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Molecular analysis of the linear 2.3 kb plasmid of maize mitochondria: apparent capture of tRNA genes

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ABSTRACT

The nucleotide sequence and transcription pattern of the linear 2.3 kb plasmid of maize mitochondria was analyzed in order to elucidate its possible function in the organelle. The plasmid has 170 bp inverted repeats at its termini composed, in turn, of shorter repetitive sequences. An open reading frame within the plasmid is transcribed and can potentially specify a 33 kD product. In addition the plasmid contains two tRNA genes homologous to chloroplast sequences; the $tRNA^{pro}$(CAA) and the $tRNA^{trp}$(UGG). Both of the tRNA genes of the plasmid are transcribed, but apparently only the $tRNA^{trp}$ is processed to the correct size. These tRNA sequences are found in the main mitochondrial genome of all higher plants tested, and in most maize relatives. An exception is the close maize relative Northern teosinte in which the $tRNA^{trp}$ gene is also carried on a plasmid. These results suggest that the 2.3 kb plasmid has acquired the tRNA sequences from the main mitochondrial DNA. It is possible that the plasmid-encoded $tRNA^{trp}$ gene is essential for organelle function thereby ensuring the maintenance of the plasmid in the mitochondrion.

INTRODUCTION

The mitochondrial genomes of higher plants have several unusual properties, including a very large and complex genome structure (570 kb for maize; 1), incorporation of chloroplast sequences as 'promiscuous' DNA (2, 3), and the presence of plasmid-like molecules (4). Although many higher plant species examined do contain circular and/or linear mitochondrial plasmids (5, 6, 7), their function has been elusive. In some cases the presence of a mitochondrial plasmid was correlated with the maternally inherited trait of cytoplasmic male sterility (*cms*) in which mitochondrial dysfunction results in pollen abortion. For example, in maize it was thought that the two linear S plasmids found only in *cms*S plants played an important role in producing the male sterile phenotype. The S-1 and S-2 molecules were lost as free episomes and integrated forms of the molecules were rearranged in some revertants to fertility (8, 9). More recently, however, it has been shown that the S plasmids can persist after cytoplasmic reversion to fertility, leaving their role unclear (10).

The only mitochondrial plasmid that is ubiquitous in maize is the linear 2.3 kb plasmid (and a related plasmid 2.1 kb in size found in some maize lines) (11, 12). This linear plasmid is tightly associated with protein(s) at the 5′ termini (13), a characteristic shared with the other linear plasmids of maize. Cloning and subsequent molecular analysis has shown that there is no integrated form of the 2.3 kb plasmid (14), in contrast to the near full-length copies of the S episomes found integrated in the main mitochondrial DNA of normal plants (15, 16, 17). These initial studies have also defined a region of homology

with chloroplast DNA; this same region is present in the main mitochondrial DNA of other plants, including some of the closest maize relatives (14). Because of its ubiquity in maize and unique sequence content, the 2.3 kb linear plasmid is the best candidate to encode an essential mitochondrial function.

To determine the possible functions of the 2.3 kb plasmid, we have analyzed its DNA sequence and RNA transcripts. We show that the region of chloroplast DNA homology in the 2.3 kb plasmid consists of two tRNA genes that are normally present integrated in the main mitochondrial genome of other higher plants. The plasmid-encoded $tRNA^{trp}$ gene is transcribed and is potentially the only functional $tRNA^{trp}$ gene in the maize mitochondrion. The capture of these tRNAs by the linear 2.3 kb plasmid from the main mitochondrial genome in domesticated maize may be a novel strategy that could select for plasmid maintenance within the organelle.

MATERIALS AND METHODS

Plant material

The original seed stock was provided by Pioneer Hi-Bred International and propagated at Stanford University by crossing male sterile B37 *cms*T, *cms*C and *cms*S lines by the fertile, B37 normal cytoplasmic (N) stock. Etiolated seedlings were grown in trays of wet vermiculite under low light conditions for 6−8 days prior to harvest. Immature cobs were obtained from field or greenhouse grown plants.

Reagents and enzymes

Radioactive deoxynucleotide triphosphates, ribonucleotide triphosphates and Hybond-N membrane were purchased from Amersham. Dideoxynucleotides were purchased from Pharmacia. Other nucleotides and pancreatic Ribonuclease A were purchased from Sigma. Restriction enzymes, Klenow polymerase, proteinase K and lambda DNA were purchased from Bethesda Research Laboratories. Nitrocellulose membrane was purchased from Schleicher and Schuell.

Mitochondrial, chloroplast and total cell nucleic acid isolation

Chloroplast DNA was prepared as described (18). Mitochondrial DNA from etiolated seedlings or immature cobs was isolated as previously described (19, 20). RNA was isolated from N and *cms*S intact mitochondria by phenol extraction (21) and total maize RNA was isolated using guanidinium isothiocyanate (22).

Plasmid preparation

E. coli were grown in Luria broth containing 100 μg/ml of ampicillin. Plasmid DNA was isolated using the alkaline lysis procedure (22).

DNA sequencing

Dideoxy sequencing (23) was performed at 42°C using M13 single strand mp18 or mp19 vectors (24) or supercoiled double-strand DNA (25). Primers used to sequence were the M13 sequencing primer (17-mer) purchased from Pharmacia and three synthetic oligonucleotides prepared on a Applied Biosystem DNA synthesizer for the single-strand sequencing, or the reverse primer (16-mer) purchased from New England Biolabs for the double-strand sequencing. Two of the synthetic oligonucleotides hybridize with each of the TIRs, nucleotides 131 to 150 (or 2182 to 2163) and 120 to 137 (or 2193 to 2176), and the third hybridizes to the left of the *Sna*B1 site (nucleotides 1719 to 1734) as shown by boxes in Fig 1.

DNA and RNA blot hybridization

Mitochondrial and chloroplast DNA were digested with the appropriate enzyme(s) and

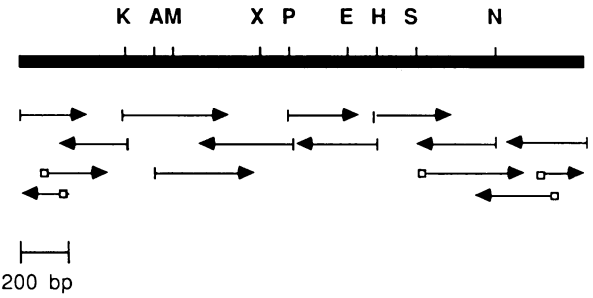

Figure 1. Restriction map and sequencing strategy.
The unique restriction sites shown are as follows: K(*Kpn*I), A(*Ava*I), M(*Mst*II), X(*Xho*II), P(*Pst*I), E(*Eco*RV), H(*Hin*dIII), S(*Sna*BI) and N(*Nsi*I). Arrows indicate the sequenced regions. Boxes represent synthetic oligodeoxynucleotide primers.

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        10         20         30         40         50         60         70         80         90        100
AAAAGTATAG CAACACACAA TTACGATTAT GATCTTATAT AAACACACAC TACATAAAGA TACCAACCGA TACACAATTA CGATTATGAT CTTATATAAA
       110        120        130        140        150        160        170        180        190        200
AAAAAACGAC CTTTTCAACC TTGAGCATAT CATTCCGAAA CACAATTACG ATTATGATCT TATATAAATA CACACTACCT TATTGAGCAT ATTCAACACA
       210        220        230        240        250        260        270        280        290        300
TACCGATACA CAATTACTAT TATGATCTTA TATATAAAAA AACTACCTTC TGAAATCCAA CCACCACATA CCGAAACACA ATTACTATTA TGATCTTATA
       310        320        330        340        350        360        370        380        390        400
TAAATACAAA CTACCTTATT GAGCATATTC AACACATATC TTCCAGGGTG TCGGATAACA TAATGTTTTT GCCCCACCCA TACTATCACA CAAGTCGTGA
       410        420        430        440        450        460        470        480        490        500
TAGAGCACAA GGGATAACTA CAAATAACAG GTACCGACAG CTCAGTCAAG AAGACAACAA CGTCTCAAAG ACTGACAGAT AAAATCCCGC CAGGAAGAGG
       510        520        530        540        550        560        570        580        590        600
CTCTTAAAAT GGAATTGAAA AATGACAAAG ACATATAGAA TATCCTCAGG ACTACTGCGG GAGGCTGTAA AAAAAATAAC GATAGCTCGG GACAGCGCCT
       610        620        630        640        650        660        670        680        690        700
TTTTTGTTGA TAAAGAAATC TTGTCAAAGA GTTCGGAACT CAAGAATAGT TTGATTAACC CGTGTCATAA TGTTGTAAGG GAGTTTGTTA GAGAGTCAAG
       710        720        730        740        750        760        770        780        790        800
AGACTGCGAC TCTGACGATT ATAAAAGAGA GTACAGGCTA AATGATTATA GGCTGGAGCT GACTCAGCCA CCCTCTAATA TAAGTTCGGG GGGTGATGTG
       810        820        830        840        850        860        870        880        890        900
GTAAAGACTG TTAGAAGGTG GGAACTTATA AATCAAAAGT ACAACGCCGA TCTATGGATA CCCCCTGCTA AAAAAGGGGT TGTGATTACA CCTGATATTA
       910        920        930        940        950        960        970        980        990       1000
TAGACAAACT CTATCTCTTC TGGGATAACT TCCTAGGAAG TTACGAAAAA GAAGGTATTA ACCGCAAACA GAACACAGAT CCGGTATCTA AGGGGAAAGT
      1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
CTTTGACTTG CTAGCCAGCC GTAGTAGTAA TAATACGAAT CAAACCCCCG AAGAGTTAAG AGAGATTCAA ACTCAAATCG AGCATTTGAC TATCCACTTT
      1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
GATGAGGGTA GCGTCCATGA GTCTGCAGGC CAGTTGAAGG GGAAGTTCTT CAATAAGGAT AGACAATCAG CTAGGGATTA CCTTCTTGAT AAATTGAAGG
      1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
ATGATAAAGA TAAAGATATT GTTAGAGGTA TGGGCACCTA TACACTGGAA TGTGTAGCAA TACATGTGTT AAGCAAGCTG TTTAATGTGT TTTCTTTGGA
      1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
AAAGACTAGT GTGCTAGCAG CCGCATTGAT ATCAGAGCTA GATATAACAG CTAAGACGGA ATATAATAAT GCACTATTAG CTAAAATGCA GCGCGAAAAG
      1410       1420       1430       1440       1450       1460       1470       1480       1490       1500
GAATAAACTG AAAAGCTAAT AAAGCGCTAT GATGAAAAGC TGGCCAAGGC TGCAATGATG AATGAACCTA CTGATACAAA TATTGATAAT ATACACGATG
      1510       1520       1530       1540       1550       1560       1570       1580       1590       1600
ATAAAAAGCT TAAGGGTATG GAATCACCTG TGGGTATTAT CCCTGTCCTG GGAACAGATG GGAGACATAT AGACCAATCT ATACAGAATA AATCGAGTAG
      1610       1620       1630       1640       1650       1660       1670       1680       1690       1700
TAATAAAAAG AGCACTAAAA AGAGTGATGT TATAAGGAAA CGAATACGTA AAGGTTAGGT TGGGTTTCTG ATGTATATCA TTTATTATAT GAAACCAAAA
      1710       1720       1730       1740       1750       1760       1770       1780       1790       1800
AGAAGAAATG ACAAGAAAGT TGTATATAGA TGGAGGATAA AATTACGATG TGGAAAACAA GACAGGGGTT TTTTTTGTAC AATGACACTG TACAACAATT
      1810       1820       1830       1840       1850       1860       1870       1880       1890       1900
TGGAAAAGGG ATGTAGCGCA GCTTGGTAGC GCGTTTGTTT TGGGTACAAA ATGTAACGGG TTCAAATCCA GTCATCCCTA CCTATTACTT CTCCTATGGG
      1910       1920       1930       1940       1950       1960       1970       1980       1990       2000
CAGTAACGAG GGATCAATTG AGATCGATTC AAATTGGACA AAATTAAGAG TTTAATTTTG CTATATGCAT GCGGTACAAA AATCATCCTT TTCTTTACTG
      2010       2020       2030       2040       2050       2060       2070       2080       2090       2100
CTTTATCTCC TGGCGTAAGA AAGCGCTCTT AGTTCAGTTC GGTAGAACGT GGGTCTCCAA AACCCAATGT CGTAGGTTCA AATCCTACAG AGCGTGATTC
      2110       2120       2130       2140       2150       2160       2170       2180       2190       2200
TTTTCTTGTT ATGTAAAATA AATAAAAGAT TAGAACAGTC TCTATTTATA TAAGATCATA ATCGTAATTG TGTTTCGGAA TGATATGCTC AAGGTTGAAA
      2210       2220       2230       2240       2250       2260       2270       2280       2290       2300
AGGTCGTTTT TTTTTATATA AGATCATAAT CGTAATTGTG TATCGGTTGG TATCTTTATG TAGTGTGTGT TTATATAAGA TCATAATCGT AATTGTGTGT
      2310
TGCTATACTT TT
```

Figure 2. Nucleotide sequence of the 2.3 kb plasmid.
Terminal inverted repeated sequences (bases 1−170 and 2143−2312) are underlined. An open reading frame (ORF), from base 522 to 1403, is boxed. tRNA sequences from base 1807−1880 ($tRNA^{pro}$) and 2023−2096 ($tRNA^{trp}$) are underlined with dots.

resolved by electrophoresis in 0.8% agarose gels in 85 mM Tris, pH7.5, 89 mM boric acid, and 2.5 mM EDTA. Blotting of gels to Hybond-N membrane filters was done by standard procedures (26). Electrophoresis of RNA was carried out in formaldehyde gels and transferred to a nitrocellulose membrane by capillary blotting (22). The membranes were baked for 2 hr and then prehybridized for 5 hr at 42°C in a hybridization buffer containing 50% formamide, 1% sodium dodecyl sulfate (SDS), 3× SSC (1× SSC is 0.15M NaCl, 0.015M sodium citrate) and 0.1% each Ficoll, polyvinyl pyrrolidone, and bovine serum albumin and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out for 16 hr at 42°C in the same buffer after addition of the labeled probes. The membranes were washed three times for 40 min at 57°C in 0.1× SSC and 0.1% SDS, and

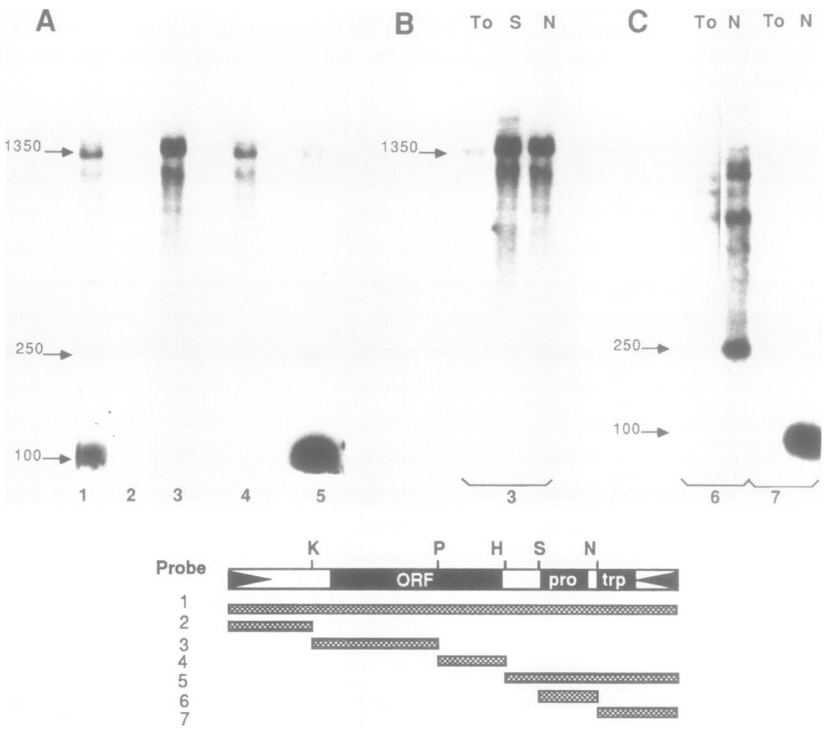

Figure 3. Transcriptional analysis of the 2.3 kb plasmid.
(A) Mitochondrial RNA from normal plants was hybridized with different portions of the 2.3 kb plasmid. The probe used in each case is shown by the numbers below each lane. The origin of each probe is indicated on the plasmid map in the lower part of the figure. The open arrows represent the TIRs. The *Pst*I sites of the ends correspond to those in the pUC8 vector of the pZm2.3 plasmid (14): 1 corresponds to the complete 2312 bp insert of the pZm2.3, 2 corresponds to a 430 bp *Pst*I-*Kpn*I fragment, 3 corresponds to a 693 bp *Kpn*I-*Pst*I fragment, 4 corresponds to a 383 bp *Pst*I-*Hin*dIII fragment, and 5 corresponds to a 806 bp *Hin*dIII-*Pst*I fragment. Approximate transcript sizes, indicated in bases, were determined using the ribosomal RNAs and the *atp9* transcript sizes as markers.
(B) Transcription of the tRNA in total maize (To) and mitochondrial RNA from normal (N) and *cmsS* (S) plants. The probe used (3) is indicated on the plasmid map and its characteristics are described above.
(C) Transcription of the tRNA genes in total maize (To) and normal mitochondria RNA (N). Probes used are indicated on the plasmid map. 6 corresponds to a 320 bp *Sna*BI-*Nsi*I fragment containing the $tRNA^{pro}$, and 7 corresponds to a 367 bp *Nsi*I-*Pst*I fragment containing the $tRNA^{trp}$.

autoradiographed at −70°C with Kodak XAR film and a Lightning Plus (DuPont) intensifying screen. The hybridization probes for DNA and RNA blots ($\sim 10^9$ cpm/μg) were labeled with [α -^{32}P]CTP by primed synthesis (27), using a mixed hexadeoxy oligonucleotide purchased from Pharmacia.

RESULTS

Sequence analysis of the 2.3 kb plasmid

To complete the molecular characterization of the 2.3 kb linear plasmid, we determined its entire nucleotide sequence. Sequencing was performed using subfragments of the plasmid cloned in M13mp18 or mp19 vectors (24) and hybridized to oligonucleotide primers as shown in Fig 1. The salient features of the sequence (Fig 2) include a low G+C content

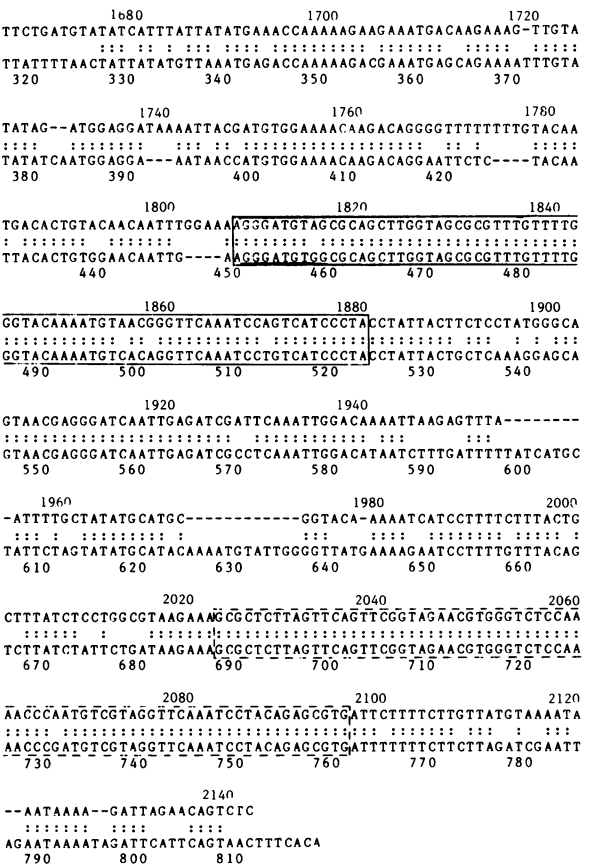

Figure 4. Nucleotide homology of the 2.3 kb plasmid to chloroplast tRNA genes.
The region of chloroplast homology extends for 463 bp, from nucleotides 1676 to 2139 on the 2.3 kb plasmid (Fig 2) and encompasses a tRNA^{pro}, an intergenic region and a tRNA^{trp} gene (upper sequence). This region is compared to tobacco chloroplast DNA (lower sequence, 33). Exact homology is indicated by double dots and deleted regions by dashes. tRNA^{pro} sequences are boxed with a solid line and tRNA^{trp} sequences are boxed with a dashed line.

Table 1. Percent Similarity of 2.3 kb Plasmid tRNA genes with other tRNA genes

Source (organelle)	$tRNA^{pro}$ CAA	Intergenic Region	$tRNA^{trp}$ UGG	Reference
bean tRNA (mitochondria)	ND	–	97*	32
wheat DNA (mitochondria)	pseudogene	92	98	31
petunia DNA (mitochondria)	ND	ND	98	Hass, *et al.*
tobacco DNA (chloroplast)	94	61	98	33
maize DNA (chloroplast)	95	52	95	34
Escherchia coli DNA	65	NL	60	35, 36
human DNA (mitochondria)	39	NL	34	37

ND = sequence not available. NL = not linked. * = modified bases in the tRNA make this a minimal estimate of homology.

(36.5%) in comparison to that of the main mitochondrial DNA of maize (47%) (28). The plasmid has identical terminal inverted repeats (TIR) 170 bp in length at each end; the TIRs have an even lower G+C content of 28%. Within the TIRs there are directly repeated sequences approximately 30 bp in length which contain *Sau*3A sites. An open reading frame (ORF) potentially encoding a protein 294 amino acids in length is located between nucleotides 522 and 1403. When the deduced amino acid sequence of the 882 bp open reading frame was compared to a protein data bank (R. Doolittle, personal communication) the only similarity found was one with the long open reading frame (ORF1) of the S-2 plasmid which encodes a 130 kD protein (29, 30). The region of homology to chloroplast DNA containing the $tRNA^{pro}$ and $tRNA^{trp}$ genes is found between nucleotides 1807 and 2096.

Transcript analysis shows that the ORF of the 2.3 kb plasmid is transcribed

Previous hybridization of cloned 2.3 kb sequences to RNA dot blots indicated that at least part of the plasmid is transcribed (14). The complete plasmid sequence hybridized to several size classes of RNA (Fig 3A, lane 1). To identify the number of transcripts and the origin of each transcript, four subfragments of the plasmid were used in separate hybridizations, as diagrammed in Fig 3A (lanes 2–5). At least three different transcripts were detected, measuring approximately 1350 b, 250 b and 100 b in length. The longest transcript hybridizes with probes containing sequences from the *Kpn*I site to the right end of the plasmid (Fig 3A, lanes 3–5). This transcript therefore includes the complete ORF sequence. Several other RNA species are detected with the same probes suggesting that processing or specific degradation of the 1350 b transcript may occur.

As previously mentioned, computer analysis demonstrated extensive similarity at the amino acid level between the ORF1 from the S-2 plasmid found in *cms*S plants and the ORF of the 2.3 kb plasmid. Therefore, we were interested in determining whether the expression of the 1350 b transcript was different in *cms*S as compared to the normal cytoplasm. As shown in Fig 3B, the 1350 b transcript was present in similar amounts in both N and *cms*S mitochondrial RNA. A slightly larger transcript was detected in RNA

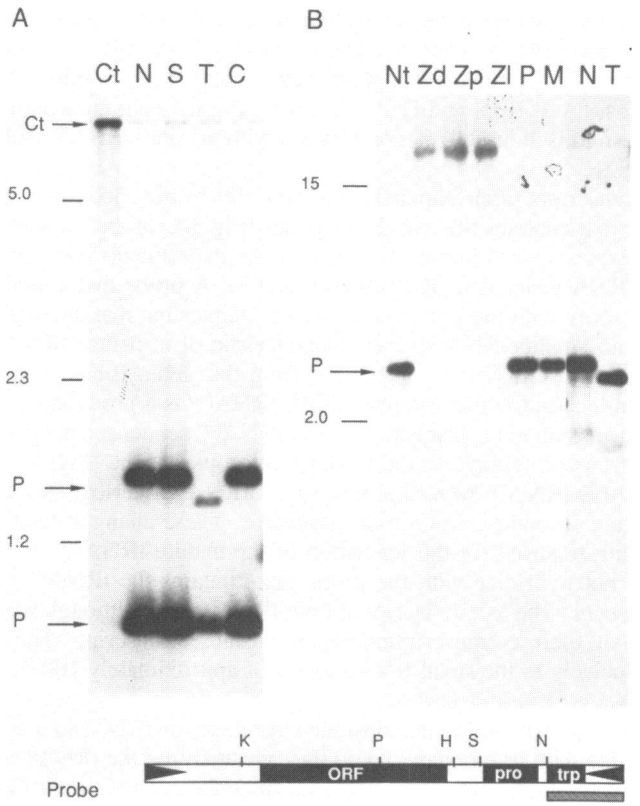

Figure 5. tRNA^{trp} gene in the mitochondrial genome of maize and maize relatives.
(A) A Southern blot of *Hin*dIII-digested mitochondrial DNA isolated from normal (N), *cms*T (T), *cms*C (C), and *cms*S (S) plants was probed with a 367 bp *Nsi*I-*Pst*I fragment containing the tRNA^{trp} and TIR sequences. Arrows shown indicate the tRNA^{trp} hybridization signal from contaminating chloroplast DNA (Ct) and hybridization to the 2.3 kb plasmid (P), which is cut into 1506 and 806 bp fragments by *Hin*dIII. It should be noted that hybridization to the 1506 bp plasmid fragment is due to the presence of TIR sequences in the probe. (B) Southern blot of undigested mitochondrial DNA isolated from Northern teosinte (Nt), *Z. diploperennis* (Zd), *Z. perennis* (Zp), *Z. luxurians* (Zl), *Z. mays ssp. parviglumis* (P), *Z. mays ssp. mexicana* (M) normal B37 (N), *cms*T B37 (T). The probed used was the same fragment described in part A. Arrows shown indicate the hybridization signal to the 2.3 kb plasmid or the shorter version found in the T cytoplasm (P). Molecular weight markers are indicated in kilobase pairs.

isolated from *cms*S mitochondria. The nature of this RNA species is currently under study.

tRNA sequences in the 2.3 kb plasmid

Sequence analysis of the region of chloroplast DNA similarity (14), demonstrated that this region contains the tRNA^{pro} and tRNA^{trp} genes. Computer analysis showed that both tRNA genes are homologous to known plastid sequences. Fig 4 shows a comparison of the 2.3 kb plasmid and tobacco chloroplast sequences that demonstrates a high degree of conservation, not only within the tRNA sequences but also in the intergenic region. Further sequence comparison of the 2.3 kb plasmid tRNAs (and intergenic region where applicable) to other related sequences is summarized in Table 1. Interestingly, the intergenic region

in the 2.3 kb plasmid shares a higher similarity with the tobacco plastid sequence than with the maize plastid (Fig 4, Table 1). There is a very high degree of similarity between the 2.3 kb plasmid tRNAs and the wheat and petunia plant mitochondrial tRNA sequences (31, J.M. de-Hass, A.J. Kool and H.J. Nijkamp, personal communication). The $tRNA^{trp}$ of the maize plasmid is at least 97% homologous with the only $tRNA^{trp}$ isolated from plant mitochondria (32).

Two small transcripts (approximately 250 and 100 b) described above were detected using the probe that contains the two tRNA genes (Fig 3A, probe 5). The nature of these two transcripts was analyzed further in Northern blot experiments using probes containing the individual tRNA genes (Fig 3C, probes 6 and 7). A probe that contains the $tRNA^{pro}$ gene hybridizes only with the transcript of 250 b, suggesting that although this sequence is transcribed, no smaller RNA species characteristic of a mature tRNA is found. It is possible that no mature $tRNA^{pro}$ is produced from this gene. It has been reported in the case of wheat mitochondria that the plastid-like $tRNA^{pro}$ is a pseudogene because part of the 5′ region is missing (31). Inspection of the $tRNA^{pro}$ sequence shows that only 3 base changes have occurred relative to the tobacco chloroplast gene (Fig 4). Although these changes permit this $tRNA^{pro}$ to fold into a typical tRNA cloverleaf structure, they could affect some of the secondary and tertiary structure. These changes could affect stability and/or processing required for the formation of the mature $tRNA^{pro}$. Because the 250 b transcript does not hybridize with the probe that contains the $tRNA^{trp}$ gene, the 250 b RNA does not seem to be a cotranscript of both tRNA genes, although we cannot exclude the possibility of their cotranscription in an unstable transcript. The $tRNA^{trp}$ probe hybridizes exclusively to the small RNA species of approximately 100 b, as expected for a mature tRNA.

In a preliminary experiment we demonstrated that there are tRNAs in maize mitochondria that are chargeable with tryptophan. To do this, we analyzed the $tRNA^{trp}$ species charged *in vitro*. Tritiated tryptophan (^{3}H-Trp) was used in conjunction with purified tryptophanyl-tRNA synthetase from *Bacillus stearothermophilus* for the charging of maize tRNAs (38). After phenol extraction and ethanol precipitation, the charged tRNAs were fractionated on RPC5 resin (39). Two peaks were resolved, a major one (80%) and a minor one (20%) (data not shown). Because of the limited quantities of the tRNAs, the sequences of the two species could not be determined. Until the charged tRNAs are analyzed further, it will not be possible to determine whether two genes are involved or whether the two species detected differ by post-transcriptional modification.

$tRNA^{pro}$ and $tRNA^{trp}$ genes in the mitochondrial genome of maize and maize relatives

Previous work indicated that there is not an integrated form of the 2.3 kb plasmid in the main mitochondrial genome analogous to the integrated forms of the S episomes found in normal and *cms*S mitochondria (14−17). To confirm that the $tRNA^{trp}$ gene is not in the main mitochondrial DNA, we probed *Hin*dIII-digested maize mitochondrial DNA from normal (N), *cms*T (T), *cms*C (C) and *cms*S (S) plants with each of the two tRNA sequences (Fig 5A). The only detectable signal resulted from hybridization to either plasmid (P) or a minor amount of contaminating chloroplast (Ct) DNA. Digestion of mitochondrial DNA with *Pst*I, *Bam*HI, and *Sal*I and probing with either the $tRNA^{trp}$ or the $tRNA^{pro}$ genes gave the same result (data not shown). We conclude that both tRNA genes are found exclusively on the 2.3 kb plasmid in maize mitochondria. Our conclusion is corroborated by a recent report indicating a lack of hybridization of a bean mitochondrial $tRNA^{trp}$ gene (and of a 27-mer oligonucleotide corresponding to the wheat mitochondrial $tRNA^{trp}$ gene)

to a cosmid library of maize mitochondrial DNA, suggesting that this tRNA gene was missing from the main maize mitochondrial chromosome (31).

We have previously reported that in *Zea diploperennis* and in *Z. luxurians*, two species in which no free plasmid form exists, homology was detected between the higher molecular weight DNA and part of the 2.3 kb plasmid (14). In an experiment using specific probes for both tRNA genes we examined the maize relatives *Z. perennis*, *Z. diploperennis* and *Z. luxurians*; in these species no plasmid equivalent to the 2.3 kb or the shorter 2.1 kb version found in the T cytoplasm (14) is present, and the $tRNA^{trp}$ is part of the main mitochondrial genome (Fig 5B). One close maize relative, Northern teosinte as well as all other *Z. mays* tested do contain the 2.3 kb plasmid and no homologous sequences can be detected in the main genome. Therefore, we propose that acquisition of the plasmid containing both tRNA genes, correlates with the loss of this sequence from the main mitochondrial genome.

DISCUSSION

One open reading frame was found in the nucleotide sequence of the 2.3 kb plasmid, and Northern analysis indicates that this region of the plasmid is transcribed (Fig 5A and 5B). The ORF could encode a 33 kD protein with a significant similarity at the amino acid level with the 130 kD protein encoded by the linear mitochondrial episome S-2 (data not shown, 30). Given this similarity, the S-2 and the putative 2.3 kb plasmid-encoded proteins may perform similar functions. It is possible that these plasmid-encoded proteins may be required for plasmid maintenance. A recent report (40) suggested that ORF1 of the S-2 plasmid might encode an RNA polymerase; the region of similarity between the 2.3 kb plasmid and this ORF is outside the portion of the ORF with characteristics of an RNA polymerase.

One striking feature of the 2.3 kb plasmid is its apparent capture of tRNA genes from the main mitochondrial genome. These tRNA sequences seem to have a plastid origin, as indicated by the high degree of sequence similarity and organization with maize and tobacco plastid DNA (Figure 4, 34). A direct transfer of tRNA genes from the plastid genome to the maize mitochondrial plasmid is doubtful given that homologous sequences are found in the high molecular weight main mitochondrial genome of maize relatives and other higher plants, including wheat and petunia (Table I, Fig 5). It is therefore more likely that the transfer of the tRNA pro and trp genes from the plastid to the mitochondria of higher plants is a much earlier event than that of the acquisition of these genes by the maize mitochondrial plasmid.

In the case of the $tRNA^{trp}$ gene, the transferred plastid sequence appears to be functional in the mitochondrion. In bean mitochondria, a homologous $tRNA^{trp}$ (Table I) is the only functional $tRNA^{trp}$ detected to date by an *in vitro* amino acid charging assay (32). In maize, the plasmid-encoded gene is transcribed, and the stable RNA product is approximately the size of a mature tRNA. From the available data we cannot exclude the possibility that a functional mitochondrial $tRNA^{trp}$ in maize could be encoded in the nuclear genome and transported into the mitochondrion as has been reported for some organelle tRNAs (41).

A paucity of plant mitochondrial protein sequence data makes it difficult to verify whether UGG is the only tryptophan codon used in plant mitochondria. With one exception the TGG codon is the only one found in protein coding genes (see references cited in Mulligan and Walbot (42), for sequences of all maize mitochondrial genes) in positions homologous

to tryptophan positions in yeast and mammalian mitochondrial proteins. The one exception is the cytochrome oxidase 2 gene of maize and *Oenothera*; in these two organisms the *cox*2 gene contains both UGG and CGG codons in positions occupied by tryptophan residues in the yeast and bovine proteins (43, 44). Only protein sequence data of the maize or *Oenothera* proteins will resolve whether CGG also serves as a tryptophan codon in plant mitochondria.

Although it is impossible at this time to prove that the plasmid-encoded $tRNA^{trp}$ gene is an essential gene, this is a plausible explanation for the ubiquitous nature of this maize mitochondrial plasmid. Plasmid maintenance would therefore be ensured because this plasmid would carry a gene required under all growth conditions. In this sense the 2.3 kb plasmid could be considered a second mitochondrial chromosome. Recently the presence of a mitochondrial gene (subunit 6 of the ATPase) on a plasmid has been demonstrated in the fungus *Cochliobolus heterostophus* (45). Although nothing has been reported about the expression of the plasmid-encoded *atp*6 gene, or whether this is the only mitochondrial copy of the gene in this organism, this finding implies that the capture of genes by mitochondrial plasmids may have occurred in organisms other than maize.

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REFERENCES

1. Lonsdale, D.M., Hodge, T.P. and Fauron, C.M.R. (1984) Nucl. Acids Res. **12**, 9249−9261.
2. Stern, D.B., and Lonsdale, D.M. (1982) Nature **299**, 698−702.
3. Sederoff, R.R., Ronald, P., Bedinger, P., Rivin, C., Walbot, V., Bland, M., and Levings, C.S.III. (1986) Genetics **113**, 469−482.
4. Pring, D.R., and Lonsdale, D.M. (1985) Int. Rev. Cytology **97**, 1−49.
5. Nikiforova, I.D., and Negruk, V.I. (1983) Planta **157**, 81−84.
6. Brennicke, A., and Blanz, P. (1982) Mol. Gen. Genet. **187**, 461−466.
7. Erickson, L., Beversdorf, W.D., and Pauls, K.P. (1985) Curr. Genetics **9**, 679−682.
8. Levings, C.S.III, Kim, B.D., Pring, D.R., Conde, M.F., Mans, R.J., Laughnan, J.R., and Gabay-Laughnan, S.J. (1980) Science **209**, 1021−1023.
9. Schardl, C.L., Lonsdale, D.M., Pring, D.R., and Rose, K.R. (1984). Nature **310**, 292−296.
10. Escote, L.J., Gabay-Laughnan, S.J., and Laughnan, J.R. (1985) Plasmid **14**, 264−267.

11. Kemble, R.J., Gunn, R.E. and Flavell, R.B. (1983) Theor. Appl. Genet. **65**, 129–144.
12. Pring, D.R., and Smith, A.G. (1985) Maize Genet. Coop. Newsletter **59**, 49–50.
13. Kemble, R.J., and Thompson, R.D. (1982) Nucl. Acids Res. **10**, 8181–8190.
14. Bedinger, P., de Hostos, E.L., Leon, P., and Walbot, V. (1986) Mol. Gen. Genet. **205**, 206–212.
15. Thompson, R.D., Kemble, R.J., and Flavell, R.B. (1980) Nucl. Acids Res. **8**, 1999–2008.
16. McNay, J.W., Pring, D.R., and Lonsdale, D.M. (1983) Plant Mol. Biol. **2**, 177–187.
17. Levings, C.S.III, Sederoff, R.R., Hu, W.W.L., and Timothy, D.H. (1983) In: Ciferri,O., Dure,L.III (eds)., Structure and Function of Plant Genomes, Plenum Publishing Corp., New York, pp 363–371.
18. Palmer, J.D. (1982) Nucl. Acids Res. **10**, 1593–1605.
19. Kemble, R.J., Gunn, R.E., and Flavell, R.B. (1980) Genetics **95**, 451–458.
20. Oro, A.E., Newton, K.J., and Walbot, V. (1985) Theor. Appl. Genet. **70**, 287–293.
21. Aiba, H., Adhya, S., and de Crombrugghe, B. (1981) J. Biol. Chem. **256**, 11905–11910.
22. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York.
23. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA **74**, 5463–5467.
24. Messing, J. (1983) Methods in Enzymology, Recombinant DNA Part C **101**, p 20–77.
25. Chen, E., and Seeburg, P.H. (1985) DNA **4**, 165–170.
26. Southern, E. (1975). J. Mol. Biol. **98**, 503–517.
27. Feinberg, A.P., and Vogelstein, B. (1983) Anal. Biochem. **132**, 6–13.
28. Ward, B.L., Anderson, R.S. and Bendich, A.J. (1981) Cell **25**, 793–803.
29. Levings, C.S.III, and Sederoff, R.R. (1983) Proc. Natl. Acad. Sci. USA **80**, 4055–4059.
30. Manson, J.C., Liddell, A.D., Leaver, C.J., and Murray, K. (1986) EMBO J. **5**, 2775–2780.
31. Marechal, L., Runeberg-Ross, P., Grienenberger, J.M., Collin, J.M., Weil, J.H., Lejeune, B., Quetier, F., and Lonsdale, D.M. (1987) Curr. Genet. **12**, 91–98.
32. Marechal, L., Guillemaut, P., Grienenberger, J.M., Jeannin, G., and Weil, H. (1985) Nucl. Acids Res. **13**,
33. Ohme,M., Kamogashira,T., Sinozaki,K., and Sugiura,M. (1984) Nucl. Acids Res. **12**, 6741–6749.
34. Lukens, J.H. and Bogorad, L. (1988) Nucl. Acids Res. **16**, 5192.
35. Young, R.A. (1979) J. Biol. Chem. **254**, 12725–12731.
36. Hsu, L.M., Klee, H.J., Zagorski, J., and Fournier, M.J. (1984) J. Bacteriol. **158**, 934–942.
37. Anderson, S., Bankier, A.T., Barrell, B.G., deBruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R., and Young, I.G. (1981) Nature **290**, 457–465.
38. Ravel, J.M. and Shorey, R.L. (1971) Methods in Enzymology **20**, 306–316.
39. Roe, B.K., Marcu, A. and Dudock, B. (1973) Biochim. Biophys. Acta **319**, 259–36.
40. Kuzmin, E.V., Levchenko, I.V. and Zaitseva, G.N. (1988) Nucl. Acids Res. **16**, 4177.
41. Marechal-Drouard, L., Weil, J.H. and Guillemaut, P. (1988) Nucl. Acids Res. **16**, 4777–4788.
42. Mulligan, R.M. and Walbot, V. (1986) Trends in Genet. **2**, 263–266.
43. Fox, T.D. and Leaver, C.J. (1981) Cell **26**, 315–323.
44. Hiesel, R. and Brennicke, A. (1983) EMBO J. **2**, 2173–2178.
45. Lin, J.J., Garber, R.C. and Yoder, O.C. (1988) Nucl. Acids Res. **16**, 9875.

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