A Subset of Conserved tRNA Genes in Plastid DNA of Nongreen Plants

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ABSTRACT

The plastid genome of the nonphotosynthetic parasitic plant *Epifagus virginiana* contains only 17 of the 30 tRNA genes normally found in angiosperm plastid DNA. Although this is insufficient for translation, the genome is functional, so import of cytosolic tRNAs into plastids has been suggested. This raises the question of whether the tRNA genes that remain in *E. virginiana* plastid DNA are active or have just fortuitously escaped deletion. We report the sequences of 20 plastid tRNA loci from *Orobanche minor*, which shares a nonphotosynthetic ancestor with *E. virginiana*. The two species have 9 intact tRNA genes in common, the others being defunct in one or both species. The intron-containing *trn*L_{UAA} gene is absent from *E. virginiana*, but it is intact, transcribed, and spliced in *O. minor*. The shared intact genes are better conserved than intergenic sequences, which indicates that these genes are being maintained by natural selection and, therefore, must be functional. For the most part, the tRNA species conserved in nonphotosynthetic plastids are also those that have never been found to be imported in plant mitochondria, which suggests that the same rules may govern tRNA import in the two organelles. A small photosynthesis gene, *psb*I, is still intact in *O. minor*, and computer simulations show that some small nonessential genes have an appreciable chance of escaping deletion.

THE plastid genomes of photosynthetic angiosperms contain 30 different tRNA loci (Wakasugi *et al.* 1986; Sugiura 1989, 1992). These specify a set of tRNAs that can read all codons using two-out-of-three and wobble mechanisms (Pfitzinger *et al.* 1990). In the nonphotosynthetic holoparasitic angiosperm *Epifagus virginiana* (Orobanchaceae), the plastid genome has been reduced to just 21 protein genes (compared to 79 in tobacco), mostly through the loss of all photosynthetic and chlororespiratory genes (dePamphilis and Palmer 1990; Wolfe *et al.* 1992a,b). The *E. virginiana* plastid DNA (ptDNA) contains only 17 intact tRNA genes (as well as five pseudogenes) and has no tRNA for six amino acids (Wolfe *et al.* 1992a,b). Because several *E. virginiana* plastid genes have been shown to be transcribed (dePamphilis and Palmer 1990; Ems *et al.* 1995) and molecular evolutionary analysis indicates that they are translated (Morden *et al.* 1991; Wolfe *et al.* 1992a; dePamphilis *et al.* 1997; see also analysis of *rps*4 in this study), it has been proposed that some nuclear-encoded tRNAs are imported from the cytosol into plastids to make up for the missing "native" genes (Morden *et al.* 1991). Import of cytosolic tRNAs into organelles is commonplace in plant mitochondria (Dietrich *et al.* 1992, 1996; Maréchal-Drouard *et al.* 1993; Kumar *et al.* 1996), but has not been documented experimentally in plastids. Morden *et al.* (1991) suggested that if such a mechanism exists, it is likely to occur in photosynthetic as well as in nonphotosynthetic species, and they speculated that loss of plastid-encoded tRNA genes might be possible only in plastids where little translation was occurring, *i.e.*, nonphotosynthetic species. A subsequent study (Taylor *et al.* 1991) showed that one tRNA gene (*trn*C_{GCA}) that is a pseudogene in *E. virginiana* is also defunct (completely absent) in a related nonphotosynthetic species (*Conopholis americana*), but is intact in all photosynthetic relatives studied, both hemiparasites and free living.

If tRNAs can be imported into plastids, one wonders why the *E. virginiana* plastid genome retains any tRNA genes at all. A special case can be made for one gene (*trn*E_{UUC}) whose product is required for heme synthesis (Howe and Smith 1991). It is possible that cytoplasmic counterparts cannot substitute some of the other remaining tRNAs for a variety of reasons (Wolfe *et al.* 1992a). Alternatively, they may remain in the ptDNA simply because they have not yet been hit by deletion. Wolfe *et al.* (1992a) argued previously that at least some of the *E. virginiana* plastid tRNA genes were unlikely to have survived by chance alone, given the extensive deletion of photosynthesis genes that has occurred adjacent to them. Also, in several cases, compensatory pairs of substitutions have occurred on either side of stems in *E. virginiana* tRNAs, maintaining Watson-Crick base-pairing and suggesting that they are functional.

In this study, we report the sequences of most of the plastid tRNA loci from a second holoparasitic plant, *Orobanche minor* (Orobanchaceae). Like *E. virginiana*, *O. minor* contains undifferentiated plastids lacking thyla-

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koid stacks (A. J. Lohan, unpublished data). Phylogenetic analysis of plastid *rps*2 showed that the common ancestor of the genera Orobanche and Epifagus was nonphotosynthetic (dePamphilis *et al.* 1997). By comparing DNA sequences between these two species, we show that several tRNA genes have been conserved in sequence, even though the DNA flanking them has diverged, and so must have been maintained by natural selection. This indicates that a subset of the 30 plastid-encoded tRNA genes is active, even in nonphotosynthetic species.

MATERIALS AND METHODS

O. minor var. *compositarum* was found growing on *Senecio greyi* plants on the campus of Trinity College Dublin, and was identified by Dr. Michael Wyse-Jackson. Total cellular DNA was extracted from above-ground tissue by the CTAB method (Doyle and Doyle 1987). Some tRNA loci were isolated as clones from a library of *Sau*3AI partial digestion products in pBluescript and screened with subcloned fragments of *E. virginiana* ptDNA. Others were isolated by polymerase chain reaction (PCR) or long-range PCR using primers designed from known *O. minor* sequences. DNA sequencing was done on an automated sequencer (model 373A; Applied Biosystems, Foster City, CA) with dye-primer and dye-terminator chemistry. RNA was extracted by the protocol described in Ems *et al.* (1995) and reverse transcriptase PCR was done using a kit from Boehringer Mannheim (Indianapolis, IN).

RESULTS

***O. minor* tRNA loci:** A total of 20 loci from *O. minor* were investigated. These are compared to their counterparts in *E. virginiana* (Wolfe *et al.* 1992b) and tobacco (Shinozaki *et al.* 1986) in Figure 1. Fourteen of these genes appear intact in *O. minor*, although this conclusion is tentative for two loci (*trn*R_{UCU} and *trn*D_{GUC}, for which only 55 and 38 bp, respectively, could be analyzed because parts of the genes were used to design PCR primers). In *E. virginiana* (Table 1), 9 of these 14 genes are also intact, 3 are completely absent (*trn*G_{GCC}, *trn*T_{UGU}, and *trn*L_{UAA}), and 2 are pseudogenes (*trn*R_{UCU} and *trn*S_{GGA}). The other six loci studied in *O. minor* are all pseudogenes, whose counterparts in *E. virginiana* are either pseudogenes (*trn*I_{GAU} and *trn*A_{UGC}), absent (*trn*G_{UCC}, *trn*V_{UAC}, and *trn*V_{GAC}), or intact (*trn*R_{ACG})[1]. Four of the *O. minor* pseudogenes contained group II introns. In the case of *trn*V_{UAC} (Figure 1G), the exons are intact and 94–95% identical to tobacco, but the intron has sustained a large deletion extending over domains IV–VI, as well as base substitutions in the exon-binding sites. Domains V and VI are crucial to proper splicing (Michel *et al.* 1989), and their loss undoubtedly prevents the intron functioning.

Expression of *trn*L_{UAA}: The gene encoding $tRNA^{Leu}$-(UAA) is apparently intact in *O. minor* (Figure 1F), and its group I intron can be folded into a normal secondary structure (with only minor length variations in unpaired loop regions, as compared to other species). This gene is absent from *E. virginiana*. Reverse transcriptase PCR showed that this gene is transcribed and the intron is spliced in *O. minor* (Figure 2A). The PCR product was sequenced and contains the expected splice junction (Figure 2, B and C).

Relative conservation of tRNA genes and intergenic spacers: Because *O. minor* and *E. virginiana* are both nonphotosynthetic, as was their common ancestor (dePamphilis *et al.* 1997), all evolutionary divergence between their plastid genomes has taken place in a nonphotosynthetic context. Therefore, when these two species are compared, any genes that show sequence conservation greater than that in noncoding regions can be inferred to have been conserved by natural selection operating in nongreen plastids.

For eight loci that are intact in both *O. minor* and *E. virginiana*, it is possible to compare the level of sequence conservation in a tRNA gene to that in the neighboring noncoding or intronic DNA. At the ninth locus (*trn*F_{GAA}), the pattern of deletion of intergenic DNA leaves no noncoding DNA available for comparison (Figure 1G). At all six usable loci that are located in the large single-copy region of the genome, the tRNA gene is conserved appreciably more strongly (≥15% higher sequence identity) than the flanking DNA; these loci are *trn*Q_{UUG}, *trn*S_{GCU}, *trn*D_{GUC}, *trn*Y_{GUA}, *trn*E_{UUC}, and *trn*fM_{CAU} (Figure 1, A–D). The two intact tRNA genes located in the IR also show conservation, but to a lesser extent (10 and 1% excess conservation for *trn*L_{CAA} and

Figure 1.—Comparisons of 20 *O. minor* plastid tRNA loci and nearby genes with their tobacco (Shinozaki *et al.* 1986) and *E. virginiana* (Wolfe *et al.* 1992b) homologs. (A–G) Genes from the large single-copy region of ptDNA; (H–J) are from the inverted repeat (IR) region. The percentage of DNA sequence identity between homologous coding and noncoding sequence pairs is shown with vertical connecting lines for each section of sequence (genic or intergenic). Solid colored boxes denote coding sequences: yellow, tRNA genes; green, photosynthetic or chlororespiratory genes; brown, genetic apparatus components; reduced-height boxes between exons (E1 and E2) are intronic sequences; V-ended boxes denote incomplete sequences; thick horizontal black lines between genes denote intergenic spacer sequences; thin lines represent major gaps in the DNA sequence alignments. Genes above the central lines are transcribed left-to-right, and those below are right-to-left. Intergenic spacers and tRNA genes are drawn approximately to scale; zig-zag lines in some genes indicate that their size is not to scale. The number of aligned nucleotides used in comparisons is shown for each intergenic region. The length of each gene cluster is shown in base pairs to the left of each panel. Alignments were made using CLUSTAL W (Thompson *et al.* 1994) for each alignable segment and adjusted by eye. Gaps were excluded in calculating percentage sequence identity. The *O. minor* sequences have been submitted to GenBank (accession numbers AJ007719–AJ007729).

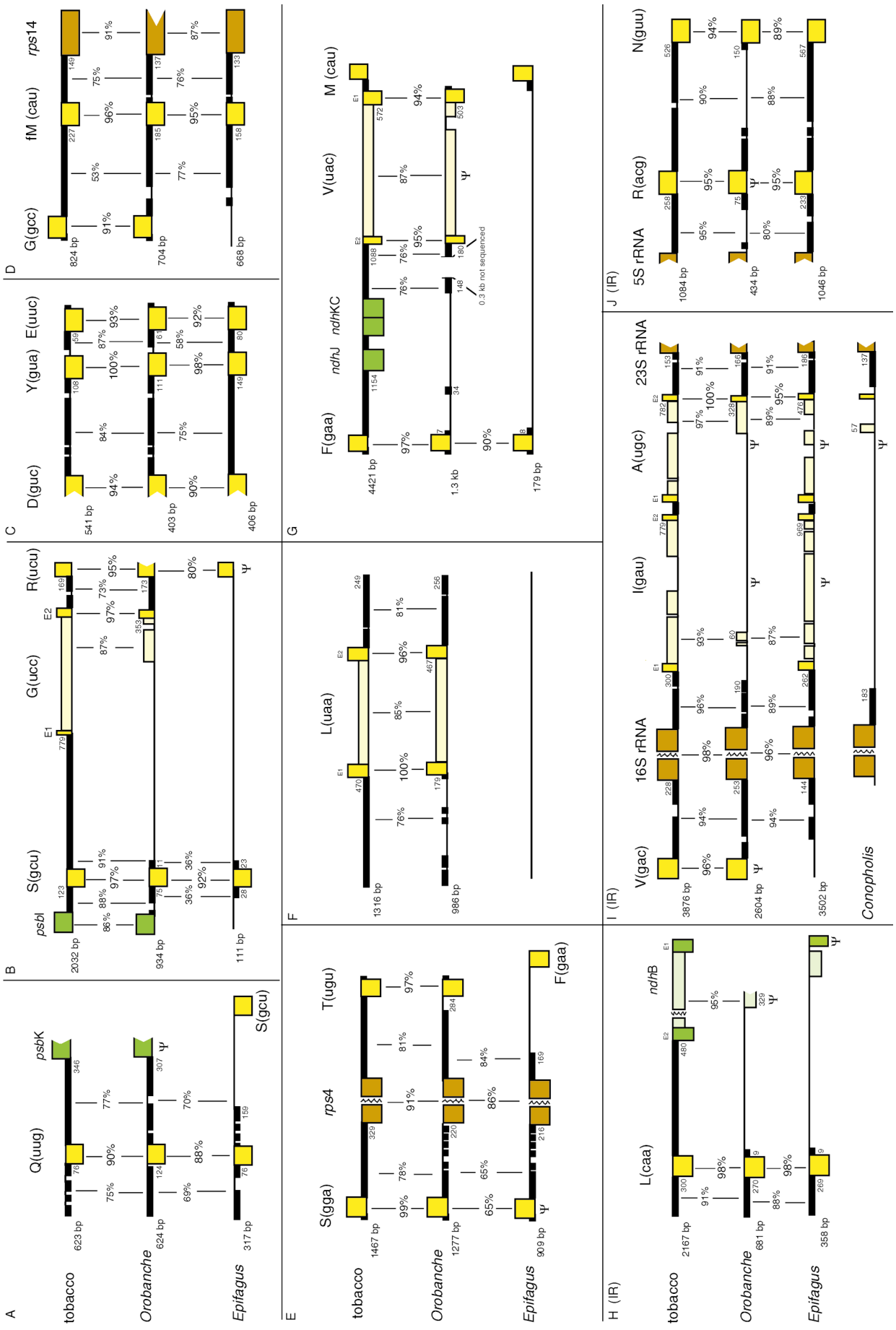

A
tobacco
Orobanche
Epifagus
Q(uug)
psbK
S(gcu)
623 bp
624 bp
317 bp
B
psbI
S(gcu)
G(ucc)
R(ucu)
2032 bp
934 bp
111 bp
C
D(guc)
Y(gua)
E(uuc)
541 bp
403 bp
406 bp
D
G(gcc)
fM (cau)
rps14
824 bp
704 bp
668 bp
E
tobacco
Orobanche
Epifagus
S(gga)
rps4
T(ugu)
F(gaa)
1467 bp
1277 bp
909 bp
F
L(uaa)
1316 bp
986 bp
G
F(gaa)
ndhJ
ndhKC
V(uac)
M (cau)
4421 bp
1.3 kb
179 bp
0.3 kb not sequenced
H (IR)
tobacco
Orobanche
Epifagus
L(caa)
ndhB
2167 bp
681 bp
358 bp
I (IR)
Conopholis
V(gac)
16S rRNA
I(gau)
A(ugc)
23S rRNA
3876 bp
2604 bp
3502 bp
J (IR)
5S rRNA
R(acg)
N(guu)
1084 bp
434 bp
1046 bp

TABLE 1

Status of tRNA genes in ptDNA of parasitic plants

	Orobanchaceae			Convolvulaceae
tRNA	*E. virginiana*	*O. minor*	*C. americana*	*C. reflexa*
Ala (UGC)[a]	ψ	ψ	ψ	+
Arg (ACG)	+	ψ		
Arg (UCU)	ψ	+[b]		
Asn (GUU)	+	+		
Asp (GUC)	+	+[b]	+[b]	
Cys (GCA)	ψ		−	
Gln (UUG)	+	+		
Glu (UUC)[a]	+	+		
Gly (GCC)	−	+		
Gly (UCC)[a]	−	ψ		
His (GUG)	+			+
Ile (CAU)	+			
Ile (GAU)[a]	ψ	ψ	−	+
Leu (CAA)	+	+		+
Leu (UAA)[a]	−	+		
Leu (UAG)	+			
Lys (UUU)[a]	−			−
Met (CAU)	+		+	+
fMet (CAU)	+	+		
Phe (GAA)	+	+	+	
Pro (UGG)	+			
Ser (GCU)	+	+		
Ser (GGA)	ψ	+		
Ser (UGA)	+			
Thr (GGU)	−			
Thr (UGU)	−	+		
Trp (CCA)	+			
Tyr (GUA)	+	+		
Val (GAC)	−	ψ		+
Val (UAC)[a]	−	ψ		ψ

+, gene is intact; −, gene is deleted; ψ, pseudogene. Data sources: *E. virginiana* (Wolfe *et al.* 1992b); *O. minor* (this study); *C. americana* (Taylor *et al.* 1991; Wimpee *et al.* 1992; Colwell 1994); *C. reflexa* (Haberhausen *et al.* 1992; Bömmer *et al.* 1993; Haberhausen and Zetsche 1994; and text).

[a] Gene contains an intron.

[b] Gene is apparently intact, but part of the gene was used to design a PCR primer.

trnN_{GUU}, respectively; Figure 1, H and J). The overall levels of noncoding sequence divergence in the IR are reduced in comparison to the large single-copy region, an observation that concurs with other studies on ptDNA (Wolfe *et al.* 1987). In contrast to the conservation of intact genes, two loci that are pseudogenes in *E. virginiana* (*trn*R_{UCU} and *trn*S_{GGA}; Figure 1, B and E) are more poorly conserved even though they seem intact in *O. minor* (80 and 65% identity, respectively, as compared to ≥88% for genes intact in both species), and *trn*S_{GGA} is no better conserved than its flanking DNA. This implies that these genes are not under selection in at least one of the species; it is not possible to say for certain that they are nonessential in both. The only piece of data that is inconsistent with the general pattern of conservation of intact genes and divergence of noncoding and pseudogene sequences is the greater conservation of ψ*trn*A_{UGC} exon 2 (95%) than its intron (89%; Figure 1I). This exon is short (22 bp compared), and the introns are so badly damaged that the gene cannot be functional in either species, so its conservation is either accidental or possibly connected to processing of the downstream 23S rRNA.

Other genes present in *O. minor* ptDNA: The sequenced regions include several genes in addition to tRNA loci. The *O. minor* plastid 16S rRNA gene appears normal. In phylogenetic trees of angiosperm plastid 16S sequences, the Orobanchaceae were weakly monophyletic (40–50% bootstrap support by different methods) and *E. virginiana* clustered with *C. americana* (98–100% bootstrap support), with *O. minor* outside (results not shown). This is consistent with other phylogenetic analyses (dePamphilis *et al.* 1997; Nickrent *et al.* 1998).

[1] We have followed Wolfe *et al.* (1992a) in designating *E. virginiana* *trn*R_{ACG} as an intact gene although this is questionable. We designated this locus as a pseudogene in *O. minor* on the basis of two mismatches in the TψC stem, and one of these is shared with *E. virginiana.*

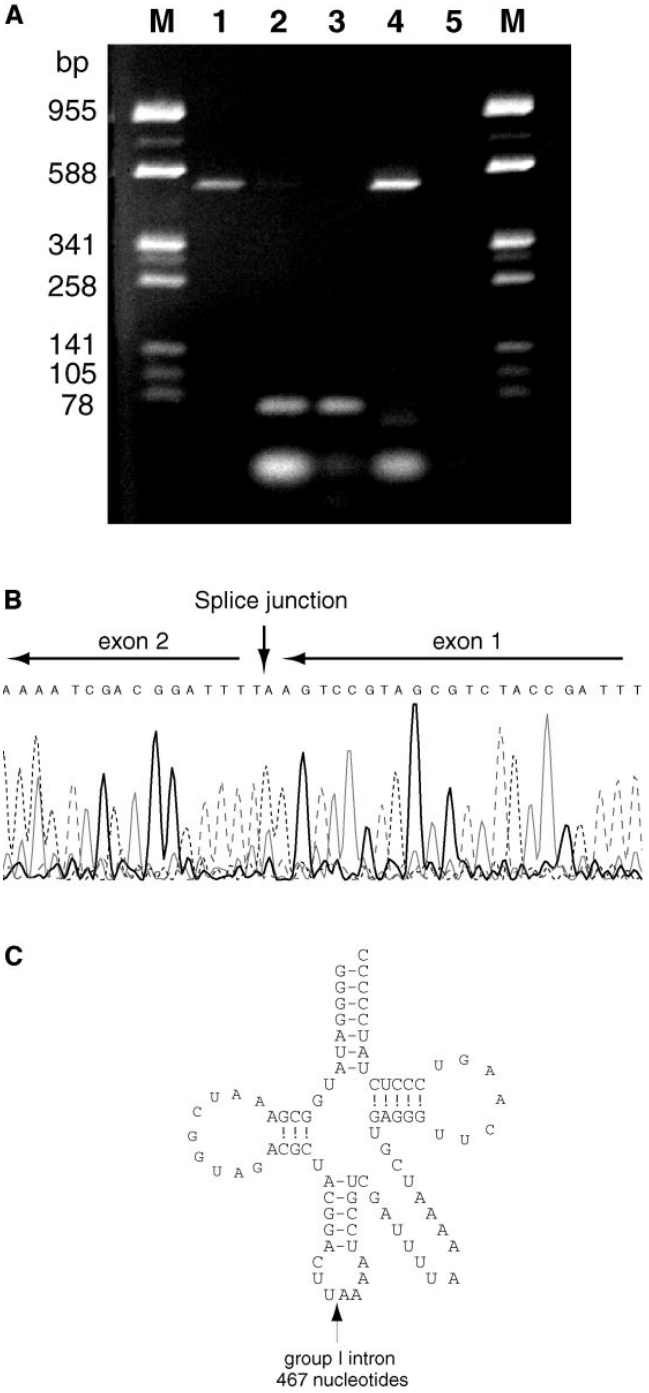


Figure 2.—Transcription and splicing of the group I intron of *trn*L_{UAA} in *O. minor*. (A) Reverse transcriptase PCR analysis. Primers (5′-TGGCGAAATCGGTAGACGCTACGGA-3′ and 5′-GATAGAGGGACTTGAACCCTCACGATTTTTA-3′) flanking the intron were used in PCR amplification of *O. minor* total cellular DNA (lane 1), total RNA (lanes 2–4), or no template (lane 5). *Taq* polymerase was included in all reactions; a previous reverse transcription was carried out for samples in lanes 2–4. Lane 3, contained DNAse; lane 4, contained RNAse. The molecular weight standard (lanes marked M) is pUC19 cut to completion with *Sau*3AI. The size of the spliced *trn*L PCR product is 75 bp and unspliced is 553 bp. (B) DNA sequencing chromatogram of the cloned PCR product showing the splice junction. The sequence is of the strand complementary to the tRNA. (C) Cloverleaf representation of *O. minor* $tRNA^{Leu}$(UAA) showing the intron site.

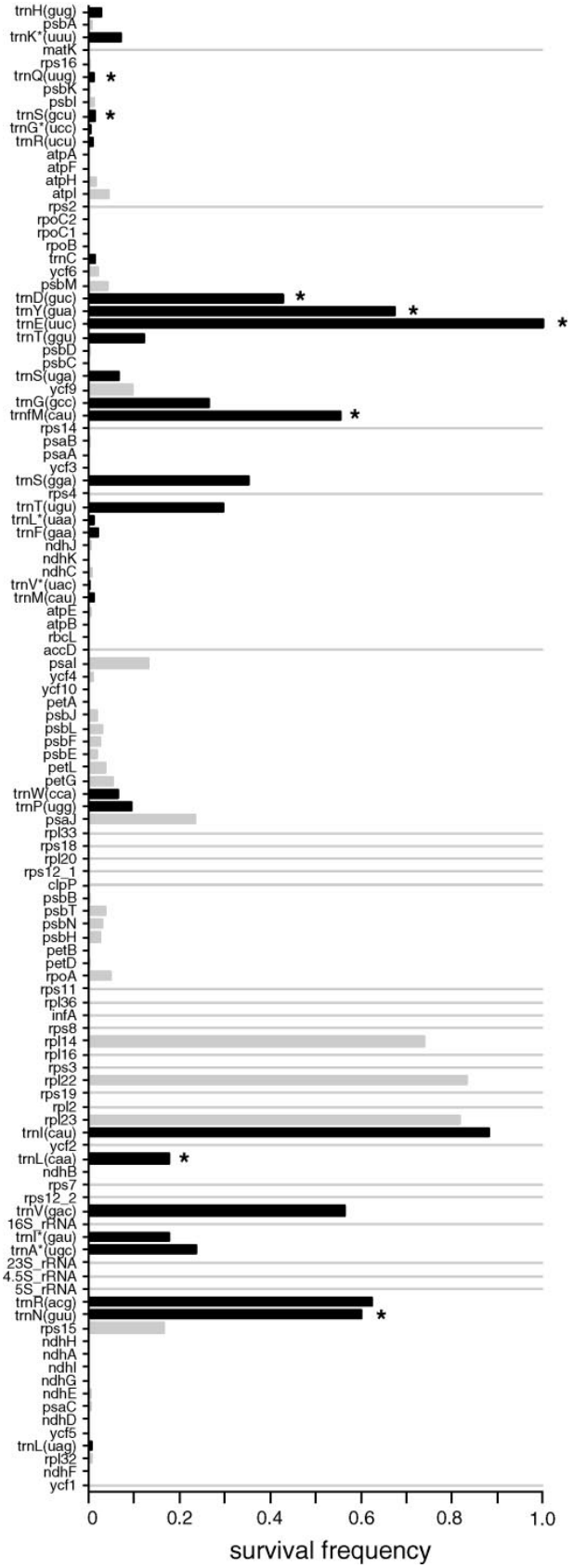
The *O. minor rps*4 gene, encoding ribosomal protein S4, is intact. When compared to its *E. virginiana* homolog, there is an excess of synonymous over nonsynonymous nucleotide substitutions in codons ($K_S = 0.366 \pm 0.067$, $K_A = 0.126 \pm 0.018$; method of Li *et al.* 1985). Because K_S and K_A should be approximately equal if the sequence is a pseudogene, this is strong evidence that the translational apparatus is active in these nonphotosynthetic plastids.

An intact open reading frame was found corresponding to the small (111-bp) gene *psb*I, which codes for the PSII-I polypeptide of photosystem II (a reaction center component; Ikeuchi and Inoue 1988; Sharma *et al.* 1997). This was unexpected in a holoparasite. The gene is located between a pseudogene of *psb*K and *trn*S_{GCU} (Figure 1, A and B). A pseudogene of the chlororespiratory gene *ndh*B was also found (Figure 1H).

Simulation of DNA deletion: The number of DNA deletions in the *E. virginiana* plastid genome, after it became nonphotosynthetic, is estimated to be in the hundreds (Wolfe *et al.* 1992b). Nevertheless, several pseudogenes exist in the ptDNA of *E. virginiana*, *O. minor*, and other nonphotosynthetic plants (Taylor *et al.* 1991; Haberhausen and Zetsche 1994; Delavault *et al.* 1995; Freyer *et al.* 1995; Wolfe and dePamphilis 1997). This suggests that some tRNA genes might still be intact in these genomes, not because they are essential, but simply because they are small and have never been hit by a deletion or other inactivating mutation. The five tRNA genes that are intact in *O. minor* but not in *E. virginiana*, as well as the incongruous *psb*I, could be this sort of *Marie Celeste* gene (lacking a purpose but still afloat).

We explored this by making a computer simulation in which the photosynthetic constraint on tobacco ptDNA (131 kb of unique sequence) was removed. Possible deletion endpoints in the circular genome were chosen at random, and deletions were permitted if they did not damage any essential genes. The genes designated as essential were the protein-coding genes that are present in *E. virginiana*, the ribosomal RNAs, and *trn*E_{UUC} (because of its role in heme synthesis). Deletions were made successively until the genome size fell below that of *E. virginiana* ptDNA (47 kb of unique sequence). Each locus in the genome was then examined to see if it was still intact, and the simulation was repeated 1000 times (Figure 3).

In the simulations, the average number of tRNA genes (including *trn*E_{UUC}) surviving was 7.4 (standard deviation 1.8). None of the 1000 replicates had >13 tRNAs (as compared to 17 in *E. virginiana*). This suggests that about half the plastid tRNA genes in *E. virginiana* are retained by chance and half are maintained by selection; some of those retained by selection have been identified in Figure 1. The frequency of survival varies enormously from gene to gene, ranging from <1% in *trn*L_{UAG}, *trn*G_{UCC}, and *trn*V_{UAC} to >60% in *trn*Y_{GUA}, *trn*I_{CAU}, *trn*R_{ACG},



and *trn*N_{GUU}, which are "sheltered" from deletion by virtue of their proximity to essential genes (Figure 3). The mean frequency of accidental survival for the 16 tRNA genes retained in *E. virginiana* (30.0%; excluding *trn*E_{UUC}) is higher than that in the 13 defunct genes (20.7%). The survival frequency of *psb*I is only 1.2%, but if the flanking tRNA genes (*trn*S_{GCU} and *trn*Q_{UUG}, which are conserved between *O. minor* and *E. virginiana*) are designated as essential, this increases to 68%.

DISCUSSION

Our computer simulations (Figure 3), as well as the discovery that five tRNA genes and one photosynthesis gene are intact in *O. minor* but not in *E. virginiana*, show clearly that some genes can remain undeleted even where they are not essential. However, the sequence comparisons (Figure 1) indicate that at least six, possibly eight, tRNA genes have not only escaped deletion in both parasites, but also have been conserved in sequence in a manner that shows that they are not pseudogenes. One of these, *trn*E_{UUC}, was expected to be conserved because of its role in heme synthesis, but there is no reason why the genes coding for other tRNAs should be conserved in sequence (despite divergence of flanking DNA), unless they are being used in translation.

The plastid tRNA genes of *O. minor* and *E. virginiana* can be classified into three groups (horizontal rows in Figure 4). The first group, those that are conserved in sequence and therefore functional, comprises 6 genes (*trn*E_{UUC}, *trn*fM_{CAU}, *trn*Q_{UUG}, *trn*Y_{GUA}, *trn*D_{GUC}, and *trn*S_{GCU}) plus possibly two others (*trn*N_{GUU} and *trn*L_{CAA}) that are conserved but have low sequence divergence in the adjacent noncoding DNA because of their location in the IR (Figure 1). The second group comprises 14 genes that are not intact in one or both species and are, therefore, nonessential and presumed to be compensated for by imported tRNAs. The third group comprises the remaining 8 genes, which are intact in *E. virginiana*, but there is no data from *O. minor* (or, in the case of *trn*F_{GAA}, there is no alignable flanking DNA).

In plant mitochondria, only a few "native" mitochondrial tRNA genes are present in all species examined

Figure 3.—Summary of computer simulation results. The histogram shows the frequency of survival of each gene, among 1000 replicate simulations in which successive deletions with random endpoints were made in tobacco ptDNA until it reached the size of *E. virginiana*-like ptDNA. Black bars represent tRNA genes; thick gray bars represent other nonessential genes; thin gray bars represent genes designated as essential. All tRNA genes except for *trn*E_{UUC} were designated nonessential. Asterisks mark the eight tRNA genes whose sequences are conserved between *O. minor* and *E. virginiana*. Genes are listed in order around the circular genome. Only one copy of the IR was modeled in these simulations.

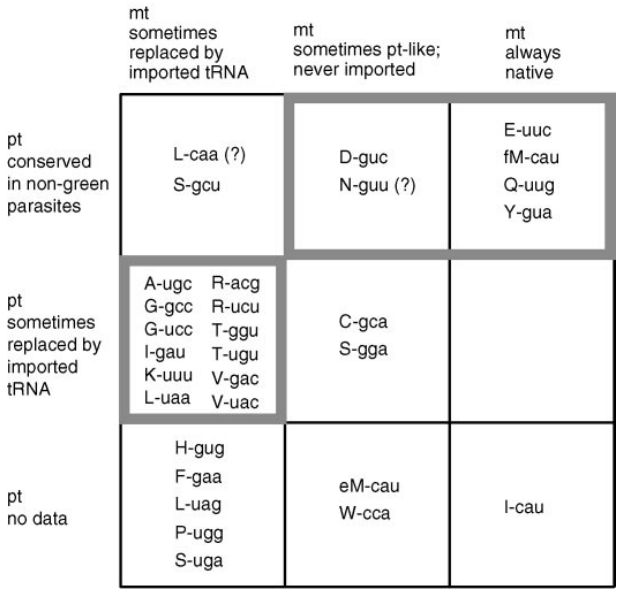


Figure 4.—Summary of the status of 30 tRNA species in plant mitochondria (mt) and nonphotosynthetic plastids (pt). The one-letter amino acid code and anticodon sequences are shown. The highlighted boxes indicate sets of tRNAs that have similar status in the two organelles. The question marks identify two genes located in the IR of ptDNA whose designation as "conserved" in plastids is not certain. Mitochondrial data (Maréchal-Drouard *et al.* 1990; Dietrich *et al.* 1992, 1996; Kumar *et al.* 1996; Veronico *et al.* 1996; Unseld *et al.* 1997) are compiled across all angiosperm species and include cases where import into mitochondria has been inferred by analysis of the complete *Arabidopsis thaliana* mitochondrial genome sequence (Unseld *et al.* 1997; J. Marienfeld, personal communication) rather than by experiment.

(Dietrich *et al.* 1996; Kumar *et al.* 1996; Unseld *et al.* 1997). Other tRNAs are imported from the cytosol or are encoded by "chloroplast-like" genes that are located in the mitochondrial genome. There are intriguing overlaps between the groups of tRNA species that apparently are (and are not) imported in the two organelles (Figure 4). The core set of mitochondrial tRNA genes that are native in all angiosperms studied consists of five genes: *trn*E_{UUC}, *trn*fM_{CAU}, *trn*Q_{UUG}, *trn*Y_{GUA}, and *trn*I_{CAU}. The first four of these are conserved between *O. minor* and *E. virginiana*, and they appear active in parasite plastids; the fifth was not examined in *O. minor*. There is no evidence that these tRNAs are imported into either mitochondria or plastids, and it is conceivable that they are "unimportable." Another conserved plastid tRNA gene, *trn*D_{GUC} (also *trn*N_{GUU} if it is conserved), is among a set of six that are native in some mitochondria and chloroplast-like in others, but never imported (Dietrich *et al.* 1992; Kumar *et al.* 1996; Unseld *et al.* 1997). Twelve tRNA species that are imported in (some) mitochondria have no genes in *E. virginiana* and/or *O. minor* ptDNA (Figure 4). Overall, the mitochondrial and plastid data concur on 18 tRNA species that are apparently either imported in both organelles or not imported in both and differ regarding only four tRNA species (Figure 4). One of these four discrepancies is *trn*L_{CAA}, which may have been wrongly identified as conserved in plastids because of its location in the IR (see results). There is not perfect agreement between the tRNA groupings in the two organelles, but they are sufficiently similar to prompt speculation that the same rules may determine which tRNAs can be imported in both plastids and mitochondria. Kumar *et al.* (1996) have suggested "idiosyncratic" reasons (essentially, differences between prokaryotic and eukaryotic translation systems) why several of the core set of tRNAs (E_{UUC}, fM_{CAU}, Q_{UUG}, and I_{CAU}) might not be importable in mitochondria; precisely the same arguments are valid for plastids.

Loss of tRNA genes from ptDNA has been documented in three holoparasitic species in the family Orobanchaceae (Wimpee *et al.* 1992; Wolfe *et al.* 1992b; this study), but the phenomenon may also occur in two other families of parasitic plants that evolved independently. In *Cuscuta reflexa* (Table 1), a hemiparasite in the family Convolvulaceae, exon 2 of the split tRNA gene *trn*K_{UUU} is not found at its normal location between *mat*K and *psb*A (Bömmer *et al.* 1993; the exon 1 region was not sequenced). Because *mat*K (encoding an intron maturase) has never been found anywhere other than inside the intron of *trn*K_{UUU} in all land plants (except in *E. virginiana*, which lacks this tRNA gene), this suggests that *trn*K_{UUU} may be completely absent from *C. reflexa* ptDNA. Also, the reported sequence of *C. reflexa trn*V_{UAC} (Haberhausen *et al.* 1992) contains a large deletion spanning intron domain V that is remarkably similar to the situation in *O. minor*. This would make *C. reflexa trn*V_{UAC} a pseudogene. A third *C. reflexa* tRNA gene (*trn*I_{CAU}) has been reported to be deleted, but the experimental data are equivocal (Bömmer *et al.* 1993; Downie *et al.* 1994). In *Cytinus ruber*, a holoparasite in the family Cytinaceae, the spacer between the 16S and 23S rRNA genes is only 200 bp and does not contain functional copies of the split tRNA genes *trn*I_{GAU} and *trn*A_{UGC} (Nickrent *et al.* 1997; R. J. Duff and D. L. Nickrent, personal communication). These additional pieces of data support the hypothesis of Morden *et al.* (1991) that import of tRNAs occurs in all plastids, but that only in the peculiar circumstances of parasitic plants does it provide sufficient quantities of tRNA to permit the loss of the native genes.

Why would a plastid require a functioning nucleic acid import system? One possibility is simply that the apparatus for importing tRNAs into mitochondria also operates at low efficiency on plastids. Another is that the mechanisms for importing the (disputed) RNA component of plastid RNAse P (Baum *et al.* 1996) or the 4.5S RNA component of the signal recognition particle (Packer and Howe 1998) also import tRNAs. The reduced selective constraints on organelle tRNAs (Lynch 1997), the reliance of organelle genomes on nuclear encoded RNA polymerase (Hedtke *et al.* 1997), and

the import of genetic apparatus components such as tRNAs are all suggestive of a lowering of the autonomy of organelles. The trend in land plant mitochondria toward lower numbers of native tRNAs (Dietrich *et al.* 1996) and, thus, a greater reliance on the nuclear genome, raises questions about the evolutionary fate of organelles. Nonphotosynthetic plants are excellent tools with which to examine whether an analogous situation exists in plastids when selection constraints are relaxed.

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