain modified bases, including inosine.[22] To avoid potential trace contamination with genomic DNA, we chose to ligate an RNA oligonucleotide to the 3' end of the tRNA of interest, to which the reverse transcription primer is targeted. For amplification, specificity was dictated by the 5' tRNA-specific primer. Total tRNA from the leaves of wheat (*T. aestivum*), soybean (*G. max*) and jack bean (*C. ensiformis*) was isolated and the nominal $tRNA^{Arg}_{ICG}$ isoacceptor analyzed. In none of the cytoplasmic $tRNA^{Arg}_{ACG}$ of the three plants could inosine be detected (**Fig. 1C–E**), in contrast to native $tRNA^{Arg}_{ICG}$ from *E. coli* (**Fig. 1B**).

In order to confirm the lack of deamination of $tRNA^{Arg}_{ACG}$, the specific isoacceptor was enriched by oligonucleotide capture[27] and subjected to mass spectrometric analysis which confirmed conclusively the absence of inosine. Discrepancies with respect to the identity of other modified bases documented by Barciszewska, et al.[10] were observed as unmodified m^2_2 G26 and unmethylated C32 (**Figs. S1 and S2**). The RNase T1 fragment containing D47 and mC49 was also observed in its unmodified form (**Fig. S9**), which is consistent with an unmodified C49 as reported by Glover et al.[14] Confirmation of Cm32[14,15] in the mitochondrial $tRNA^{Arg}_{ACG}$ pool may point to a modification which is characteristic for the imported species (see below).

In angiosperms, the mitochondrial genome codes for a certain number of tRNAs[34] and represent a subclass of tRNAs none of which have an A34 and hence do not require A-to-I editing to fulfill their decoding function. Cytoplasmic $tRNA^{Arg}_{ACG}$ is imported into the mitochondria of angiosperms[31] but is encoded by the mitochondrial genome of, e.g., mosses and some algae.[32,35] The fraction of cytoplasmic tRNA that is imported into the mitochondria varies greatly[36] but represents less than 0.15% for $tRNA^{Arg}_{ACG}$ in Chlamydomonas. Imported tRNAs have been characterized in wheat[14] and in potato.[15] In both these plants, the presence of I34 in $tRNA^{Arg}_{ICG}$ was conclusively and independently shown.

Should an inosine in the wobble position of $tRNA^{Arg}_{ACG}$ be essential for mitochondrial protein synthesis, one would expect to find evidence for this modification in the mitochondrial cDNA of plants that do not rely on its import. Of the non-vascular plants that encode $tRNA^{Arg}_{ACG}$ in their mitochondrial genome,[32] we have examined this isoacceptor in the moss *P. patens*. Using primers specific for the $tRNA^{Arg}_{ACG}$ encoded by the mitochondrial DNA an RT-PCR product was generated whose sequence is in accordance

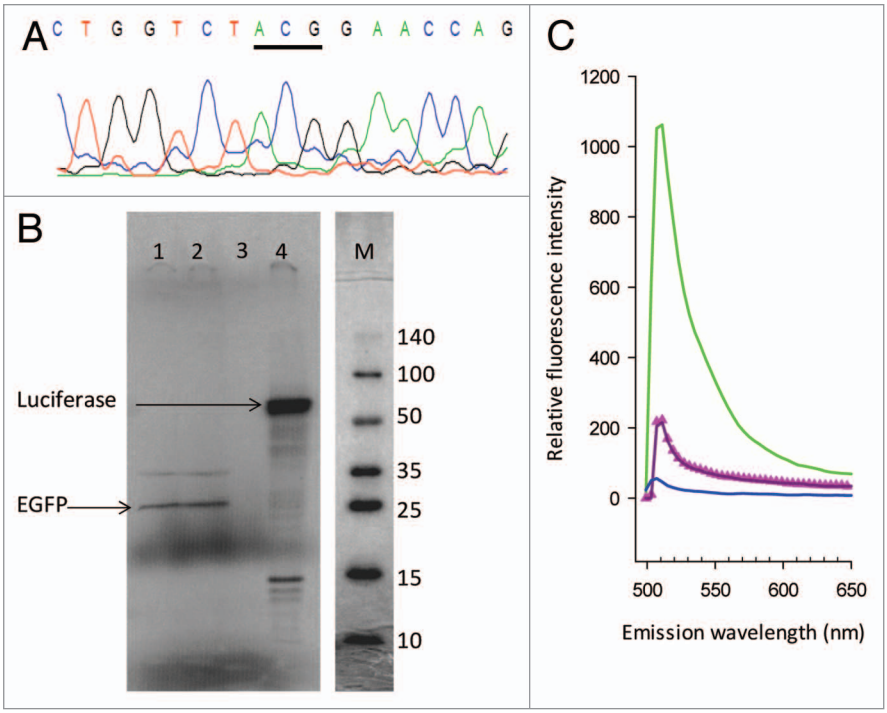

Figure 3. In vitro translation of CGC encoded arginine by a wheat germ extract. (**A**) Sequence of RT/PCR product from $tRNA^{Arg}_{ACG}$ extracted from commercial wheat germ cell free translation extract. The anticodon is underlined. (**B**) SDS gel analysis of in vitro translation using wheat germ extract. Product formation was monitored by phosphorimager analysis of the incorporation of [^{35}S]-Met into EGFP in whose gene all arginine codons had been replaced by CGC. Lanes 1 and 2, EGFP protein (predicted, 26.9 kDa); 3, Flexi® Vector without an integrated gene sequence; 4, Luciferase positive control (predicted, 61 kDa); M, Protein Ladder. Samples and marker were run on the same gel. The unlabelled protein ladder was visualized optically and the separate images scaled to the identical size. The red molecular weight bands of 40, 70 and 260 kDa of the marker were not visible under the conditions of the image capture. (**C**) Fluorescence emission spectrum of the translation products EGFP (green), translation vector only (pink triangles), luciferase (purple) and no translation template (blue). Excitation was at 470 nm.

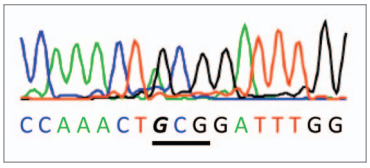

Figure 4. DNA sequence of the RT/PCR product derived from moss mitochondrial $tRNA^{Arg}_{ACG}$. Anticodon stem/loop sequence is shown. The anticodon is indicated by a bar and the A-to-I deaminated base 34 (read as G by the reverse transcriptase) is in italics.

with the gene sequence (Acc. No. AB251495, position 54682–54609) with the exception of an A34I conversion (**Fig. 4**) whose efficiency resembles that documented for the chloroplast encoded $tRNA^{Arg}_{ACG}$ editing in Arabidopsis.[8]

Evidently, inosine in the first position of the anticodon is required by the plant mitochondrial decoding system with the example of moss disproving the long-held supposition that inosine does not occur in mitochondrial tRNAs.[37,38] In moss the three genetic compartments encode individual $tRNA^{Arg}_{ACG}$ isoacceptors of which the chloroplast and the mitochondrial transcripts are deaminated by organelle-specific deaminases. The cytoplasmic translation machinery of plants has evolved to dispense with this requirement yet the mitochondria of angiosperms rely on the post-import modification of the nuclear encoded $tRNA^{Arg}_{ACG}$ to provide a functional decoding ability. Post-import alterations in the structure of tRNAs have been recorded for plant isoacceptors of Leu (G18 to Gm18),[39] for trypanosome Leu, Lys and Tyr (C32 to Cm32[40]), Glu (U34 dethiolation[41]), Trp (C34 to U34 and U33 thiolation[42]) and for Leishmania Glu, Gln, Lys (U34 to Um34 and dethiolation[43]). The mitochondrion-specific A-to-I conversion reported here is the first example of an adjustment of the decoding properties within the plant organelle's genetic system. The originally published sequence of $tRNA^{Arg}_{ICG}$,[10] which has been the single source for globally specifying the presence of I34 in plant cytoplasmic $tRNA^{Arg}$, makes no explicit claim as to its subcellular origin but has been assumed to be cytoplasmic.[2] On the basis of its inosine content, it is now apparent that it should be re-assigned as originating from the mitochondrial RNA pool.

In eukaryotes 7–8 cytoplasmic tRNAs contain I at the first position of the anticodons.[24] If, as is proposed for the A34I conversion in chloroplasts,[44] the editing of $tRNA^{Arg}_{ACG}$ in mitochondria is a remnant from the prokaryotic origins of the organelles, those cytoplasmic tRNAs which are targets for other deaminases should still reflect their inosine content following RT/PCR. In accordance with this, inosine could be found in $tRNA^{Ser}_{IGA}$ (which is not imported into mitochondria) in total jack bean tRNA (**Fig. 1F**), in line with the published sequences of these spinach and tobacco cytoplasmic tRNAs.[25,26] $tRNA^{Val}_{IAC}$, which has been characterized as a component of the imported mitochondrial tRNA pool,[14] exists in its IAC-anticodon form in the plant cytoplasm.[45]

Characterization of the deaminase in yeast[17,33] provided evidence for the presence of a single enzyme for all A34-containing tRNAs. Since we confirmed A-to-I editing in the jack bean $tRNA^{Ser}_{IGA}$, and the imported $tRNA^{Val}_{IAC}$ is performed in the cytoplasm,[45] it would appear that in plants the cytoplasmic deaminase specifically avoids $tRNA^{Arg}_{ACG}$ modification. The yeast TAD2 activity is dependent on the presence of a purine at the central position of the anticodons but, exceptionally, also edits the ACG anticodon.[33] This exception appears to have been lost by the corresponding deaminase in plants. The only A-to-I deaminase found in plants to date, TADA, has been shown to be targeted exclusively to the chloroplasts[8,38] and is responsible for I34 formation in the plastid-encoded $tRNA^{Arg}_{ACG}$ transcript. An additional,

mitochondrial targeted, tadA-like deaminase would have to be responsible for the post-import A34I conversion in the mitochondrial isoacceptor.

The absence of inosine in cytoplasmic $tRNA^{Arg}_{ACG}$ raises the question of how CGC codons in the nuclear genome are translated. CGC codons are widespread in the plant genome, yet no gene for a functional tRNA with a GCG anticodon exists.[24] The decoding functionality of an unmodified A34 was examined in an in vitro translation system. If inosine is required to read a C in the wobble position, as demanded by the Wobble Hypothesis, the presence of CGC codons might be expected to prevent or reduce the efficiency of translation or induce mistranslation. A gene for GFP in which all arginine codons had been replaced by CGC was, nevertheless, efficiently translated by a commercial wheat germ extract in which an unmodified wobble base in $tRNA^{Arg}$ had been established (**Fig. 3**). Furthermore, in GFP the presence of native Arg96 or mutant Lys96 has been shown to be critical for fluorophore formation.[46] The fact that the product of in vitro translation expressed the optical properties of GFP suggests that mistranslation of the codon has not occurred. Although we cannot claim that $tRNA^{Arg}_{ACG}$ rather than another arginine isoacceptor is responsible for the translation of the CGC codon, it is evident that its decoding is independent of the presence of inosine. Other examples in which decoding occurs by an unmodified A34 are nematode[6] and yeast mitochondrial $tRNA^{Arg}_{ACG}$[7] although their genomes use the CGC codon (www.kazusa.or.jp/codon/). Alternative codon reading mechanisms, such as two-out-of-three,[47] provide a potential solution, especially since two G:C base pairs are available. The plausible existence of an A:C base pair which would be formed by the unmodified A34 has been emphasized[48] and such structures are known from other regions of the tRNA[49] enabling the stabilization of the two-out-of-three mechanism. This would also be consistent with the situation in the Salmonella mutant in which the $tRNA^{Pro}_{GGG}$ had been replaced by a $tRNA^{Pro}_{ACG}$ but the CCC codon reading ability was not impaired by the A:C mismatch.[4] With the $tRNA^{Thr}_{AGU}$ of mycoplasma, the only reported cytoplasmic tRNA with an unmodified A34, to date,[5] the translation of all threonine codons except ACA was demonstrated in vitro.[50] The functionality of a partially unmodified cytoplasmic trypanosome $tRNA^{Thr}_{AGU}$ has been proposed.[51] In contrast, Cantara, et al.,[52] recently showed by an in vitro assay that in the context of an *E.coli* ribosome, a $tRNA^{Arg}$ is not bound by a CGC codon unless A34 is modified to I34.

In generalizing our observation, it is now apparent that the few exceptions to an inosine in the first position of the $tRNA^{Arg}$ anticodon that had been documented to date were merely indicative of a much more widespread phenomenon encompassing the plant kingdom.

Materials and Methods

Oligonucleotides (**Table 1**) were synthesized by Sigma-Aldrich and the polymerase chain reaction system was purchased from Genaxxon BioScience. All chemicals used were commercially available pro analysis quality unless stated otherwise. DNA sequence analysis was performed using BigDye version 1.1 chemicals (Applied Biosystems/Life Technologies) in combination with an ABI Prism 310 Genetic Analyzer. Concentration of nucleic acids was determined by measuring the absorbance at 260 nm using a NanoDrop 1000 (Thermo Fisher Scientific). *E. coli* tRNA, enriched in $tRNA^{Arg}_{ACG}$ to an arginine acceptance

Table 1. Primers used for reverse transcription, PCR and sequencing. The following combination of primers was used to characterize the tRNAs described, after ligation of an RNA tail to the 3′ end.

tRNA	Reverse transcription	PCR	Sequencing
Soybean $tRNA^{Arg}_{ACG}$	1	1 + 3	7
Jack bean $tRNA^{Arg}_{ACG}$	1	1 + 3	7
Wheat $tRNA^{Arg}_{ACG}$	1	1 + 3	7
E. coli $tRNA^{Arg}_{ICG}$	1	1 + 4	1
Jack bean $tRNA^{Ser}_{IGA}$	1	1 + 5	8
Moss mitochondrial $tRNA^{Arg}_{ICG}$	1	2 + 6	7
E.coli $tRNA^{Arg}_{ACG}$ gene		4 + 9	4

Primer	Sequence
1	M13 universal primer
2	ACG GCC AGT GCA GTG AGT GGC ATG
3	TTT AAT ACG ACT CAC TAT AGA CTC CAT GGC CCA ATG GAT
4	TTT AAT ACG ACT CAC TAT AGC ATC CGT AGC TCA GCT GGA
5	TTT AAT ACG ACT CAC TAT AGT GGA CGT GCC GGA GTG GTTA
6	TTT AAT ACG ACT CAC TAT AGT GCT TGT AGC TCA ATC GGATA
7	TAC AGG AAA CAG CTA TGA CCC ATC GTG ATC CTT AGA CTT CAT ACA CTT ACT TTA ATA CGA CTC ACT ATA
8	ACG GCC AGT GCA GTG AGT GG
9	CCC TGC AGT GGT GCA TCC GGG AGG ATT CGA

of 760 pmol/A260, was obtained from an expression construct described by Wu, et al.[23]

Isolation and enrichment of native plant $tRNA^{Arg}_{ACG}$ isoacceptor. Plants for total RNA extraction were grown in the greenhouse (jack bean—*C. ensiformis*) or in the field (wheat-*T. aestivum* and soybean—*G. max*) under standard growth conditions (Dachswanger Mühle). Total tRNA from plants was isolated as described[53] with some minor modifications and the wheat $tRNA^{Arg}_{ACG}$ isoacceptor purified according to Spears, et al.[27] A DEAE cellulose column (DE52—Whatman) was prepared by suspending 5 g DEAE cellulose in 150 ml 0.1 M NaOH and carefully stirring it for 1h at 65°C. The cellulose was washed three times with H_2O, resuspended and stirred in 150 ml 0.2 M acetic acid for 15 min and washed again five times with H_2O. After washing it was taken up in 150 ml 0.14 M sodium acetate, pH 4.8 and equilibrated at 4°C overnight. For purification, total tRNA was bound to the DEAE cellulose column, washed once with 150 ml 0.14 M sodium acetate, pH 4.8 and a second time with 200 ml 0.14 M sodium acetate /0.25 M NaCl until A260 < 0.1, tRNA was then eluted with 0.14 M sodium acetate, pH 4.8/1 M NaCl. The isoacceptor corresponding to $tRNA^{Arg}_{ACG}$ was isolated from total plant tRNA using biotinylated oligonucleotides covering the anticodons loop/stem region [(Btn)AGA ATC TCT GGT TTC GTA GAC CAG CGC CTT] on a streptavidin-Sepharose column (GE Healthcare). Purification to homogeneity was achieved by preparative denaturing PAGE on a 10% polyacrylamide gel containing 7 M urea. After ethanol precipitation the concentration of the $tRNA^{Arg}_{ACG}$ isoacceptor was determined, yielding 3–6 μg (species-dependent) of the isoacceptor from 200–300 g leaves.

Ligation, reverse transcription, PCR and sequencing. In order to sequence tRNA and to avoid trace DNA contamination, an RNA primer was ligated to the 3' end of the isolated native tRNA. About 1 μg of total RNA was ligated to 20 pmol of a 5'-phosphorylated, 3'-periodate-oxidized RNA oligonucleotide [5'(Phos)CUC ACU GCA CUG GCC GUC GUU UUA CCU_{ox}]. The oligonucleotide was designed as described[9] to enable the use of the universal M13 primer for reverse transcription. Ligation was performed in ligation buffer using 10 units of T4 RNA ligase (Affymetrix-USB) and 40 U RNasin (Promega) in a total volume of 50 μl. The reaction was incubated for 90 min at room temperature. The total volume of the ligation was vacuum concentrated to 11 μl for reverse transcription and the ligation product was annealed to 20 pmol of universal M13 primer for 10 min at 65°C. Reverse transcription was performed under standard conditions using 10 U transcriptor reverse transcriptase (Roche) as described in the company's manual. Incubation was for 30 min at 55°C and the reaction was terminated by heating to 85°C for 5 min. PCR was performed with 2 μl of the cDNA using a combination of 3' tail and 5' specific primers for wheat, jack bean, soybean and *E.coli* $tRNA^{Arg}_{ACG}$ (**Table 1**). The amplicons were analyzed on 2% agarose gel, purified with the QIAquick PCR purification Kit (Qiagen) and sequenced using primers from either end (**Table 1**).

RNase T1 digestion of tRNA and LC-MS/MS. Purified $tRNA^{Arg}_{ACG}$ from wheat was digested with RNase T1 by mixing 1 μg of the sample with 50 U of RNase T1 in 20 mM ammonium acetate and incubating for 2 h at 37°C. Digestion products were separated using a Thermo Surveyor HPLC system with an Xbridge C18 1.0 × 150 mm column (Waters) at room temperature with a flow rate of 30 μL/min. Before each run the column was equilibrated for 15 min at 95% Buffer A (200 mM HFIP, 8.15 mM TEA pH 7.0) and Buffer B (200 mM HFIP, 8.15 mM TEA:methanol 50:50 v:v pH 7.0). The gradient used was 5% B for 5 min, 30% B at 7 min and 95% B at 50 min and held at 95% B for 5 min. The eluent was directed into a LTQ-XL (Thermo Scientific) for mass spectral analysis with a capillary temperature of 275°C, spray voltage of 4.5 kV, sheath, auxiliary and sweep gases were set to 25, 14 and 10 arbitrary units, respectively. Collision induced dissociation tandem mass spectrometry set to 35% with a Q value of 0.35 for 30 msec was used to obtain sequence information of the digestion products in data dependent mode. The data dependent settings will select each individual ion for CID for 15 scans or 30 sec before placing it on an exclusion list for 30 sec.

In vitro translation. In vitro translation was conducted with the TNT® Coupled Wheat Germ Extract Systems from Promega. As DNA template for in vitro translation the Flexi® Vector system from Promega was used. The EGFP coding sequence was amplified from pEGFP-N3 and was verified by sequencing. The restriction sites within the pF3A WG (BYDV) Flexi® Vector (Promega) as well as those appended to the PCR product were digested with *Sfa*I (isoschizomer of *Sgf*I) and *Mss*I (isoschizomer of *Pme*I) from Fermentas using the Fermentas digestion protocol with buffer B for double digestions. Approximately 100 ng digested PCR product was ligated with 200 ng Flexi® Vector in a 20 μl reaction containing 400 U T4 DNA Ligase (New England BioLabs) for 4 h at room temperature. Transformation of competent *E.coli* cells and sequence verification was accomplished according to the Flexi® Vector systems protocol from Promega. Plasmids containing the correct sequence were used for in vitro translation. In vitro translation was performed as described in the TNT® Coupled Wheat Germ Extract Systems users' protocol with [^{35}S]-methionine for protein detection. Translation products were analyzed on a 12% SDS (w/v) polyacrylamide gel and compared with a protein molecular mass marker (Spectra™ Multicolor Broad Range Protein Ladder, Fermentas). Newly translated proteins were detected by exposure of the dried gel to phosphorimager plates followed by visualization using the molecular imager PharosFX system from BioRad. The fluorescence of EGFP was measured with a NanoDrop 3300 Fluorospectrometer (Thermo Scientific).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/rnabiology/article/21839

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