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Short Communication

Identification of Two Methionine Transfer RNA Genes in the Maize Mitochondrial Genome[1]

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ABSTRACT

Two methionine transfer RNA (tRNA) genes were identified in the maize mitochondrial genome by nucleotide sequence analysis. One tRNA gene was similar in nucleotide sequence and secondary structure to the initiator methionine tRNA genes of eubacteria and higher plant chloroplast genomes. This tRNA gene also had extensive nucleotide homology (99%) with an initiator methionine tRNA gene described for the wheat mitochondrial genome. The other methionine tRNA gene sequence was distinct and more closely resembled an elongator methionine tRNA.

The mitochondria of eukaryotic cells have a semiautonomous protein synthesizing system which depends on rRNAs and tRNAs encoded by mitochondrial genes. In mammalian systems (human [1], mouse [5], and bovine [2]) the nucleotide sequence of the entire mitochondrial genome has been determined permitting identification of two rRNA genes and 22 tRNA genes. The nucleotide sequence, predicted secondary structure and number of mammalian mitochondrial tRNAs are distinct from eubacterial or nuclear-encoded tRNAs. The limited number of tRNA genes in mitochondria results from an expanded codon usage in which some tRNAs are able to read additional codons (4). The mitochondrial genome of maize is considerably more complex than the mammalian mitochondrial genome and its organization is not well understood. Three maize mitochondrial genes encoding 5S, 18S, and 26S rRNAs have been identified and characterized; these genes appear more closely related to eubacterial rRNA genes than are their mammalian counterparts (9, 10, 12). Maize tRNA gene sequences have not been described.

This study was initiated to characterize the tRNAs of maize mitochondria and gain an understanding of codon usage. We report the presence of two nucleotide sequences in the maize mitochondrial genome that could encode methionine tRNAs. One gene resembles the initiator methionine tRNA found in eubacteria and chloroplast genomes and also displays 99% homology with the initiator methionine tRNA gene described for wheat mitochondria (15). The other methionine tRNA gene more closely resembles elongator methionine tRNAs.

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MATERIALS AND METHODS

Purification of tRNAs. Mitochondria were isolated from etiolated shoots and RNA was extracted as described by Bonen and Gray (7). tRNAs were isolated from the total RNA sample by making the sample 2 M in LiCl and holding at 4°C overnight. The sample was centrifuged at 12,000*g* for 10 min and the supernatant, which contained the small mol wt tRNAs, was removed. tRNAs were concentrated by precipitation with 95% ethanol. The RNA was resuspended in water and electrophoresed in a 10% polyacrylamide gel containing 7 M urea using the buffer system of Peacock and Dingman (24). The gel band containing the tRNAs was excised, and the RNA eluted by crushing the gel slice with a mortar and pestle and incubating the crushed gel for 16 h at 45°C in 3 to 5 ml of elution buffer (250 mM NaCl, 10 mM Tris-HCl [pH 7.5], 10 mM EDTA). tRNAs were purified further by NACS-52 column chromatography per the manufacturer's instructions (Bethesda Research Labs, Bethesda, MD). The tRNA fractions were collected and precipitated with 95% ethanol.

Labeling of tRNAs. Purified tRNA was heated at 55°C for 5 min, treated with bacterial alkaline phosphatase (21), extracted with phenol and precipitated with 95% ethanol. tRNAs were resuspended in water and the 5′ termini were labeled using T4 polynucleotide kinase and [^{32}P] gamma-labeled ATP (21).

tRNAs were labeled at their 3′ termini with [5′-^{32}P]pCp by T4 RNA ligase as described by England and Uhlenbeck (14). Unincorporated nucleotides were removed by column chromatography.

Identification of Clones Containing tRNA Sequences. A genomic library of maize mitochondrial EcoRI DNA fragments in the vector pUC9 was screened by colony hybridization (17) using purified [^{32}P] end-labeled tRNAs. Putative positive clones were selected and plasmids purified (6). The various plasmid DNAs were digested with the restriction enzyme EcoRI and the DNA fragments separated by gel electrophoresis on 1.5% agarose gels. The DNA fragments were transferred to nitrocellulose (27) and hybridized independently to four different ^{32}P-labeled probes. The hybridization probes were: (a) tRNAs labeled at the 5′ termini; (b) tRNAs labeled at the 3′ termini; (c) a nick-translated recombinant DNA molecule containing the mitochondrial 18S and 5S ribosomal RNA gene sequences; and (d) a nick-translated recombinant molecule containing the 26S mitochondrial ribosomal gene.

Nucleotide Sequence Determination. Plasmid DNA fragments that hybridized only with the tRNA probes were separated by electrophoresis in 1.5% agarose gels. Fragments were purified by electroelution onto NA-45 membrane (Schleicher and Schuell, Keene, NH) (13). The DNA was eluted from the paper in a high

salt buffer and concentrated by precipitation with 95% ethanol. The purified fragment was then digested with either PstI, Xba I, TaqI, or Sau3AI and the resulting fragments were ligated into the appropriate M13 bacteriophage vector as described by Messing *et al.* (22).

Plaques were screened by hybridization and DNA was isolated and its sequence determined (25).

RESULTS

Fifty-four colonies of the EcoRI maize mitochondrial library hybridized with the ^{32}P-labeled maize mitochondrial tRNA probe. Of these original 54 colonies, 12 contained unique fragments based on restriction enzyme analysis. These 12 contained nucleotide sequence(s) which hybridized with the end-labeled tRNA probes and lacked sequence homology with ribosomal RNA gene probes. Hybridization results were the same when either the 5′ or 3′ end-labeled tRNA was used as the probe.

Two of these 12 recombinant DNA molecules, clones pDE-1 and pDE-4, were selected based on small insert size. DNA sequences were determined, 1759 nucleotides in pDE-1 and 1312 nucleotides in pDE-4.[2] Nucleotide sequences which could be folded into a cloverleaf secondary structure, characteristic of a tRNA, were identified (Fig. 1). Clone pDE-1 contained a nucleotide sequence for a putative methionine tRNA gene, which was 74 nucleotides in length and contained the anticodon -C-A-T-. Clone pDE-4 also contained a nucleotide sequence for a methionine tRNA gene, also of 74 nucleotides and possessing the anticodon -C-A-T-. However, the latter sequence was distinct from the methionine tRNA gene found in pDE-1. The 3′ terminal nucleotides -C-C-A-OH, characteristic of all tRNAs, were not encoded in these two gene sequences. Intervening sequences were not evident in the DNA sequence of these two tRNA genes.

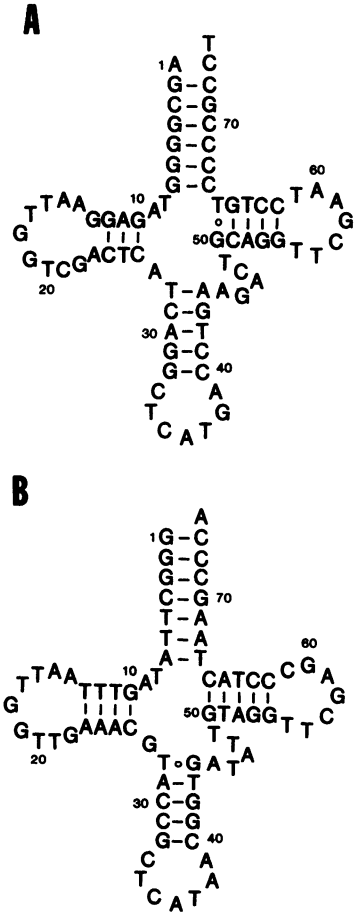

FIG. 1. The predicted secondary structure of the two methionine tRNA gene sequences from maize. Watson-Crick base pairing (—) and T,G base pairing (O) are presented for the initiator-like methionine tRNA (A), and for the elongator-like methionine tRNA (B).

[2] Nucleotide sequences of pDE-1 and pDE-4 can be obtained from C. S. Levings III, Department of Genetics, North Carolina State University, Raleigh, NC 27695.

These two tRNA gene sequences were compared to the nucleotide sequences of methionine tRNAs and methionine tRNA genes described for other organisms and other mitochondrial systems (28, 29). Comparison involved examining the particular stems and loops of each tRNA for common nucleotide sequences (Table I). The methionine tRNA gene of pDE-4 displayed homology with initiator methionine tRNAs from other organisms. Sequence homology of the maize initiator methionine tRNA gene was 71% with eubacterial and chloroplast genes, 61% with fungal mitochondrial initiators, and 52% with mammalian mitochondrial methionine genes. This tRNA differed by only one base from the methionine tRNA gene recently described for wheat mitochondria (15). The methionine tRNA sequence contained in pDE-1 shared sequence homology with the elongator methionine tRNA found in other organisms. These homologies were less than those of the initiator, ranging from 61% with one of the *Aspergillus* elongators, to 41% with chloroplast elongator genes. The nucleotide sequences of both methionine tRNA genes reported here are different from the methionine tRNA gene recently described for the maize chloroplast genome (30).

DISCUSSION

The two maize mitochondrial methionine tRNA genes are similar to eubacterial tRNAs. This is in contrast to the structure that has been proposed for a number of tRNAs found in various mammalian mitochondrial systems, where differences in standard loop sizes and conserved nucleotides have been demonstrated (1, 2, 5, 31).

The primary sequence of the putative initiator methionine tRNA gene and its predicted secondary structure share a number of characteristics with eubacterial initiator methionine tRNAs (Fig. 1A; Table I). Identification of this sequence as an initiator methionine tRNA gene is based on its greater homology with other initiator tRNAs than with elongator methionine sequences. The number of nucleotides that comprise the various loops and stems of the tRNA structure is identical to that found in eubacteria. Also, nucleotides at positions 1 and 73, located at the terminal ends of the aminoacyl stem, are not base paired. This characteristic is believed to contribute to the inability of the initiator tRNA to bind the elongation factor, Tu, during translation (26). The high percentage of guanidine-cytosine base pairs in the aminoacyl stem is also characteristic of initiator tRNAs (16). The primary sequence and secondary structure of the initiator methionine tRNA gene recently described for the wheat mitochondrial system display 99% homology with this maize methionine tRNA. The only nucleotide change is the substitution of a guanidine for an adenosine at position 22. This is unusual, since an adenosine residue in this position is conserved in most tRNA sequences. The maize mitochondrial initiator methionine tRNA sequence differs in some respects from eubacterial methionine initiator tRNAs. The D stem has unpaired guanidine and adenosine at positions 13 and 23, whereas in a eubacterial initiator tRNA, the adenosine is replaced by a cytosine resulting in a guanidine-cytosine base pair. In the D loop, a guanidine replaces an adenosine at position 22 (16). In eubacterial initiator tRNAs, the presence of three guanidine-cytosine base pairs adjacent to the anticodon loop is conserved and has been implicated to be important in tRNA stability (32). The maize mitochondrial

Table I. *Comparison of Methionine tRNA Sequences*[a]

	AMINOACYL STEM		D STEM	D LOOP	D STEM		ANTICODON STEM	ANTICODON LOOP	ANTICODON STEM	EXTRA ARM	T STEM	T LOOP	T STEM	AMINOACYL STEM	MAIZE INITIATOR[b] NO. MATCHES	MAIZE INITIATOR[b] % HOMOLOGY	MAIZE ELONGATOR[b] NO. MATCHES	MAIZE ELONGATOR[b] % HOMOLOGY
MAIZE (MT) INITIATOR	A6CG666	TA	GA66	AATT 66TC6	ACTC	A	TCA66	CTCATGA	CCTGA	AGACT	6CA66	TTC6AAT	CCT6T	CCCC6CCT	--	--	40	54
MAIZE (MT) ELONGATOR	G66CTTA	TA	6TTT	AATT 66TT6	AAAC	G	TACC6	CTCATAA	C66T6	ATATT	GTA66	TTC6AGC	CCTAC	TAA6CCCA	40	54	--	--
INITIATORS																		
E. COLI	C6CG666	TG	GA6C	A6CCT66TA	GCTC	G	TC666	CTCATAA	CCC6A	A66TC	GTC66	TTCAAAT	CC66C	CCCC6CAA	52	70	35	47
B. SUBTILUS	C6CG666	TG	GA6C	A6TTC66TA	GCTC	G	TC666	CTCATAA	CCC6A	A66TC	GCA66	TTCAAAT	CCT6C	CCCC6CAA	56	76	37	50
N. CRASSA (MT)	T6CG6AT	TA	TTGT	AATA G TA	ACAT	A	TTT66	CTCAT6?	CC6AA	T6AC	ATA66	T6CAAAT	CCT6T	ATCC6CAT	48	65	32	43
A. NIDULANS (MT)	A6CG66T	TG	ATGT	AATA G TA	ACAT	A	TATG6	CTCAT6C	CCATA	ATA T	TTA66	T6CAACT	CCTAA	ATCC6CTA	44	59	38	51
YEAST (MT)	T6CAATA	TG	ATGT	AATT 66TTA	ACAT	T	TTA66	GTCATGA	CCTAA	TTA T	ATAC6	TTCAAAT	C6TAT	TATT6CTA	42	57	39	53
WHEAT (MT)	A6CG666	TA	GA66	AATT 66TCA	ACTC	A	TCA66	CTCATGA	CCTGA	AGACT	GCA66	TTC6AAT	CCT6T	CCCC6CCT	73	99	39	53
BEAN (CHL.)	C6CG6A6	TA	GA6C	AACTT66TA	GCTC	G	CAA66	CTCATAA	CCTT6	AA6TT	AC666	TTCAAAT	CCC6T	CTCC6CAA	52	70	38	51
SPINACH (CHL.)	C6CG666	TA	GA6C	A6TTT66TA	GCTC	G	CAA66	CTCATAA	CCTT6	A66TC	AC666	TTCAAAT	CCT6T	CTCC6CAA	53	72	37	50
ELONGATORS																		
E. COLI	G6CTAC6	TA	GCTC	A6TT66TTA	GA6C	A	CATCA	CTCATAA	TGAT6	G66TC	ACA66	TTC6AAT	CCC6T	C6TA6CCA	40	54	41	55
B. SUBTILUS	G6CG6TG	TA	GCTC	A6CT66CTA	GA6C	G	TAC66	TTCATAC	CC6T6	A66TC	G666G	TTC6ATC	CCCTC	C6CC6CTA	39	53	43	58
A. NIDULANS (MT)#1	GCCAAA6	TA	GTTT	AAT 66T A	GAAC	A	ATAAT	TTCAT6A	ATTAA	GAAT	GA6AA	TTC6ATT	TTCTC	CTTT66CT	35	47	31	42
A. NIDULANS (MT)#2	AAGACTA	TA	GCTT	AATC66T A	AA6C	G	AACCA	CTCAT6A	T66TT	T6A6T	AAAT6	TTCAA6T	CATTT	TA6TCTTA	32	43	45	61
MAIZE (CHL.)	GCCTACT	TA	ACTC	A6T 66TTA	GA6T	A	TT6CT	TTCATAC	G6CG6	GA6TC	ATT66	TTCAAAT	CCAAT	A6TA66TA	27	36	32	43
SPINACH (CHL.)	ACCTACT	TA	ACTC	A6C 66TTA	GA6T	A	TT6CT	TTCATAC	G6CG6	GA6TC	ATT66	TTCAAAT	CCAAT	A6TA66TA	27	36	30	41
TOBACCO (CHL.)	ACCTACT	TA	ACTC	A6T 66TTA	GA6T	A	CT6CT	TTCATAC	G6CG6	GA6TC	ATT66	TTCAAAT	CCAAT	A6TA66TA	27	36	30	41
MAMMALIAN																		
BOVINE (MT)	A6TAA66	TC	A6CT	AATTA	A6CT	A	TC666	CCCATAC	CCC6A	AAAT	6TT66	TTTATAT	CCTTC	CC6TACTA	39	53	31	42
MOUSE (MT)	A6TAA66	TC	A6CT	AATTA	A6CT	A	TC666	CCCATAC	CCC6A	AAAC	6TT66	TTTAAAT	CCTTC	CC6TACTA	40	54	31	42
HUMAN (MT)	A6TAA66	TC	A6CT	AAATA	A6CT	A	TC666	CCCATAC	CCC6A	AAAT	6TT66	TTATAC	CCTTC	CC6TACTA	37	50	31	42

[a] SEQUENCES OTHER THAN THOSE FROM MAIZE MITOCHONDRIA ARE FOUND IN REFERENCES 28 AND 29.

[b] % HOMOLOGIES CALCULATED AS: $\frac{\text{NUMBER OF MATCHES}}{74}$

(74 = NUMBER OF NUCLEOTIDES IN MAIZE MITOCHONDRIAL METHIONINE tRNA SEQUENCES).

tRNA only has two such guanidine-cytosine nucleotide pairs. This is similar to the sequence and structure of fungal and wheat mitochondrial methionine initiator tRNAs (8, 15, 18, 19).

In the wheat mitochondrial genome, the initiator methionine tRNA gene is proximal to the 5′ terminus of the 18S ribosomal RNA gene with a single nucleotide separating the two genes (15). The genomic location of the initiator methionine tRNA in maize mitochondria has not been elucidated. However, the nucleotide sequences flanking the methionine tRNA (unpublished data) and the 18S ribosomal genes (10) have been determined and the results suggest a minimum of 500 nucleotides must separate these two genes. Therefore, the precise genomic location of the initiator methionine tRNAs is not conserved in these grasses.

The putative elongator methionine tRNA gene from the maize mitochondrial genome shares sequence and structural similarities with elongator methionine tRNAs from other organisms (Fig. 1.). Among these similarities are (a) guanidine-cytosine pairing at the terminus of the aminoacyl stem, with A-U pairing at the base, and (b) a pyrimidine at position 11 pairing with a purine at position 25 (16). In addition, the elongator sequence and structure do not exhibit several characteristics common to initiator sequences. The terminal bases of the aminoacyl stem are guanidine-cytosine paired, and the remainder of the stem lacks the strong guanidine-cytosine pairing typical of eubacterial initiator tRNA sequences. Other deviations occur in (a) the D-stem, where the usual sequence GAG (positions 10–12) is GTT; (b) the anticodon stem where a G-T pair in position 28–44 replaces the usual Watson-Crick base pair; and (c) the T-loop, where a C replaces the T normally found in position 61 (16). This maize tRNA gene displays maximum nucleotide homology (61%) with one of the two elongator methionine tRNA genes described for *Aspergillus nidulans* (19) (Table I). The maize mitochondrial genome may be similar to *Aspergillus* where more than one isoaccepting methionine elongator tRNA species may exist.

The presence of these tRNA genes complements the findings of Chao *et al.* (9) who suggest by analysis of rRNA genes that the genome of maize mitochondria is more closely related to eubacteria than are the mitochondria of yeast or mammals.

The presence of two genes coding for methionine tRNA has implications on the potential number of tRNAs in the maize mitochondrial genome. Although Aujame and Freeman (3) show the presence of both formylated and nonformylated methionine tRNAs, DNA sequence determinations of several complete mammalian mitochondrial genomes (human [1], mouse [5], bovine [2]) reveal only one identifiable methionine tRNA gene. This can possibly be explained by a posttranscriptional modification of a single gene product. This is in contrast to the distinct methionine initiator and elongator tRNA genes found in fungal mitochondrial genomes (yeast and *Aspergillus*) and in bacteria.

In the mammalian systems, 22 tRNAs have been identified (1, 2, 5) while 23 tRNA genes have been described for the *Neurospora* mitochondrial genome (18) and 24 for the yeast (8) and *Aspergillus* (19, 23) mitochondrial genomes. These systems require less than the 32 tRNAs found in bacteria and predicted by the 'Wobble Rules' of Crick (11). It remains to be determined how many tRNA genes will be found in the maize mitochondrial genome. However, in view of (a) the large mitochondrial genome size (20), (b) the apparent close evolutionary relationship to eubacteria, and (c) the presence of two methionine tRNA genes, maize mitochondria may possess a more complete complement of the 32 tRNAs predicted by Crick.

These sequences and structures represent putative tRNA methionine genes in the maize mitochondrial genome. Direct RNA sequencing of maize mitochondrial tRNAs that can be aminoacylated with methionine will be necessary to determine if either or both of the putative genes described in this paper are tran-

scribed and plan an integral role in the translation of maize mitochondrial messenger RNAs.

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