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Localization, sequence and expression of the gene coding for tRNA^{Pro} (UGG) in plant mitochondria

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Abstract

The four Sal I fragments of wheat mitochondrial DNA containing the 18S and 5S ribosomal RNA genes were screened for the presence of tRNA genes. Upon sequencing, a tRNA^{Pro} (UGG) gene was found in two of these four fragments. The localization of the corresponding gene on the maize mitochondrial genome was established. Transcriptional studies have shown that this gene is transcribed in wheat and maize mitochondria. The sequence of the corresponding tRNA^{Pro} (UGG) of bean mitochondria was determined using *in vitro* post-labeling techniques.

Introduction

The plant mitochondrial (mt) genome has been shown to consist of a number of circular molecules, deriving from a master chromosome by recombination events, plus a set of small plasmid-like molecules [1, 2]. Together, these DNA molecules constitute large genomes, the size of which differ depending on the plant considered (200–2500 kb) [1, 3, 4], much more complex than animal or fungal mt genomes [5, 6].

Only a few plant mt tRNA genes and their flanking regions have been sequenced so far [7–9]. Due to the high homology between these genes and their chloroplast (cp) counterparts (up to 98%), it was postulated that the plant mt and cp tRNA genes have evolved from common ancestor genes [10]. In addition, it has been shown that the plant mt genome contains cp DNA insertions sometimes harbouring cp tRNA genes [11]. For instance, the maize mt genome contains a 12 kb cp DNA insertion [12] which carries cp tRNA genes coding for tRNA^{Val}, tRNA^{Ile} and the 3′ half of tRNA^{Ala}. The distinction between unexpressed pseudogenes present in cp DNA insertions and functional plant mt tRNA genes is therefore not evident.

In wheat mitochondria, a sequence harbouring the 5S and 18S rRNA genes and a tRNA^{fMet} gene has been shown to be repeated four times in the genome. The organization of the flanking regions of this repeated sequence suggests that a recombination event is responsible for the presence of these four fragments [13].

We report the localization and the sequence of a tRNA^{Pro} (UGG) gene which is present in two of the four clones containing this repeated fragment. We also provide evidence that this tRNA^{Pro} (UGG) gene is expressed, in contrast to another, partial or complete, tRNA^{Pro} (UGG) gene present as a cp insertion in the mt genome of wheat and maize,

respectively [14]. That the $tRNA^{Pro}$ (UGG) gene mapped and sequenced in this study is transcribed was confirmed by the fact that its sequence is 100% homologous to that of bean mt $tRNA^{Pro}$ (UGG) which was also determined in this work.

Material and methods

Isolation of wheat mt tRNAs

Wheat mt tRNAs were extracted from mitochondria purified by sucrose gradient centrifugation [15]. The mitochondria were lysed with 1% Triton X100 and the membranes were pelleted. The tRNAs were purified from the supernatant by phenol extraction, 1 M NaCl precipitation of the high molecular weight RNAs, DEAE-cellulose chromatography of the supernatant and ethanol precipitation.

Localization and sequencing of the wheat $tRNA^{Pro}$ gene

For the localization of the tRNA genes in the wheat mt genome, the total wheat tRNAs were specifically labeled at their 3′ end, using α-^{32}P-ATP, in the presence of yeast tRNA nucleotidyl-transferase, after limited digestion of the terminal CCA sequence with snake venom phosphodiesterase [16]. The labeled tRNAs were hybridized as already described [17] to a dot blot of the 53 Sal I cloned fragments of wheat mt DNA covering the whole genome [18]. These clones were obtained from F. Quetier and B. Lejeune, Orsay (France). The $tRNA^{Pro}$ gene was located in a 750 bp Hind III fragment of the F2 Sal I clone. This fragment was cloned in M13mp10 and sequenced by the dideoxynucleotide chain termination method [19].

Localization of the maize mitochondrial $tRNA^{Pro}$ gene

The mt $tRNA^{Pro}$ gene was localized on the maize mt genome using an oligodeoxynucleotide (26 nucleotides long) complementairy to the 3′ end of the wheat mt $tRNA^{Pro}$ gene. This oligodeoxynucleotide was labeled with γ-(^{32}P)-ATP as previously described [14] and hybridized to a set of maize mt cosmid clones digested with Sma I or Xho I and blotted on GeneScreen Plus. These blots were obtained from D. Lonsdale (PBI, Cambridge, UK). The hybridization and subsequent washings were carried out as already described [14], except that the washing temperature was raised to 70 °C in relation to the Td of the oligodeoxynucleotide probe which was used [20].

Characterization of the transcripts of the $tRNA^{Pro}$ genes

Wheat and maize mt and cp total RNAs were isolated as previously described [21]. The RNAs were separated on a denaturating formaldehyde agarose gel and blotted on GeneScreen Plus. These "Northern" blots were hybridized with labeled oligodeoxynucleotides complementary to the mt or cp $tRNA^{Pro}$ genes.

Isolation and purification of bean mt $tRNA^{Pro}$

Total bean mt tRNAs were prepared from dark-grown bean (*Phaseolus vulgaris*) hypocotyls as previously described [17] and fractionated on a RPC-5 column [10]. Using *E. coli* aminoacyl-tRNA synthetases, $tRNA^{Pro}$ were identified by aminoacylation; fractions containing the $tRNA^{Pro}$ were pooled, concentrated and subjected to two-dimensional polyacrylamide gel electrophoresis. The spot containing $tRNA^{Pro}$ was identified and the pure tRNA was eluted. From 3.2 mg of total bean mt tRNAs, 1.8 μg of pure $tRNA^{Pro}$ was purified.

Sequencing of bean mt $tRNA^{Pro}$

One μg of pure $tRNA^{Pro}$ was partially hydrolysed in deionized formamide at 80 °C for 3 min. After

5′-labeling under standard conditions using 2 units of T4 polynucleotide kinase and γ^{32}P-ATP, the resulting fragments were separated on a thin 15% polyacrylamide slab gel. Each fragment was completely digested with nuclease P1 and the 5′ terminal nucleotide was identified by mono- and two-dimensional thin layer chromatography on cellulose plates. Since the fragments with 5′-ends corresponding to the D-loop and the anticodon loop did not separate properly on polyacrylamide gels, these clusters containing a mixture of oligoribonucleotides were eluted and run again on a hot gel. This allowed the separation of the oligoribonucleotides of the anticodon loop, but not those of the D-loop. The clustered oligoribonucleotides of the D-loop were therefore digested with RNase T1 or RNase A and analysed by eletrophoresis on DEAE-cellulose using oligoribonucleotides of known sequences as standards. To confirm the sequence, the small oligoribonucleotides were eluted from the DEAE-cellulose paper and further digested with nuclease P1 to determine the 5′-end nucleotide of each of them. The technique used to sequence the D-loop was also used to confirm the sequence of specific regions of the tRNA. The nucleotide sequence of the 3′-end of the tRNA was determined by homochromatography [16].

Results and discussion

(1) Localization of the tRNA^{Pro} (UGG) gene on the wheat mt genome

Total wheat mt tRNAs, specifically labeled at their 3′-end, were hybridized to the plasmid bank consisting of the 53 Sal I fragments of wheat mt DNA [18]. Among the clones hybridizing to tRNAs were the four clones carrying the genes coding for 5S and 18S rRNAs and tRNA^{fMet} [13], which were used for further studies. These four clones (F2, G2, R1 and T) were digested with Hind III, blotted and these blots were probed with labeled total mt tRNAs.

As can be seen in Fig. 1A, the following results were obtained:

- on clone F2, 3 Hind III fragments hybridize to mt tRNAs. Their size is 5.1, 2.6 and 0.75 kb respectively,
- on clone G2, 2 Hind III fragments hybridize to mt tRNAs, namely the 5.1 kb and the 2.6 kb fragments,
- on clone R1, 2 Hind III fragments hybridize to mt tRNAs, namely a 7.5 kb and a 0.75 kb fragment,
- on clone T, only the 7.5 kb Hind III fragment hybridizes to mt tRNAs.

The position of these fragments on the four clones is shown in Fig. 1B. Hind III fragment 5.1 kb (in F2 and G2 clones) and Hind III fragment 7.5 kb (in R1 and T clones) were already shown to contain a tRNA^{fMet} gene [8]. Hind III fragment 2.6 kb (in F2 and G2 clones) contains a tRNA gene which was not investigated in this study. Hind III fragment 0.75 kb (F2 and R1 clones) contains a tRNA gene which was further studied by sub-cloning and sequencing (see below).

This tRNA gene is located, both in F2 and R1 clones, just outside the region common to all four clones (see vertical dotted lines in Fig. 1B). The distribution of the tRNA genes in the four clones confirms the organization of the regions flanking the 5S and 18S rRNA genes in these clones. This organization is probably the result of recombination events [13].

(2) Sequence of the wheat mt tRNA^{Pro} (UGG) gene

The Hind III fragment 0.75 kb of the F2 clone was purified, blunt-ended, sub-cloned in the Sma I site of phage M13mp10 and sequenced by the Sanger method [19]. This fragment is only 710 bp long (Fig. 2). A 75 bp tRNA gene was found, thanks to its GTTC sequence, extending from nucleotides 531 to 605. The CCA terminal triplet is not encoded by the gene. The anticodon is TGG (UGG), which codes for a tRNA^{Pro}. It can be folded as a cloverleaf, absolutely identical to that shown in Fig. 5 for bean mt tRNA^{Pro} (UGG) except that the post-transcriptional modifications are not known in the case of the wheat mt tRNA^{Pro} (UGG).

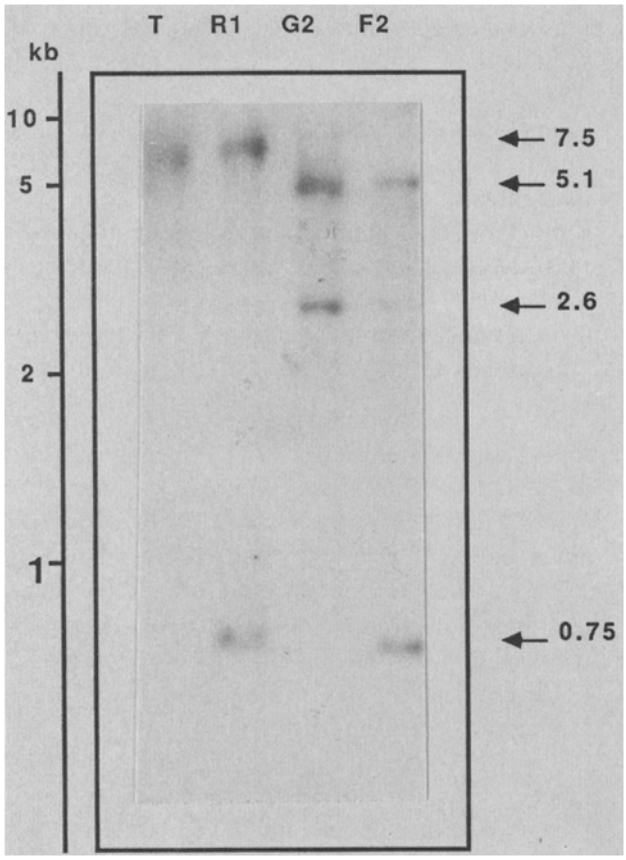

Fig. 1A

Fig. 1. Localization of tRNA genes on the wheat mitochondrial cloned fragments F2, G2, R1 and T. (1A) Southern hybridization of these four clones, cut with Hind III, with 3′-labeled wheat total mt tRNAs. (1B) Localization of the tRNA gene-containing fragments on the Hind III restriction maps of the four clones [13]. The positive fragments are shown by hatched boxes. The 7.5 kb Hind III fragments of R1 and T include vector (pBR 322) sequences as shown by dotted boxes. The limits of the repeated sequences common to all four clones are shown by vertical dotted lines. Vertical bars marked "H" represent Hind III sites, those marked "S" represent the Sal I cloning sites.

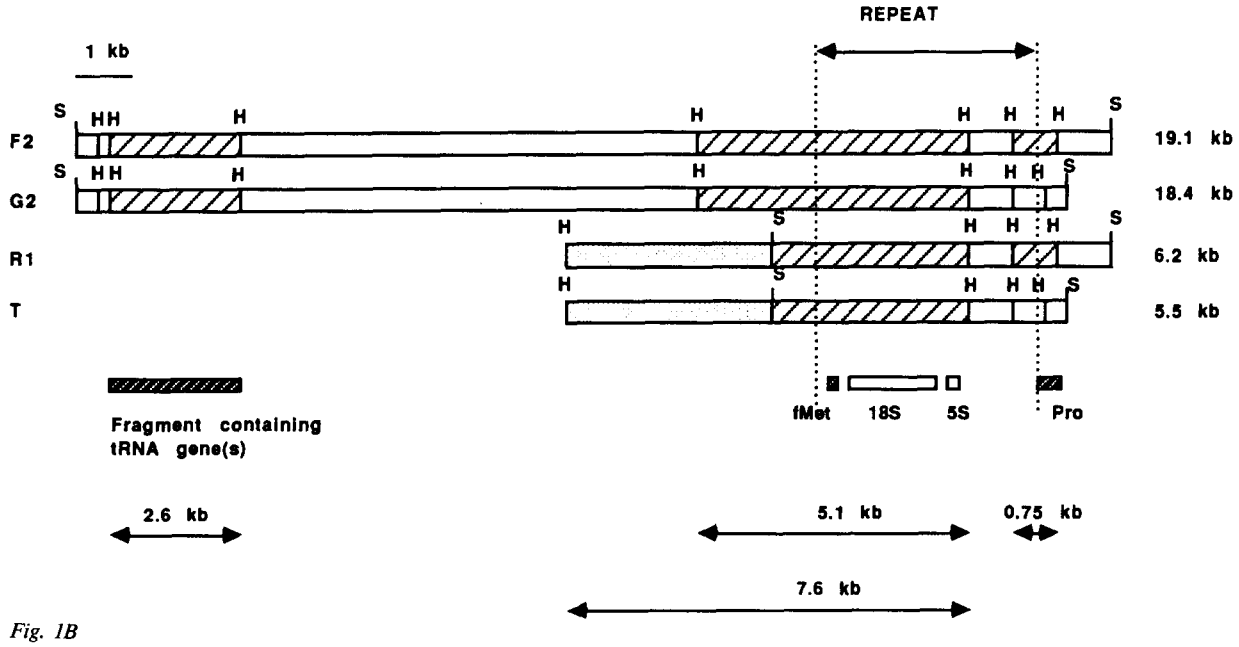

Fig. 1B

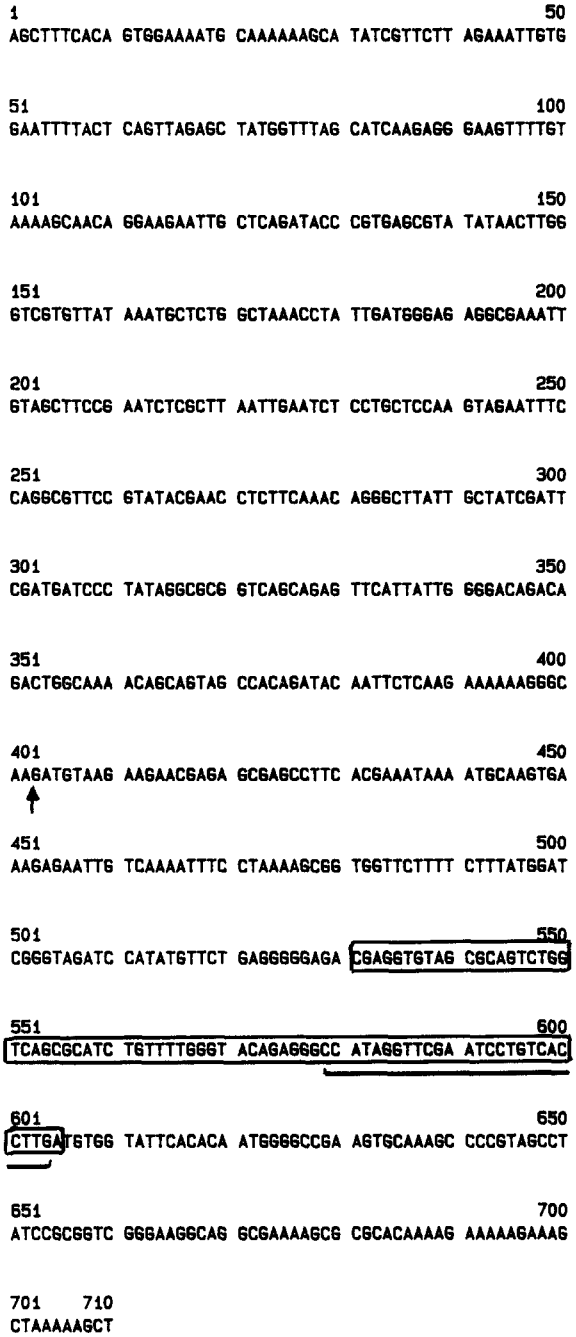

Fig. 2. Nucleotide sequence of the 710 bp Hind III fragment of clone F2 of wheat mitochondrial DNA containing the $tRNA^{Pro}$ (UGG) gene. The tRNA gene is boxed. The sequence of the oligodeoxynucleotide used for localization of this gene in maize mitochondrial DNA and for transcription studies is underlined. The arrow indicates the limit of the repeat show in Fig. 1B.

(3) Localization of the $tRNA^{Pro}$ (UGG) gene on the maize mt genome

A radioactive oligodeoxynucleotide complementary to the 3′ end of wheat mt $tRNA^{Pro}$ (UGG) gene (nucleotides 579 to 604 on Fig. 2) was hybridized to a set of cosmid clones covering the whole maize mt genome [1]. The results (not shown) indicate that the corresponding gene is present twice in the maize mt genome, because it is located on the 14 kb inverted repeats. It is found in two 7.7 kb Sma I fragments extending from position 39.2 to 46.9, and from position 117 to 124.7, respectively [1].

(4) Identification of the transcription products of wheat and maize cp and mt $tRNA^{Pro}$ (UGG) genes

Total RNA extracted from wheat mitochondria was fractionated on a denaturing formaldehyde agarose gel, blotted on GeneScreen Plus and hybridized to the above-described radioactive oligodeoxynucleotide complementary to nucleotides 579 to 604 of wheat mt $tRNA^{Pro}$ (UGG). Only one band was observed, whose size is that of a mature tRNA, which reveals the presence of the transcription product of the wheat mt $tRNA^{Pro}$ (UGG) gene (Fig. 3A). A similar band is observed in the maize mt RNA (Fig. 3B), showing that the corresponding gene, which has been localized on the 14 kb repeat (see above), is transcribed in maize mitochondria.

In both wheat and maize, the hybridization reveals a very faint band in total cellular RNA (the proportion of mt RNA in total RNA is small) and in chloroplast RNA (due probably to a small contamination by mt RNA).

As shown in Fig. 3C and 3D, a strong band is observed however with wheat and maize chloroplast RNA using as radioactive probes an oligodeoxynucleotide complementary to the first 26 nucleotides of the 5′ end of wheat cp $tRNA^{Pro}$ (UGG) gene, which has been sequenced in our laboratory [14]. But when this oligodeoxynucleotide was hybridized to wheat or maize mt RNAs, no signal was observed (except for a faint one, probably due to a contamination of mt RNAs by cp RNAs). It

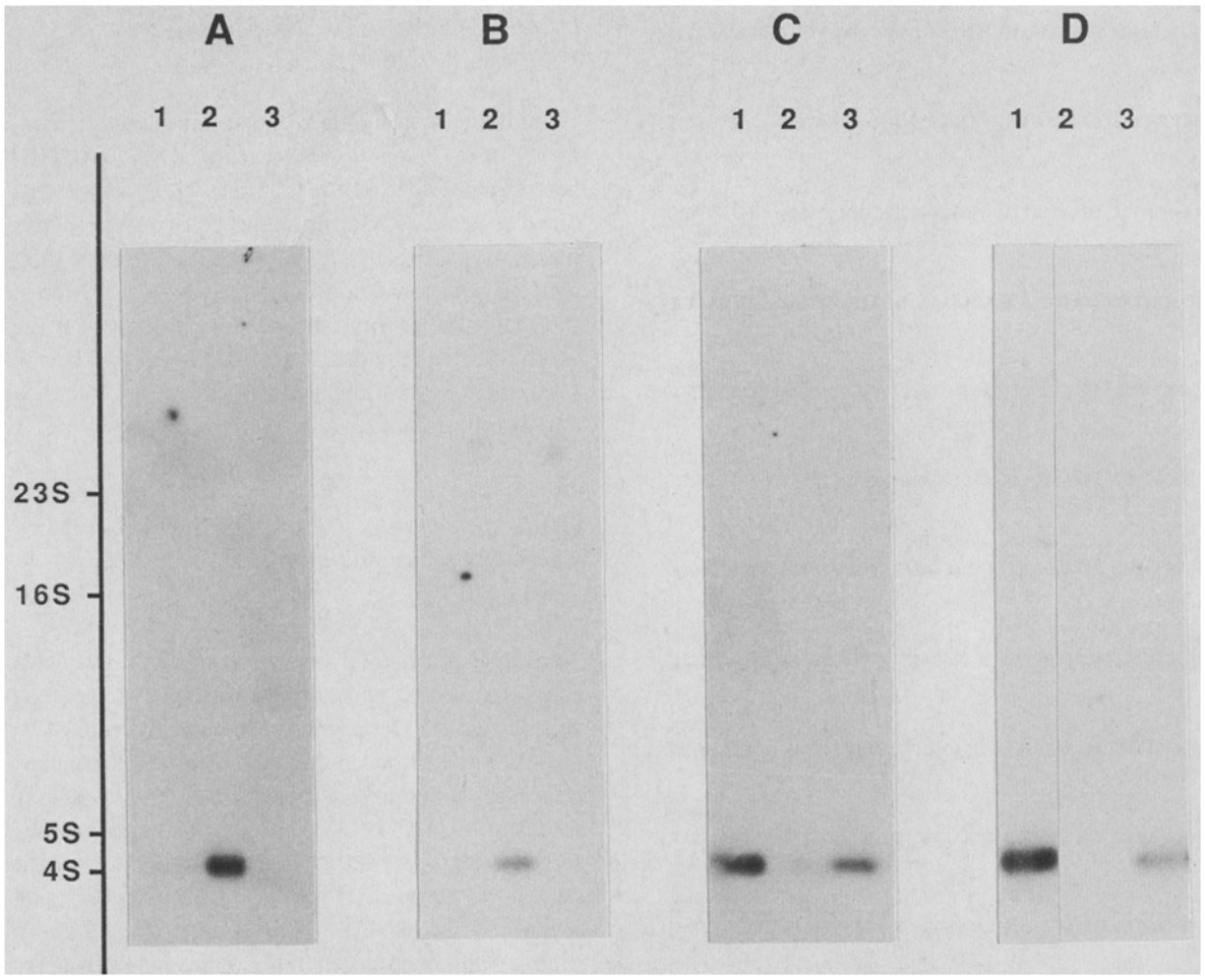

Fig. 3. Identification of the transcription products of wheat and maize cp and mt $tRNA^{Pro}$ (UGG) genes. Total cell RNA (lane 1), mt RNA (lane 2) and cp RNA (lane 3) from wheat (A and C) and from maize (B and D) were electrophoresed on formaldehyde agarose gels and blotted on GeneScreen Plus. Individual filters were hybridized with 5'-labeled oligodeoxynucleotides homologous to wheat mt $tRNA^{Pro}$ (UGG) gene (A and B) or wheat cp $tRNA^{Pro}$ (UGG) gene (C and D). Size markers were from *E. coli* total RNAs.

has been shown [14] that the maize and wheat mt genomes contains a chloroplast DNA insertion including, respectively, a complete or partial copy of the cp $tRNA^{Pro}$ (UGG) gene. The fact that the corresponding transcription product is not revealed using the oligodeoxynucleotide probe complementary to the cp $tRNA^{Pro}$ (UGG) gene, shows that the inserted gene is not expressed in the mitochondria, even in the case of maize mitochondria where the inserted tRNA gene is complete.

(5) Sequence of bean mt $tRNA^{Pro}$

As it is unfortunately very difficult to obtain sufficient amounts of pure individual species of mt tRNA from wheat or maize, we decided to study the transcription product of the $tRNA^{Pro}$ (UGG) gene present in bean (*Phaseolus vulgaris*) mitochondria, because these mitochondria have already provided enough material to allow sequencing of several tRNAs [10, 17, 22, 23].

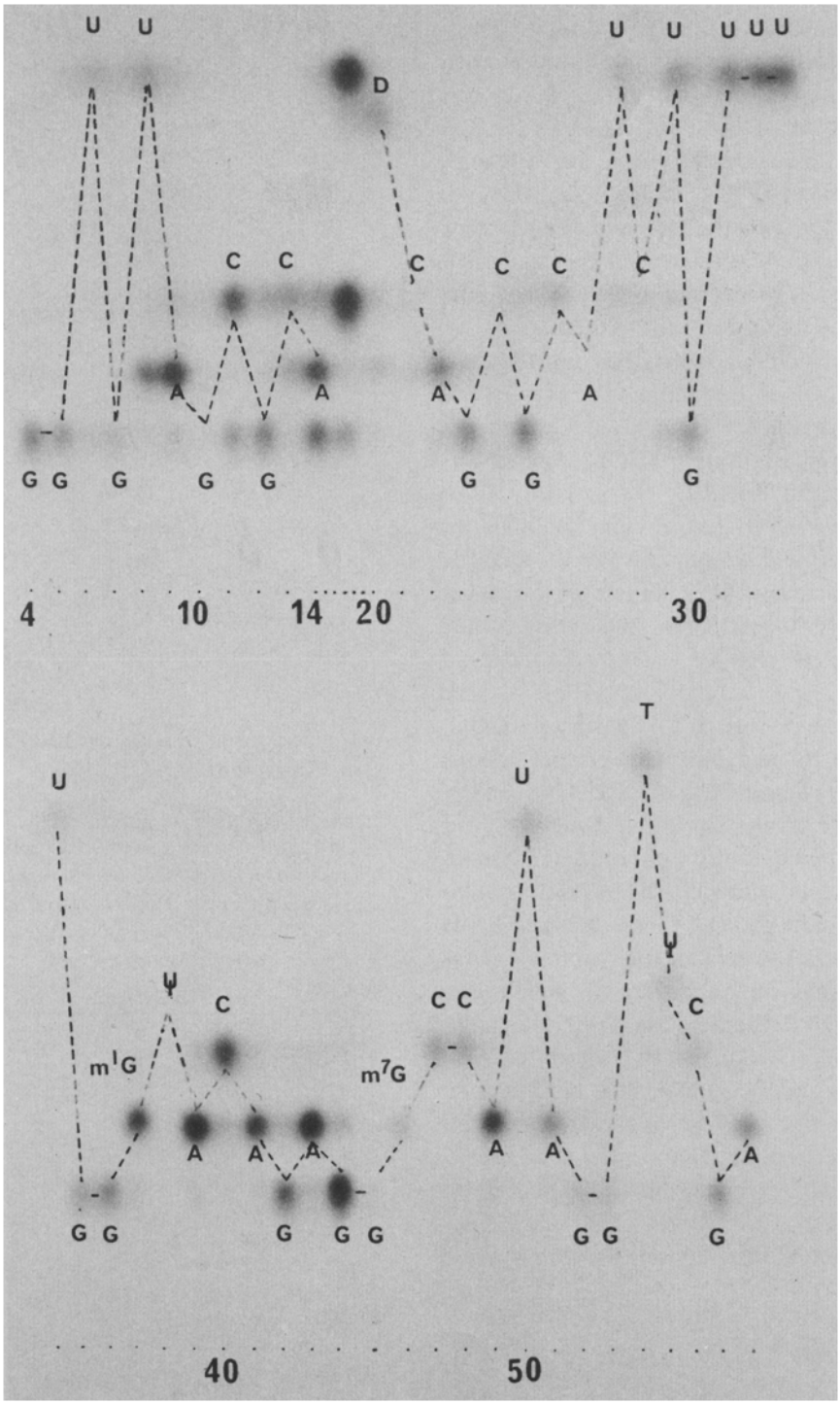

Fig. 4. Sequence analysis of bean mitochondrial $tRNA^{Pro}$ (UGG) using the Stanley and Vassilenko technique [28]. The numbers refer to the position of nucleotides on the cloverleaf structure [29]. Nucleotides 15 to 19 were not separated on this gel, due to the D-loop compression, and were determined as described in Material and Methods.

Figure 4 shows the results of thin-layer chromatography identifying the 5′ nucleotide of each fragment (from fragment 4 to 58) generated by formamide hydrolysis of bean mt tRNA^{Pro} (UGG). The complete sequence of bean mt tRNA^{Pro} (UGG) is shown in Fig. 5. This tRNA has 100% sequence homology with the tRNA^{Pro} (UGG) gene of wheat mitochondria sequenced in this study, showing a complete conservation of this gene in the mitochondria of a monocot and a dicot.

As shown in Table 1, this bean mt tRNA^{Pro} (UGG) has 69 to 76% homology with prokaryotic and chloroplast tRNAs^{Pro} (UGG) (including the complete chloroplast tRNA^{Pro} (UGG) gene inserted in the maize mt genome), but has only 41 to 47% homology with fungal and animal mt tRNAs^{Pro} (UGG) and with eukaryotic cytoplasmic tRNAs^{Pro}, particularly 44% homology with bean nuclear tRNA^{Pro} (UGG) gene [24] which is the only plant nuclear tRNAs^{Pro} gene sequenced so far.

The anticodon of this tRNA^{Pro}, namely UGG, should be able to read only two proline codons CCA and CCG, but not CCU or CCC. One can imagine that there is another mt tRNA^{Pro} to read CCU and CCC, unless a "two out of three" mechanism [25] operates, allowing tRNA^{Pro} (UGG) to read all four proline codons. This is apparently the case in chloroplasts, where all four proline codons are used but where only one tRNA^{Pro} gene exists, which also has a UGG anticodon [26, 27]. In spinach cp tRNA^{Pro} (UGG), the U is modified [31], although it is generally assumed that modification of the U in the first position of the anticodon restricts codon recognition. On the other hand, this U is unmodified either in bean (this study) or in yeast [32] mt tRNA^{Pro} (UGG), which would allow reading of the four proline codons by this unique tRNA.

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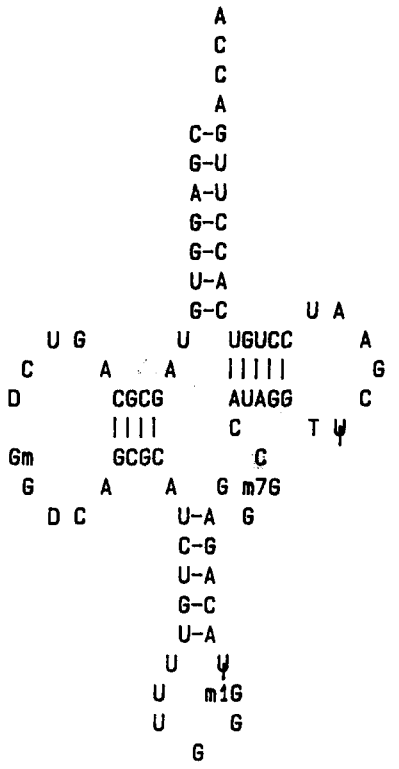

Fig. 5. Nucleotide sequence and secondary structure of the bean mitochondrial tRNA^{Pro} (UGG).

Table 1. Percentage of sequence homology between wheat tRNA^{Pro} gene and various tRNAs^{Pro} and tRNA^{Pro} genes. All these tRNA and tRNA gene sequences can be found in refs. [29] and [30] except those which are marked *, ** and *** which can be found in refs. [14], [24] and [27] respectively.

Species	Percentage
Salmonella typhimurium	76%
Marchantia polymorpha (chloro)***	76%
Escherichia coli	73%
Zea mays (chloro)*	73%
Nicotiana tabacum (chloro)	73%
Spinacia oleracea (chloro)	73%
Bacillus subtilis	69%
Spiroplasma sp.	64%
Aspergillus nidulans (mito)	55%
Saccharomyces cerevisiae	52%
Halobacterium volcanii	48%
Mouse (mito)	47%
Rat (mito)	45%
Bovine (mito)	44%
*Phaseolus vulgaris***	44%
Saccharomyces cerevisiae (mito)	43%
Xenopus leavis (mito)	43%
Drosophila melanogaster (mito)	43%
Human (mito)	41%
Drosophila yacuba (mito)	37%

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