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Coexistence of nuclear DNA-encoded tRNA^{Val}(AAC) and mitochondrial DNA-encoded tRNA^{Val}(UAC) in mitochondria of a liverwort *Marchantia polymorpha*

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ABSTRACT

The liverwort *Marchantia polymorpha* mitochondrial DNA encodes almost all tRNAs required for mitochondrial translation except for the isoleucine (AUU, AUC) and threonine (ACA, ACG) codons, while the missing tRNAs are supplied in part by the nucleus and imported in mitochondria. In this paper, we report a finding of two radically different nuclear tRNA^{Val}(AAC) genes and import of the corresponding tRNA isoacceptors in *M.polymorpha* mitochondria. This finding is surprising since the mtDNA encodes the gene for tRNA^{Val}(UAC), which alone was considered sufficient for translating all four valine codons GUN by the U/N wobble mechanism. The present results suggest for the first time that the import of ncDNA-encoded tRNAs may result in decoding overlaps in plant mitochondria. The coexistence of nuclear DNA-encoded tRNA^{Val}(AAC) and mitochondrial DNA-encoded tRNA^{Val}(UAC) in liverwort mitochondria and the significance for the decoding mechanism as well as evolution of tRNA import are discussed.

INTRODUCTION

Although tRNA import into mitochondria has been observed in many organisms including angiosperms (1,2), gymnosperm (3), protozoa (4–9) and yeast (10,11), the liverwort *Marchantia polymorpha* is the plant system available that would permit thorough examinations of both mitochondrially- and nuclearly-encoded tRNAs within mitochondria. Previously, from the complete nucleotide sequence of the liverwort mitochondrial genome, 29 tRNA genes representing 27 different tRNA species have been deduced (12,13), but the genes for tRNA^{Ile} decoding the AUU and AUC codons and tRNA^{Thr} decoding the ACA and ACG codons are missing in the mitochondrial DNA (mtDNA). The recent study of Akashi *et al*. confirmed the presence of nuclear DNA (ncDNA) encoded tRNA^{Ile}(AAU) in mitochondria (14). This tRNA species should satisfy decoding the three isoleucine codons (AUU, AUC, AUA), provided that the first adenosine nucleotide residue (A) of the anticodon AAU is modified to inosine (I), as this universally occurs from *Saccharomyces cerevisiae* to humans (15). Additionally, the ncDNA-encoded tRNA^{Thr}(AGU) was found to be accumulated in liverwort mitochondria (16). The above tRNA and the native tRNA^{Thr} (GGU) in fact result in decoding overlap, but both tRNAs together are not even sufficient and thus, at least one additional tRNA^{Thr} recognizing the ACG codon is needed to translate all four threonine codons used in mitochondria.

To fully elucidate the mechanisms and the biological significance of tRNA import in *M.polymorpha* and other organisms, we began searching for other nuclear tRNA genes whose transcripts might be imported. In this report, we identified two nuclear tRNA^{Val} genes with the same anticodon AAC but with radically different nucleotide sequences. Northern blot analysis showed that the corresponding tRNA^{Val} species were imported into mitochondria. This was surprising since we assumed previously that the mtDNA-encoded tRNA^{Val} (UAC) would be sufficient to read all four valine codons (GUN) by the two out of three (17) or the U/N wobble mechanism (18). The present finding suggests that liverwort mitochondria are able to import tRNAs which *a priori* appear unnecessary. Our results may throw a new light for understanding the mechanisms and evolution of tRNA import.

MATERIALS AND METHODS

Isolation of mitochondria and nucleic acids

Cell suspension cultures of the liverwort *M.polymorpha* were maintained as previously described (19). Liverwort total and mitochondrial RNAs were isolated from the 7–10 day old cells in suspension cultures as described (14,16). For isolating mitochondria, originally a French press (19) or later a glass-beads homogenizer was used to break cells in homogenization buffer. Mitochondrial suspension was further purified through a Percoll stepwise gradient in a buffer containing 0.25 M sucrose and 0.2% BSA in 20 mM HEPES–KOH (pH 7.5). In order to isolate mitochondria free of cytosolic RNA, mitochondrial suspensions were treated with RNaseA (50 µg/ml) for 30 min and subsequently either with proteinase K (50 µg/ml) or pronase A (1 mg/ml) in buffer containing 0.4 M mannitol, 50 mM HEPES–KOH (pH 7.5), 10 mM $MgCl_2$ and 6 mM β-mercaptoethanol for 1 h at 4°C.

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Polymerase chain reaction (PCR) for cloning nuclear tRNA^{Val} genes

The tRNA^{Val} sequence was fortuitously discovered during the process of isolating tRNA^{Ile} genes. This was done by the method (20) of labeling tRNA^{Ile} selectively with [5′-^{32}P]pCp and RNA ligase. Labeled tRNAs produced eight radioactive bands on a polyacrylamide gel, each of which was then sequenced (data not shown) by partial enzymatic digestion (21). Two bands were identified to be tRNA^{Val}. One was the mtDNA-encoded tRNA^{Val} (UAC) according to the alignment of its sequence with the gene (13). The other was a novel tRNA^{Val} species. Accordingly the PCR primers below were constructed from the partial RNA sequences of the latter for amplifying the novel tRNA^{Val} genes: D-loop sense (5′-AA*TCTAGA*GTTTYCGTNGTGCANNTGG-YT-3′), and TψC-loop antisense (5′-AA*GGATCC*TGTTTCCG-TCCRGANTYGAAC-3′), in which the added *Xba*I and *Bam*HI restriction sites are italicized, and the abbreviations are: N = A, C, G or T; Y = C or T; R = A or G. The conditions of PCR amplification and cloning the amplified fragment were described previously (14,16). The resulting plasmid DNA encoding the tRNA^{Val} sequence was designated as pTV-PCR1.

Library screening and DNA sequencing

Construction of the liverwort genomic library and screening for liverwort nuclear tRNA^{Val} genes were performed as described previously (14,16). We used the insert of the plasmid pTV-PCR1 or the oligonucleotide pV1 (see below) as screening probes. The sequences of the plasmid pTV-1 and pTV-2 were determined by the dideoxynucleotide method (22) with the Auto-read Sequencing Kit and the A.L.F. DNA sequencer (Pharmacia).

Northern hybridization

Liverwort total and mitochondrial RNAs (0.5 µg per lane) were electrophoresed in 0.7% denaturing agarose gels and blotted onto nylon membranes (23). Hybridization was performed as described previously (14,16). For polyacrylamide gel electrophoresis, mitochondrial and total cellular RNAs (~15–20 µg per lane) were separated by electrophoresis on a gel (50 cm in length, 10% polyacrylamide, 0.5% bisacrylamide, 1× TBE buffer and 3 or 7 M urea) for ~22 h at 12 mA in the cold (4°C). After electrophoresis, the tRNA region was electroblotted onto a nylon membrane in 1× TAE buffer. After blotting, membranes were irradiated for cross-linking in a UV-Stratalinker (Stratagene). For northern hybridization, the following oligonucleotide probes were used (see details in text). For liverwort ncDNA-encoded tRNA^{Val}(AAC), pV1: 5′-AGTGTGTTAGACTGACGTGATAA-3′; pV2: 5′-GGA-GACCTTCAGTGTGTTAG-3′; pV3-1: 5′-TGTTTCCGTCCAG-GTTCGAA-3′; and pV3-2: 5′-TGGCTTTATCCAGGCTCG-AA-3′. For liverwort mtDNA-encoded tRNA^{Val}(UAC), pmV: 5′-GCTGACTCTCTCGGTGTAAA-3′(15); and for tobacco cytosolic tRNA^{Tyr}(GUA), pNY: 5′-TCCGACCTGCCGGATTC-GAACC-3′(24), were used, respectively.

The oligonucleotides (1–2 pmol) were labeled with [γ-^{32}P]ATP (5000 Ci/mmol) using a 5′-end labeling kit (MEGALABEL™, TaKaRa). Hybridization with individually labeled probes was performed in a buffer consisting of 6× SSPE, 1% SDS and 1× Denhardt's solution at 37°C overnight. The filters were then washed three times with 100 ml of 2× SSPE containing 1% SDS buffer briefly at room temperature and finally at 37°C for 20 min, and autoradiographed on X-ray films. To test hybridization strength and specificity, the membranes were washed at 37°C with 0.2× SSPE, 1% SDS and, if necessary, washed at 42, 47 or 55°C for 20 min and autoradiographed. For probes with 20-nucleotide length of ~50% (G+C/A+G+C+T) composition, non-specific bands were completely removed by the first washing step with 2× SSPE at 37°C. When radioactivity levels were low, blots were exposed to the Bioimaging Plate BAS2000 (Fuji photo film Co. Ltd) and hybridized band images were obtained.

RESULTS

Isolation and identification of a liverwort nuclear tRNA^{Val} gene

In order to isolate the gene encoding the novel tRNA^{Val}, PCR primers were designed from the partial RNA sequence and amplification was then carried out using liverwort total DNA as a template. The PCR product of 90 bp was detected and subcloned into a plasmid vector. The resulting plasmid DNA had an insert of the tRNA^{Val} sequence (data not shown). This insert was then used as a hybridization probe for screening ~3.6 × 10^4 recombinant phages of the liverwort genomic library. One λ clone was obtained, yielded Southern hybridization signals with the *Xba*I (4.3 kb), *Kpn*I (14 kb) and *Hin*dIII (11 kb) restriction fragments (data not shown). Further subcloning yielded a clone pTV-1, having a 0.2 kb *Sac*II–*Pst*I insert exhibiting hybridization with the tRNA^{Val} probe.

The nucleotide sequence from the pTV-1 fragment revealed the tRNA^{Val} (AAC) gene sequence (designated *val-1*) (Fig. 1). The deduced tRNA sequence can be folded into the classical cloverleaf structure with stems and loops of expected sizes. Database homology search revealed that the *val-1* sequence had 89% identity with that of *Arabidopsis thaliana* ncDNA-encoded tRNA^{Val}(IAC) (25) and 84% with the human nuclear tRNA^{Val} (AAC) gene (26), respectively. The fact that the *val-1* sequence is not found on the complete sequences of liverwort mitochondrial and chloroplast DNAs (12,19) confirms its nuclear origin. The *val-1* gene showed relatively low levels of sequence similarity with the following tRNA genes, i.e., 49% with liverwort chloroplast DNA-encoded tRNA^{Val}(UAC) (19), 47% with liverwort mitochondrial DNA-encoded tRNA^{Val}(UAC) (13) and 47% with *Escherichia coli* tRNA^{Val}(UAC) (27), respectively.

The liverwort *val-1* gene had the AAC anticodon, which is a typical triplet for the major tRNA^{Val} gene in eukaryotic nuclear genomes, in contrast to prokaryotic GAC and UAC (15,28). In many cases the first letter (A residue) of the anticodon is modified to an inosine (I) residue, and the generated IAC anticodon interacts with the U, C and A residues of the third letter in the valine codons (GUN) (28). Hence, the tRNA^{Val}(AAC) gene should code for a tRNA capable of reading the codons GUU, GUC and GUA.

Localization of ncDNA-encoded tRNA^{Val}(AAC) in mitochondria

Total cellular and mitochondrial RNAs were run on formamide denaturing agarose gels and northern blots were obtained. The oligonucleotide probe pV1 represents the complementary nucleotide sequence from the D-loop to the 3′ end of the anticodon stem of *val-1* (Fig. 1a). This probe hybridized with both liverwort mitochondrial tRNA and total tRNA preparations (Fig. 2, lanes 1

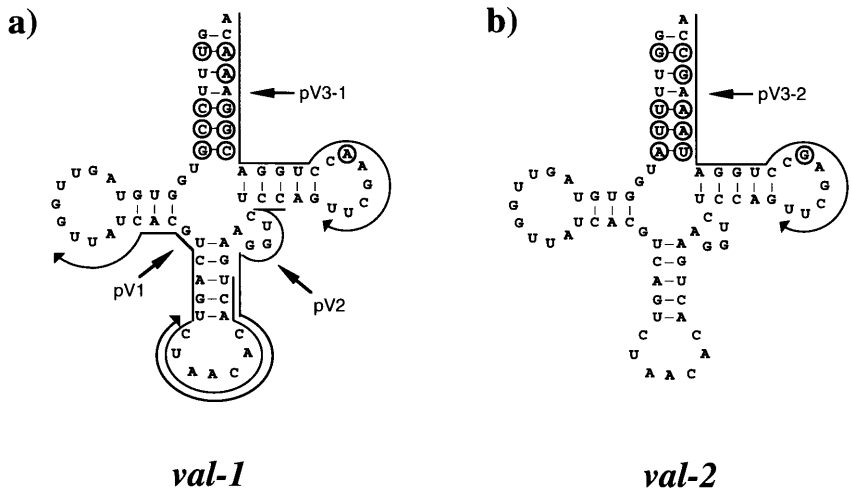

Figure 1. Potential clover-leaf structures deduced from (**a**) *val-1* and (**b**) *val-2* genes for liverwort nuclear tRNA^{Val}(AAC) genes. The nucleotides differing between *val-1* and *val-2* are circled. The regions complementary to the oligonucleotides (pV1, pV2, pV3-1, pV3-2) used for northern analysis are indicated by lines along the nucleotides. These data will appear in the NCBI, EMBL and DDBJ databases under the accession numbers U81145 for pTV-1 (0.2 kb *Sac*II–*Pst*I fragment encoding *val-1*) and AF016367 for pTV-2 (1.0 kb *Kpn*I–*Sma*I fragment encoding *val-2*), respectively.

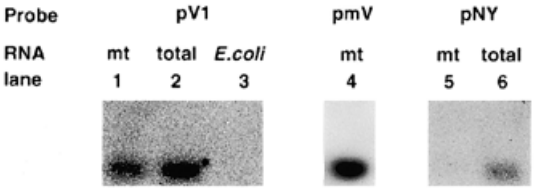

Figure 2. Northern analysis of tRNAs developed by agarose gels. RNAs from purified mitochondria (mt), total RNA from liverwort cells (total) and total RNA from *E.coli* (*E.coli*), were hybridized with the ^{32}P-labeled oligonucleotide probes specific to liverwort ncDNA-encoded tRNA^{Val}(AAC) (pV1), liverwort mtDNA-encoded tRNA^{Val}(UAC) (pmV), and tobacco cytosolic tRNA^{Tyr} (GUA) (pNY), respectively.

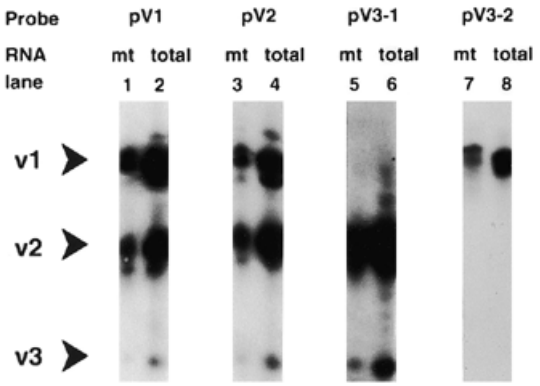

Figure 3. Northern hybridization of tRNAs separated by polyacrylamide gels. Probes with four different oligonucleotides (pV1, pV2, pV3-1 and pV3-2) represent three sections of the tRNA sequences of *val-1* and *val-2* (Fig. 1). Each blot set contains mitochondrial (mt) and total (total) tRNAs, respectively.

and 2). The conditions of hybridization and washing were stringent enough to eliminate any cross-hybridization signal to *Escherichia coli* tRNA (Fig. 2, lane 3). Purity of the mitochondrial preparation was confirmed by the probe pNY of tobacco cytosolic tRNA^{Tyr}, which hybridized with liverwort total tRNA (Fig. 2, lane 6) but did not hybridize with mitochondrial RNA (Fig. 2, lane 5). This demonstrated that the mitochondrial preparation was free from cytosolic tRNAs. Furthermore, an oligonucleotide probe for mtDNA-encoded tRNA^{Val}(UAC) showed considerably strong hybridization signals with mitochondrial tRNA (Fig. 3, lane 4). The above results indicate that ncDNA-encoded tRNA^{Val}(AAC) is imported into mitochondria and concurrently, 'native' tRNA^{Val}(UAC) is expressed, thus resulting in coexistence of two tRNA^{Val} isoacceptors of distinct genetic origins within the same mitochondria. The expression of the liverwort tRNA^{Val} species was further examined by northern analysis using several oligonucleotide probes. The probe pV1 showed three major hybrid signals widely separated with both mitochondrial and total cell RNAs, designated v1, v2 and v3 from the gel top to the bottom, respectively (Fig. 3, lanes 1 and 2). In addition, each of v1 and v2 was composed of more than one band. Similarly, the probe pV2, which is complementary from the anticodon loop to the end of the variable loop, showed the identical hybrid pattern (Fig. 3, lanes 3 and 4). In contrast, the probe pV3-1, complementary from the 3′ end of the amino acid acceptor stem to the TψC region, hybridized only with the bottom two bands, v2 and v3 (Fig. 3, lanes 5 and 6). These results indicated that v1 did not share the same amino acid acceptor stem as v2 and v3.

Isolation and identification of another nuclear tRNA^{Val} gene with the anticodon AAC

To determine if there exists a liverwort tRNA^{Val} gene corresponding to band v1, which did not hybridize with pV3-1 probe, we used pV1 as a hybridization probe and screened ~3.6×10^4 recombinant phages of the liverwort genomic library. Two positive clones were obtained. Dot hybridization analysis of these two clones showed that one hybridized with pV2 but not with pV3-1, suggesting it to be a likely candidate clone for v1. This λ clone produced a *Hin*dIII (4.3 kb) fragment which showed Southern hybridization signals with pV1. Further subcloning yielded a clone pTV-2, having a 1.0 kb

*Kpn*I–*Sma*I insert exhibiting hybridization with pV1. The second clone was not examined further since it showed no hybridization with pV2.

The nucleotide sequence from the pTV-2 fragment revealed a new species of tRNA^{Val}(AAC) gene (designated *val-2*) (Fig. 1). The sequences of *val-2* and *val-1* were remarkably different in the amino acid acceptor stem, in fact, differing at nine nucleotide positions, plus one nucleotide in the TψC-loop. The degree of the differences between them is unprecedented between two isoacceptor tRNAs found in a single organism. Homology search revealed that the *val-2* sequence had 87% identity with *val-1*, although the 5′- and 3′-flanking regions of *val-1* and *val-2* did not show any significant sequence similarity to each other (data not shown). The *val-2* showed 91% sequence similarity with the *A.thaliana* ncDNA-encoded tRNA^{Val}(IAC) (25), and 71% with the human nuclear tRNA^{Val}(AAC) gene (26), respectively. The fact that the *val-2* sequence is also not found on the complete sequences of liverwort mitochondrial and chloroplast DNAs (12,19) confirms its nuclear origin.

Identification of tRNA^{Val} isoacceptors in mitochondria

In order to examine whether the northern band v1 corresponds to the *val-2* gene product, we constructed a new oligonucleotide probe pV3-2, which is complementary from the 3′ end of the amino acid acceptor stem to the TψC region of *val-2* (Fig. 1b). The probe pV3-2 hybridized only with the top band v1 (Fig. 3, lanes 7 and 8), but showed no hybridization signal with the bottom bands v2 and v3. This result clearly shows that the band v1 represents the products of *val-2*.

The bands v2 and v3 cannot be differentiated by the three different oligonucleotide probes pV1, pV2 and pV3; thus, the northern bands v2 and v3 could be same or similar in their primary sequences. Moreover, we have observed that each of v1 and v2 is composed of at least two discrete but adjacent tRNA bands in northern analysis (Fig. 3, v1 and v2). It is not uncommon to find an individual tRNA species producing more than one band under our high resolution polyacrylamide gel conditions. This may be due to variable secondary structures and/or base modifications. Since there normally exist multiple gene copies for a tRNA species, sequence variances of one or more nucleotides for a tRNA species with the same anticodon are commonly found, and these tRNA variants can even migrate to different gel positions. Needless to say, the variances due to nucleotide sequences and/or modified bases of these individual tRNA species must be fully elucidated.

DISCUSSION

Our results demonstrated the presence of at least two ncDNA-encoded tRNA^{Val} isoacceptors in liverwort, having the identical anticodon AAC. It is interesting to note that these isoacceptors have strikingly different amino acid acceptor stems in terms of the nucleotide sequence. Nevertheless, both are imported into liverwort mitochondria.

The coexistence of both the imported tRNA^{Val}(AAC) and the mtDNA-encoded native tRNA^{Val}(UAC) raises a novel question about the possible decoding overlap within mitochondria. Theoretically, all four valine codons could be decoded by the native tRNA^{Val}(UAC) alone by the two out of three (17) or U/N wobble mechanism (18), as have been observed in the vast majority of mitochondrial tRNA^{Val} (15). Since the usage frequency of all valine codons is not especially high among all the codons used in liverwort mitochondria (13), there appears to be no compelling reason why ncDNA-encoded tRNA^{Val} must be imported into mitochondria at all. Therefore, the import of ncDNA-encoded tRNA^{Val}(AAC) could bring tRNA redundancy for at least three mitochondrial valine codons, GUU, GUC and GUA, provided that the common base modification at the first anticodon position from adenine to inosine really occurrs in liverwort.

Alternatively, there exists a possibility that the ncDNA-encoded tRNA^{Val} is in fact required to compensate for decoding inefficacy of the native tRNA^{Val}(UAC). For example, codon recognition of the native tRNA^{Val}(UAC) could be restricted due to the posttranscriptional modification of the U in the first position of the anticodon (28), or the influence of nucleotides flanking the anticodon (29). In order to solve the problem, the structures and decoding properties of both the imported and native tRNA^{Val} should be investigated in more detail.

A similar situation has been reported in *S.cerevisiae*, where the mitochondrial genome encodes 25 tRNA species that satisfy the coding requirement within mitochondria. Nevertheless, a ncDNA-encoded tRNA^{Lys} is imported (10,11). Since this tRNA is imported in the aminoacylated form, it may likely be used in intramitochondrial translation, as such providing decoding overlaps. In liverwort, however, it remains to be investigated whether the two imported tRNAs^{Val} can be aminoacylated by the mitochondrial valyl-tRNA synthetase and/or they are aminoacylated as they are imported.

The fact that multiple tRNA isoacceptors are imported supports the idea that a common factor capable of recognizing these isoacceptors may be responsible for mediating import. It is likely that the corresponding aminoacyl-tRNA synthetase may serve for such purposes (30). The present knowledge of tRNA import suggests that tRNA-import determinants are coincident with the tRNA-aminoacylation identity elements (2), tRNA sequence itself (31) or anticodon (32). All suggest involvement of the cognate synthetase at some point in import processes (11). On the other hand, there may exist a radically different import mechanism in the protozoan *Leishmania* (33), where tRNA import is apparently mediated solely by a mitochondrial receptor.

It was suggested earlier that ‘the ability to import different tRNAs has been acquired at different times in different lineages’ during the evolution of plant mitochondria (3). This proposal is based on the assumption that the imported tRNA species in lower plant mitochondria can be inferred from the genes (or anticodons) which are missing from their mtDNAs. However, the present data show that the anticodon of imported cytosolic tRNA species is not strictly correlated with the absence of the corresponding tRNA gene(s) in the mtDNA. Thus, we propose an alternative hypothesis. The ability to import a variety of tRNAs existed in all ancestral eukaryotes. During evolution, import of redundant tRNAs was gradually lost more or less randomly and independently in different eukaryotes.

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