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In vivo import of a normal or mutagenized heterologous transfer RNA into the mitochondria of transgenic plants: towards novel ways of influencing mitochondrial gene expression?

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Evidence that nuclear-encoded RNAs are present inside mitochondria has been reported from a wide variety of organisms, and is presumed to be due to import of specific cytosolic RNAs. In plants, the first examples were the mitochondrial leucine transfer RNAs of bean. In all cases, the evidence is circumstantial, based on hybridization of the mitochondrial RNAs to nuclear and not mitochondrial DNA. Here we show that transgenic potato plants carrying a leucine tRNA gene from bean nuclear DNA contain RNA transcribed from the introduced gene both in the cytosol and inside mitochondria, providing proof that the mitochondrial leucine tRNA is derived from a nuclear gene and imported into the mitochondria. The same bean gene carrying a 4 bp insertion in the anticodon loop was also expressed in transgenic potato plants and the transcript found to be present inside mitochondria, suggesting that this natural RNA import system could eventually be used to introduce foreign RNA sequences into mitochondria.
Key words: in vitro tRNA transcript aminoacylation/ mitochondrial RNA import/*Phaseolus vulgaris*/*Solanum tuberosum*/tRNA^{Leu}

Introduction

The import of some nuclear-encoded RNAs into mitochondria has been implied in a variety of organisms (Nagley, 1989). In yeast (Martin *et al.*, 1979), protozoans (Benne, 1985; Suyama, 1986; Simpson *et al.*, 1989; Hancock and Hajduk, 1990) and plants (Green *et al.*, 1987; Gray and Boer, 1988; Maréchal-Drouard *et al.*, 1988, 1990a; Joyce and Gray, 1989; Sangaré *et al.*, 1990), the RNAs concerned are transfer RNAs, and can represent either a minority (higher plants) or a large majority (some protozoans) of mitochondrial tRNAs. In mammals, the RNA components of mitochondrial RNase MRP and RNase P appear to be of nuclear origin (Doersen *et al.*, 1985; Chang and Clayton, 1989). The evidence for import is based on hybridization of the mitochondrial RNAs to nuclear and not mitochondrial DNA and, for tRNAs, on the inability to find a complete set of genes in the mitochondrial genome. The mechanism by which such an import might occur is unknown, but the involvement of mitochondrial aminoacyl-tRNA synthetases, which have been shown to be nuclear-encoded in fungi (Pape *et al.*, 1985; Natsoulis *et al.*, 1986; Koerner *et al.*, 1987; Chatton *et al.*, 1988), in tRNA import has been suggested (Suyama, 1986).

It has not yet proved possible to demonstrate import of specific RNAs into isolated mitochondria (L.Maréchal-Drouard and A.Dietrich, unpublished results), as can be done routinely with mitochondrially imported proteins. Direct proof of this phenomenon is therefore still lacking, as is a system in which it can be studied. Alternative origins for these mitochondrial RNAs can be envisaged. For instance, the existence of RNA editing in protozoan (Simpson and Shaw, 1989; Van der Spek *et al.*, 1990) and plant (Covello and Gray, 1989; Gualberto *et al.*, 1989; Hiesel *et al.*, 1989) mitochondria has made it impossible formally to rule out the possibility that they are produced by extensive modification of transcripts from cryptic mitochondrial genes.

We have attempted to develop a system to demonstrate and study the mitochondrial import of heterologous tRNAs *in vivo*. Such a system may turn out to be of more than academic interest, as the genetic transformation of mitochondria varies from difficult to currently impossible (depending on the organism), and the ability to import RNAs transcribed from gene constructs inserted into nuclear DNA could allow the manipulation of mitochondrial gene expression in ways which are presently unavailable.

Results

*Expression of bean tRNA^{Leu}(C*AA) in transgenic potato plants*

The basis for our experiments was a cloned bean (*Phaseolus vulgaris*) gene encoding tRNA^{Leu}(C*AA) (Figure 1), a tRNA found in both the cytosol and the mitochondria of bean plants, the cytosolic and the mitochondrial species differing only by a methylation of the ribose in position 18 (Green *et al.*, 1987). This gene has the advantage (unlike most tRNA genes) of being present in low (probably single) copy number (Green *et al.*, 1987), thus implying that it contains all the requisite transcription signals and that it should be capable of producing detectable levels of transcript when reintroduced into plants in low copy number by transformation.

The gene, together with either 1.5 kb or 150 bp of flanking sequences, was placed in a plasmid containing sequences designed to express neomycin phosphotransferase (conferring resistance to certain aminoglycoside antibiotics). These plasmids (tRL and tRLD, respectively) were used to transform potato (*Solanum tuberosum* × *S.chacoense*) protoplasts by electroporation. Colonies of antibiotic-resistant cells were selected, and subsequently plants were regenerated and grown *in vitro*. Different transformants were screened for expression of the introduced gene by hybridization of an oligonucleotide complementary to the variable loop of

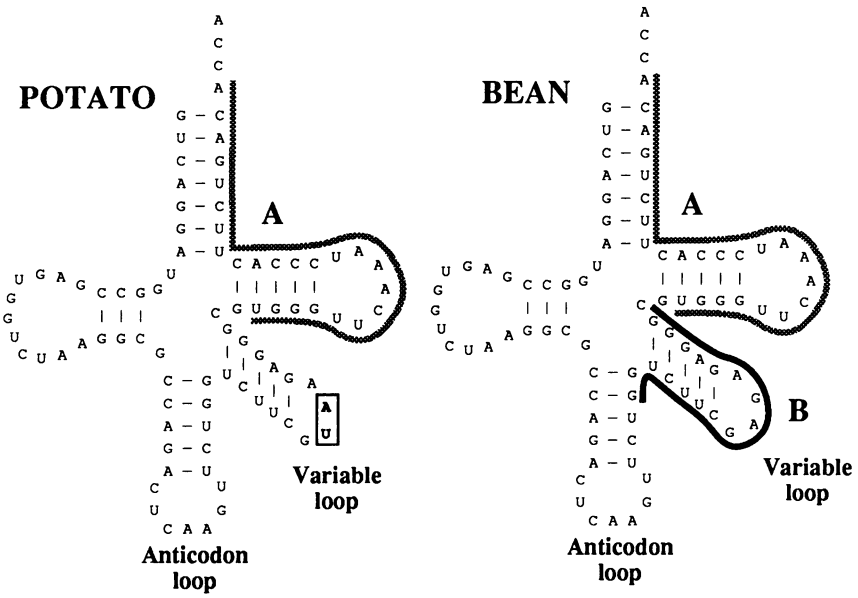

Fig. 1. Primary sequences of the tRNAs^{Leu}(C*AA) from potato and bean. The sequences are represented in the cloverleaf secondary structure without the post-transcriptional modifications. Positions in the variable loop of the potato tRNA where the sequence differs from that of bean are boxed. The regions complementary to the oligonucleotides (A and B) used for hybridization (Figures 2, 5 and 6) are indicated. A, 5′-TGTCAGAA-GTGGGATTTGAACCCA-3′; B, 5′-GCCCTCTCTCGAAGACC-3′.

bean tRNA^{Leu}(C*AA) to Northern blots of crude total tRNA preparations (Figure 2). The majority of transformed potato plants expressed the introduced tRNA gene at levels comparable to that seen in bean, showing that the 50 bp 5′ and 100 bp 3′ of the tRNA gene are sufficient for efficient transcription. Potato plants showing high levels of bean tRNA^{Leu}(C*AA) transcript were multiplied *in vitro* and then transferred to a greenhouse.

*Expression of mutagenized bean tRNA^{Leu}(C*AA) in transgenic potato plants*

The oligonucleotide used for the detection of the bean tRNA^{Leu}(C*AA) often showed weak cross-hybridization to the endogenous potato tRNA^{Leu}(C*AA), which differs by only two nucleotides (in the variable loop) from its bean counterpart (Figure 1) and is also present in both the cytosol and the mitochondria (Maréchal-Drouard *et al.*, 1990b). Therefore, oligonucleotide mutagenesis was used to create unique restriction sites in the anticodon or variable loop regions of the bean gene (Figure 3) into which extra sequences could be inserted. It was felt that these regions, which are outside the potential internal promoters, would best tolerate alterations without too greatly affecting expression of the gene. As an initial step, the four base insertion TCGA was made by filling in of the restriction sites (Figure 3). The intention was to use the TCGA sequence as a tag for the transgene transcript, and to see whether the imported tRNA would carry the extra sequence (UCGA) into mitochondria. These constructs were used for protoplast transformation in the same way as the unmodified gene, and transformants were screened for expression of the modified tRNA genes by RNase protection assays. It turned out that the XhoAL4 construct, containing the TCGA insertion in the anticodon loop, was expressed at easily detectable levels in transgenic potato plants and appeared to be correctly processed (Figure 4), whereas modification of the variable region gave rise to constructs (SalVL and SalVL4) which were expressed at very low or undetectable levels (not

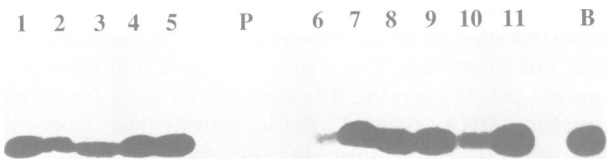

Fig. 2. Expression of bean tRNA^{Leu}(C*AA) in transgenic potato plants, as analysed by hybridization. Lanes 1–11, tRNA from 11 independent potato transformants; P, tRNA from an untransformed potato plant; B, tRNA from bean. The probe was oligonucleotide B (legend to Figure 1) ^{32}P-labeled with polynucleotide kinase.

shown). This strongly decreased expression is probably due to the effect of the alterations on tRNA stability *in vivo*, as small insertions (e.g. introns) or mutations in tRNA genes outside the internal promoter regions generally have little effect on tRNA gene transcription in *in vitro* assays (Geiduschek and Tocchini-Valentini, 1988).

*In vivo import of unmodified and mutagenized bean tRNA^{Leu}(C*AA) into the mitochondria of transgenic potato plants*

Potato transformants carrying the unmodified bean tRNA^{Leu}(C*AA) gene (tRL or tRLD) or the XhoAL4 construct were investigated in more detail: total leaf tRNA or tRNA extracted from purified mitochondria was fractionated on polyacrylamide gels, transferred to nylon filters and hybridized with various specific oligonucleotide probes (Figure 5). The identity of the mitochondrial tRNA fractions was confirmed by hybridization with an oligonucleotide specific for potato mitochondrial tRNA^{Ile}(L*AU) (Weber *et al.*, 1990) and lack of contamination by cytosolic tRNAs was verified by using a consensus probe (see oligonucleotide E in the legend to Figure 5) corresponding to typical cytosolic plant tRNAs^{Phe}(GAA) (Sprinzl *et al.*, 1989). The absence of

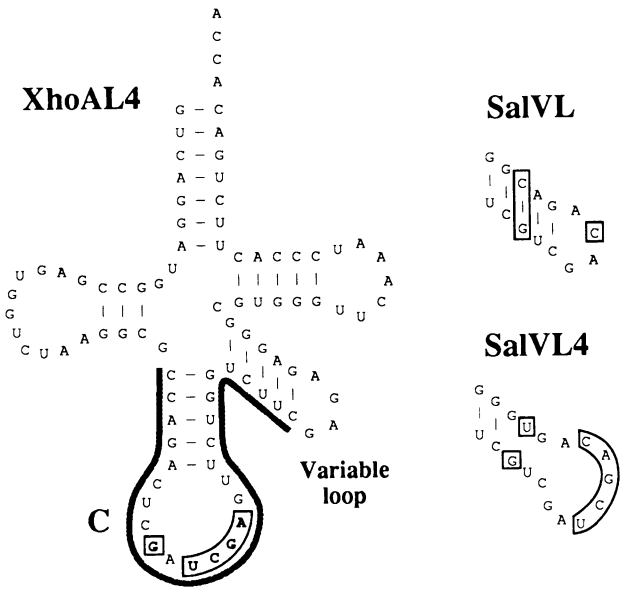

Fig. 3. Primary sequence of the modified tRNA XhoAL4. The variable regions of the modified tRNAs SalVL and SalVL4 are also shown. Positions in the anticodon loop or in the variable loop where the sequences differ from the bean $tRNA^{Leu}$(C*AA) are boxed. The region complementary to the oligonucleotide C used for hybridization (Figures 5 and 7) is indicated. C, 5′-GAAGACCAGAACTCGATCG-AGTCTGG-3′.

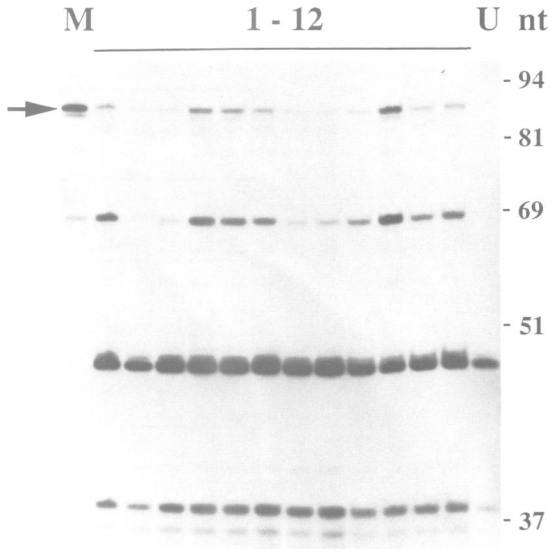

Fig. 4. Expression of XhoAL4 in transgenic potato plants, as analysed by RNase protection. M, marker obtained by protection of the probe by an *in vitro* produced RNA with the XhoAL4 sequence shown in Figure 3; 1–12, independent XhoAL4 transformants; U, untransformed potato; nt, size in nucleotides estimated from the migration of *in vitro* transcribed RNAs of known sequence (not shown). A protected fragment of the expected size (91 nucleotides, arrowed) indicative of XhoAL4 expression is seen with the tRNA samples from all the transformed plants, and is lacking in the untransformed control. The comigration of this protected fragment with that seen in the marker track suggests that the XhoAL4 transcript is probably correctly end-processed in the transgenic plants. The smaller protected fragment (ca. 69 nucleotides) specific to the transformed plants is probably due to overdigestion of the probe/XhoAL4 hybrid with RNase, as it varied in intensity according to the digestion conditions. The major protection products of less than 50 nucleotides seen with all the plant tRNA samples but not with the marker RNA are probably due to protection of the probe by endogenous $tRNA^{Leu}$.

signal when hybridizing the potato mitochondrial $tRNA^{Ile}$(L*AU) probe to total leaf tRNA showed that the proportion of mitochondrial tRNAs in total leaf tRNA preparations does not interfere with the analysis of cytosolic species.

The transcript from the unmodified bean $tRNA^{Leu}$ (C*AA) gene can be easily detected both in total leaf tRNA and in the mitochondrial tRNA fraction from transgenic potato plants (Figure 5), indicating that the product of the nuclear gene is indeed partitioning between the cytosol and the mitochondria. Interestingly, despite the alterations present in the XhoAL4 transcript, and the fact that it is present in significantly lower amounts than the unmodified leucine tRNA, it seems nevertheless to be efficiently imported into the mitochondria of the transgenic plants (Figure 5). The import was further analysed by submitting mitochondria isolated from the transformants to an osmotic shock leading to the disruption of the external membrane. Transfer RNA was extracted from the re-isolated mitoplasts and hybridization analysis (Figures 6 and 7) confirmed the presence of the unmodified heterologous bean leucine tRNA and of the XhoAL4 tRNA inside the mitochondria of the corresponding transgenic potato plants. Finally, mitoplasts obtained after osmotic shock were also incubated with RNase A and re-isolated. RNase A treatment of mitoplasts led to lower yields of tRNA, but the presence of the unmodified bean tRNA and of the XhoAL4 tRNA inside the mitochondria of the transformed potato plants could again be clearly confirmed by specific hybridization (Figures 6 and 7).

Due to higher resolution, two hybridization bands were often observed for a given tRNA when using non-radioactive labelling with digoxigenin (Figure 5, B and D). This probably reflects minor variations in the tRNA structure (for instance in the level of post-transcriptional modifications), as also revealed by the presence of two or more spots corresponding to a given mitochondrial tRNA after two-dimensional polyacrylamide gel electrophoresis (Maréchal-Drouard *et al.*, 1990a). Such structural differences have been described in more detail for bean mitochondrial $tRNA^{Tyr}$ and chloroplast $tRNA^{Phe}$ (Maréchal *et al.*, 1985a; Pfitzinger *et al.*, 1990). For the latter, the variations correlate with leaf development (Pfitzinger *et al.*, 1990).

*Aminoacylation of in vitro transcripts from the unmodified or mutagenized bean $tRNA^{Leu}$(C*AA) gene*

The original and the mutagenized bean leucine tRNAs were produced by *in vitro* transcription and tested for their ability to be aminoacylated by purified bean cytosolic leucyl-tRNA synthetase. Aminoacylation of *in vitro* transcripts lacking all post-transcriptional modifications has been described previously for other tRNAs (see for example Sampson and Uhlenbeck, 1988; Himeno *et al.*, 1989; Perret *et al.*, 1990). The bean $tRNA^{Leu}$(C*AA) *in vitro* transcript can be aminoacylated, although less efficiently than the natural tRNA (Figure 8). The results obtained with the mutagenized tRNAs correlate with the *in vivo* expression data. The XhoAL4 transcript is efficiently aminoacylated (and is expressed correctly in transformed plants), whereas the transcripts containing modified variable regions (SalVL and SalVL4) are poorly aminoacylated (and are poorly expressed), although both the anticodon and the variable region appear not to be in close contact with the enzyme

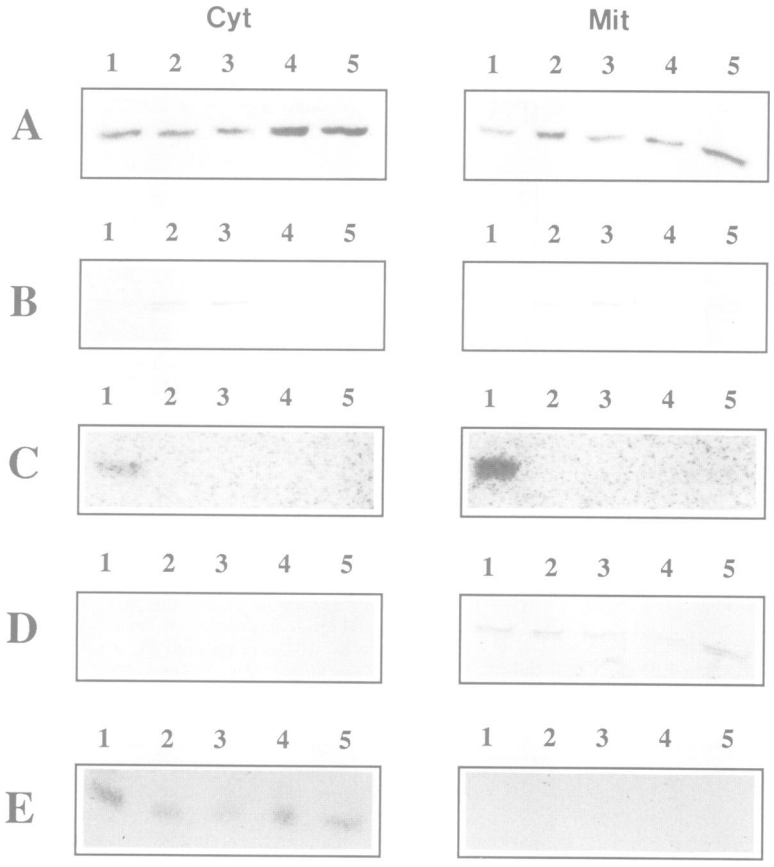

Fig. 5. Presence of bean tRNA^{Leu}(C*AA) and of the modified tRNA XhoAL4 in the total leaf and in the mitochondrial tRNA fractions of transgenic potato plants, as analysed by hybridization. Probes specific for bean and potato tRNAs^{Leu}(C*AA) (A), bean tRNA^{Leu}(C*AA) (B), tRNA XhoAL4 (C), potato mitochondrial tRNA^{Ile}(L*AU) (D) and plant cytosolic tRNA^{Phe}(GAA) (E) were hybridized to equal amounts of total (Cyt) or mitochondrial (Mit) tRNA from potato plants transformed with the XhoAL4 (1), tRL (2) and tRLD (3) plasmids, from potato plants transformed with a plasmid containing no bean tRNA^{Leu} gene (4) and from untransformed potato plants (5). Oligonucleotide B cross-hybridizes weakly with the endogenous potato tRNA^{Leu}(C*AA), see B, lanes 1, 4 and 5. Oligonucleotides A, B and C are given in the legends to Figures 1 and 3. D, 5′-TGGGCTTAGTAGGGCTCGAACCTACAA-3′; E, 5′-TTCAGTCTGACGCTCTCCCAACTGAGCT-3′. Oligonucleotides A, C and E were ^{32}P-labeled with polynucleotide kinase. Oligonucleotides B and D were non-radioactively labeled with digoxigenin-11-dUTP.

when leucine tRNAs are complexed to bean cytosolic leucyl-tRNA synthetase (Dietrich *et al.*, 1990). This suggests that the modifications in the variable loop have perturbed the tertiary structure of the bean tRNA^{Leu}, causing a loss in its amino acid accepting activity.

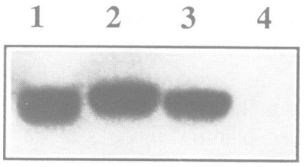

Fig. 6. Presence of the heterologous bean tRNA^{Leu}(C*AA) in mitoplasts from transgenic potato plants, as revealed by hybridization. Mitochondria isolated from potato plants expressing the bean tRNA^{Leu}(C*AA) were submitted to osmotic shock and the mitoplasts recovered by centrifugation (see Materials and methods). The probe specific for bean tRNA^{Leu}(C*AA) (oligonucleotide B, legend to Figure 1, ^{32}P-labeled with polynucleotide kinase) was hybridized to the tRNAs of mitochondria (1) or mitoplasts (2, 3) isolated from potato plants transformed with the tRLD plasmid (1−3) and to the tRNAs of mitochondria extracted from potato plants transformed with a plasmid containing no bean tRNA^{Leu} gene (4). Mitoplasts were recovered directly (2) or after RNase A treatment (3).

Discussion

This research has produced several interesting findings. Firstly, it is possible to transfer a tRNA gene between different plant species by transformation and obtain easily detectable transcripts from the introduced gene, implying high conservation of the sequences necessary for expression. Secondly, it is clearly possible to obtain mitochondrial tRNA^{Leu} from a nuclear gene, providing the final proof that RNA import, rather than the presence of cryptic mitochondrial genes, is the explanation for the previous observations of cytosolic-like tRNA^{Leu} inside mitochondria. Thirdly, we have shown that it is possible, in principle at least, to efficiently import into mitochondria tRNAs carrying additional inserted sequences.

The aminoacylation data obtained with the *in vitro* transcripts of the mutagenized bean tRNA^{Leu} genes suggest that the ability to efficiently interact with the aminoacyl-tRNA synthetase might be important for *in vivo* stability of mutated tRNA transcripts. On the other hand, these data also show that an efficient interaction with the enzyme is still possible for leucine tRNAs after extensive modification of the anticodon loop. It is striking that the XhoAL4 mutagenized tRNA transcript is as well aminoacylated as the corresponding unmodified tRNA transcript. Finally, our observations also leave open the possibility that

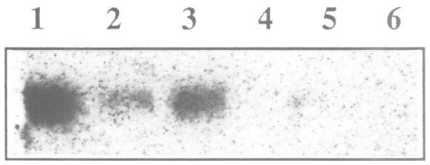

Fig. 7. Presence of the modified tRNA XhoAL4 in mitoplasts from transgenic potato plants, as revealed by hybridization. Mitochondria isolated from potato plants expressing the XhoAL4 construct were submitted to osmotic shock and the mitoplasts recovered by centrifugation (see Materials and Methods). The probe specific for tRNA XhoAL4 (oligonucleotide C, legend to Figure 3, ^{32}P-labeled with polynucleotide kinase) was hybridized to the tRNAs of mitochondria (1) or mitoplasts (2, 3) isolated from potato plants transformed with the XhoAL4 plasmid (1−3) and to the tRNAs of mitochondria extracted from potato plants transformed with a plasmid containing no bean tRNA^{Leu} gene (4). Mitoplasts were recovered directly (3) or after RNase A treatment (2). As a control for RNase treatment efficiency, mitochondria isolated from potato plants transformed with the XhoAL4 plasmid were resuspended in water (5) or isotonic buffer (6) and submitted to RNase A digestion in the presence of 2% (v/v) Triton X-100.

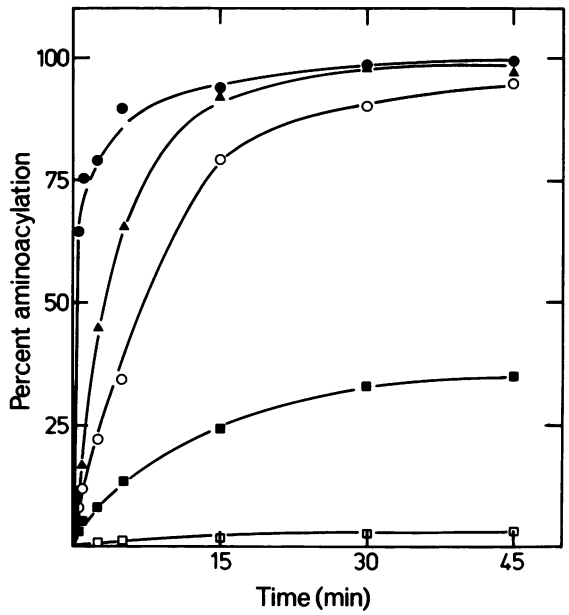

Fig. 8. Aminoacylation of *in vitro* transcripts from the unmodified or mutagenized bean tRNA^{Leu}(C*AA) gene. Purified natural bean tRNA^{Leu}(C*AA) (●) and *in vitro* transcribed bean tRNA^{Leu}(C*AA) (○), tRNA XhoAL4 (▲), tRNA SalVL (■) and tRNA SalVL4 (□) (see Figures 1 and 3 for the corresponding sequences) were aminoacylated in the presence of pure bean cytosolic leucyl-tRNA synthetase.

aminoacylation, or at least interaction with the leucyl-tRNA synthetase, is involved in the import process.

In the immediate future, we hope to use our system to investigate the means by which specific tRNAs are recognized for import by creating mutations which specifically block the import process *in vivo*. In the longer term, the ability to import foreign RNAs into mitochondria might provide a means specifically to alter gene expression inside mitochondria, for example by using antisense RNA sequences. Any approach of this sort would require the ability to insert longer sequences into the imported tRNAs. In principle, this should be possible; many organisms (including plants) contain tRNA genes with introns (generally ~10−30 nucleotides in length and with few sequence constraints) which appear to have little effect on transcription or on the tertiary structure of the transcript (e.g. Lee and Knapp, 1985; Stange *et al.*, 1988). Our initial attempts at expressing tRNA genes with larger insertions (from 12 to 113 nucleotides) have been unsuccessful (I.Small and J.Masson, unpublished results), probably due to rapid turnover of the transcripts, but in these cases no attempt was made to maintain the secondary or tertiary structure of the tRNA. With careful attention to this point, these problems should be surmountable. In yeast, one group has successfully expressed a synthetic tRNA gene carrying an insertion modelled on a natural intron; alterations to the splice junctions ensured that the transcript accumulated in its long, unspliced form (Krieg *et al.*, 1991). Although considerable progress is clearly necessary before this becomes a practical means of influencing mitochondrial gene expression, given the current difficulties with direct transformation of mitochondria (it has yet to be achieved with higher plant mitochondria), this alternative approach seems to be worth pursuing.

Materials and methods

*Bean tRNA^{Leu}(C*AA) gene subcloning and mutagenesis*

The 1.5 kb stretch of bean nuclear DNA containing the tRNA^{Leu}(C*AA) gene described by Green *et al.* (1987) or a 220 bp *Hin*cII−*Kpn*I fragment thereof were cloned using classical techniques (Ausubel *et al.*, 1990) into BlueScript SK+ (Stratagene), to give plasmids tRL and tRLD respectively. Oligonucleotide-directed mutagenesis (McClary *et al.*, 1989) was used to introduce unique *Xho*I or *Sal*I restriction sites into the anticodon loop (tRNA XhoAL) or variable region (tRNAs SalVL and Sal2VL) of the bean tRNA^{Leu}(C*AA) gene in plasmid tRLD using the following primers: 5′-AGAACTCGAGTCTGGCGCC-3′ (XhoAL); 5′-CGCCGTCTGTCG-ACGACCAG-3′ (SalVL) and 5′-CGCCCACTGTCGACGACCAG-3′ (Sal2VL).

Transfer RNA genes XhoAL4 and SalVL4 were obtained by insertion of the sequence TCGA into the sites in XhoAL and Sal2VL respectively by filling in and religation using standard techniques (Ausubel *et al.*, 1990). All the alterations made to the gene were verified by sequencing using fluorophore-labelled primers and an Applied Biosystems 373A sequencing system.

*Transformation of potato with the unmodified or mutagenized bean tRNA^{Leu}(C*AA) gene*

The plasmids tRL, tRLD and modifications thereof, including XhoAL4, were digested with *Xba*I and *Bam*HI and ligated to the *Xba*I−*Bgl*II fragment of pABD1 (Paszkowski *et al.*, 1984), containing a neomycin phosphotransferase gene under the control of a plant promoter and poly(A)$^+$ signals. These constructs were used to obtain transgenic potato plants (*S.tuberosum* × *S.chacoense*, clone 217) using methods which have been described in detail elsewhere (Masson *et al.*, 1989).

*Analysis of the expression of the unmodified or mutagenized bean tRNA^{Leu}(C*AA) gene in transgenic potato*

Total nucleic acid was extracted from independent transformants by guanidine hydrochloride and phenol−chloroform extraction (Logemann *et al.*, 1987). After ethanol precipitation, total tRNA was obtained from the pellet by selective redissolving in 1 M NaCl.

Expression of the unmodified bean gene was checked by specific hybridization. Two micrograms of total tRNAs per sample were electrophoresed in a 1.5% (w/v) agarose non-denaturing mini-gel and dry-blotted to a nylon membrane (Hybond-N, Amersham). Prehybridization and hybridization were carried out in 6×SSC (1 M NaCl, 0.1 M sodium citrate), 0.2% (w/v) bovine serum albumin, 0.2% (w/v) Ficoll, 0.2% (w/v) polyvinylpyrrolidone, 0.1% (w/v) SDS, 100 μg/ml sonicated denatured salmon sperm DNA at 42°C overnight. The probe was a 17mer oligonucleotide complementary to the bean tRNA^{Leu}(C*AA) variable loop (oligonucleotide B, legend to Figure 1), radioactively labelled with ^{32}P using T4 polynucleotide kinase. After hybridization, the filters were washed at 50°C for 30 min in 6×SSC and autoradiographed.

Expression of the mutagenized genes was checked by RNase protection according to Goodall *et al.* (1990). RNase digestion (~2 μg of total tRNA per sample) was done at 50°C in the presence of ^{32}P-labelled probes generated by T7 RNA polymerase transcription of XhoAL4, SalVL and

SalVL4 constructs cut with *Sca*I. Marker fragments were produced by protecting the probe with the non-radioactive *in vitro* T7 RNA polymerase transcripts used for aminoacylation studies [see section 'Aminoacylation of *in vitro* transcripts from the unmodified or mutagenized bean $tRNA^{Leu}$(C*AA) gene']. The protected products were run on a 12% (w/v) polyacrylamide gel in the presence of 8 M urea.

*Analysis of the subcellular localization of the unmodified or mutagenized bean $tRNA^{Leu}$(C*AA) expressed in transgenic potato plants*

Purified mitochondria were prepared from potato leaves according to Neuburger *et al.* (1982) using polyvinylpyrrolidone–Percoll gradients with sucrose as an osmoticum. Organelle integrity was checked by measuring exogenous cytochrome *c* reduction (Douce *et al.*, 1972). For osmotic shock, isolated mitochondria were first resuspended in 50 μl isotonic buffer and then diluted in 650 μl sterile water. After 30 s, osmotic pressure was readjusted using 650 μl of two times concentrated buffer and mitoplasts were recovered by centrifugation (3 min at 8000 *g*). In some experiments, mitoplasts were incubated for 3 min at 25°C in the presence of 10 μg RNase A prior to centrifugation. Breakage efficiency of the external mitochondrial envelope was also checked by measuring exogenous cytochrome *c* reduction.

Transfer RNA was extracted from mitochondria as described previously (Maréchal *et al.*, 1985b). Total tRNA was prepared from potato leaves using the same method. Mitochondrial or total leaf tRNAs were fractionated by electrophoresis on 15% (w/v) polyacrylamide gels under denaturing conditions (Green *et al.*, 1987) and transferred onto nylon membranes (Hybond-N, Amersham) by electroblotting (15 min at 150 mA and then 30 min at 500 mA, in a Trans-Blot™-Cell apparatus from Bio-Rad) in 10 mM Tris–acetate (pH 8.0), 0.5 mM EDTA buffer. Hybridization was carried out overnight, in 6×SSC (1 M NaCl, 0.1 M sodium citrate), 0.2% (w/v) bovine serum albumin, 0.2% (w/v) Ficoll, 0.2% (w/v) polyvinylpyrrolidone, 0.1% (w/v) SDS, 100 μg/ml sonicated denatured salmon sperm DNA at 60°C for oligonucleotides A, C, D and E and 42°C for oligonucleotide B. The sequences of the oligonucleotides are given in the legends to Figures 1, 3 and 5. Oligonucleotides were radioactively labelled with ^{32}P using T4 polynucleotide kinase or non-radioactively labelled with digoxigenin-11-dUTP (Boehringer). After hybridization, the filters were washed at 60°C successively in 2×SSC and in 2×SSC, 0.1% (w/v) SDS. For oligonucleotide B, washing was carried out at 50°C in 6×SSC. Radioactive hybridizations were submitted to autoradiography. Non-radioactive hybridizations were revealed using an anti-digoxigenin, alkaline phosphatase conjugated antibody (Boehringer) in the presence of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

*Aminoacylation of in vitro transcripts from the unmodified or mutagenized bean $tRNA^{Leu}$(C*AA) gene*

Oligonucleotide-directed mutagenesis (McClary *et al.*, 1989) was used to produce constructs in BlueScript SK+ (Stratagene) suitable for *in vitro* run-off transcription with T7 RNA polymerase after digestion with *Bst*NI. The final plasmids contained the unmodified or mutagenized tRNA sequences fused directly to a T7 RNA polymerase promoter at the 5′ terminus and included a *Bst*NI site (CCAGG) at the 3′ terminus of the tRNA coding sequence to obtain tRNA transcripts with a 3′ CCA end. Transcripts were prepared according to Perret *et al.* (1990) and their ends checked by RNA sequencing (Pillay *et al.*, 1984). Natural bean $tRNA^{Leu}$(C*AA) used as a control and bean cytosolic leucyl-tRNA synthetase were isolated as previously described (Green *et al.*, 1987; Dietrich *et al.*, 1983). Aminoacylation of the tRNA and of the transcripts (8 μM) was conducted under optimal conditions (Dietrich *et al.*, 1983) in the presence of the pure enzyme (0.1 μM).

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