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Genes for Two Mitochondrial Ribosomal Proteins in Flowering Plants Are Derived from Their Chloroplast or Cytosolic Counterparts

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Often during flowering plant evolution, ribosomal protein genes have been lost from the mitochondrion and transferred to the nucleus. Here, we show that substitution by a duplicated, divergent gene originally encoding the chloroplast or cytosolic ribosomal protein counterpart accounts for two missing mitochondrial genes in diverse angiosperms. The *rps13* gene is missing from the mitochondrial genome of many rosids, and a transferred copy of this gene is not evident in the nucleus of Arabidopsis, soybean, or cotton. Instead, these rosids contain a divergent nuclear copy of an *rps13* gene of chloroplast origin. The product of this gene from all three rosids was shown to be imported into isolated mitochondria but not into chloroplasts. The *rps8* gene is missing from the mitochondrion and nucleus of all angiosperms examined. A divergent copy of the gene encoding its cytosolic counterpart (*rps15A*) was identified in the nucleus of four angiosperms and one gymnosperm. The product of this gene from Arabidopsis and tomato was imported successfully into mitochondria. We infer that *rps13* was lost from the mitochondrial genome and substituted by a duplicated nuclear gene of chloroplast origin early in rosid evolution, whereas *rps8* loss and substitution by a gene of nuclear/cytosolic origin occurred much earlier, in a common ancestor of angiosperms and gymnosperms.

INTRODUCTION

Mitochondrial genomes are derived from the genome of a bacterial endosymbiont, with many genes having been lost or transferred to the nucleus early in mitochondrial evolution (reviewed by Gray, 1992; Gray et al., 1999). The transfer of mitochondrial genes to the nucleus and functional activation has been an ongoing and frequent process during flowering plant evolution. Several separate transfers of the same gene have been documented (Adams et al., 2000, 2001b). The process of functional gene transfer in angiosperms involves several steps, including reverse transcription of mRNA (usually; the cellular location in which reverse transcription occurs is not known), movement of the nucleic acid to the nucleus, chromosomal integration, gain of a nuclear promoter and other regulatory elements, gain of a mitochondrial targeting sequence (usually), and silencing and loss of the mitochondrial copy (reviewed by Brennicke et al., 1993; Thorsness and Weber, 1996; Martin and Herrmann, 1998; Adams et al., 1999; Palmer et al., 2000). Nonfunctional gene transfers (i.e., transfers that do not result in functional activation of the transferred gene) omit many of these steps and can occur by direct DNA transfers of varying sizes (Knoop and Brennicke, 1994; Blanchard and Schmidt, 1995), including the extraordinary transfer of most of a mitochondrial genome in an ecotype of Arabidopsis (Arabidopsis Genome Initiative, 2000).

Repeated transfer to the nucleus has led to a highly variable distribution of ribosomal protein and succinate dehydrogenase genes among mitochondrial genomes of angiosperms. This variability is highlighted by the complete sequence determination of the mitochondrial genomes of Arabidopsis (Unseld et al., 1997) and sugar beet (Kubo et al., 2000) and by comprehensive DNA gel blot hybridization surveys of mitochondrial gene contents across angiosperms (Adams et al., 2002). For maize, there is complete correspondence between gene loss from the mitochondrion and gene transfer to the nucleus: eight ribosomal protein and succinate dehydrogenase genes that are present in at least one other angiosperm mitochondrial genome have been inferred to have been lost from the maize mitochondrial genome (Adams et al., 2002), and transferred copies of all eight genes have been discovered in the nucleus (Figueroa et al., 1999a; Adams et al., 2000, 2001a, 2001b, 2002). In contrast, the completely sequenced Arabidopsis mitochondrial genome (Unseld et al., 1997) lacks functional copies of nine genes that are present in other angiosperm mitochondria,

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but only seven of these genes have been discovered in the nucleus of Arabidopsis (Wischmann and Schuster, 1995; Sánchez et al., 1996; Perotta et al., 1998; Figueroa et al., 1999b; Adams et al., 2001b; *rps11* expressed sequence tags [ESTs] in the National Center for Biotechnology Information [NCBI] databases), despite its nearly complete sequence. Furthermore, there are two genes (*rps8* and *rpl6*) in the mitochondrial genome of the liverwort *Marchantia polymorpha* that are not present in the mitochondrion of Arabidopsis or any other angiosperm examined. *rpl6* has been identified in the Arabidopsis nucleus (Arabidopsis Genome Initiative, 2000), but a transferred *rps8* gene has not been found.

We have studied the fate of two genes, *rps13* and *rps8*, that are missing from the mitochondrion of diverse angiosperms. Our results indicate that these two genes have been lost entirely from the cell and replaced by duplicated nuclear genes of cytosolic or chloroplastic origin.

RESULTS

Arabidopsis, Cotton, and Legumes Have Lost *rps13* from the Mitochondrion but Contain an *rps13*-Like Gene in the Nucleus

As part of a DNA gel blot hybridization survey of mitochondrial gene losses in 280 angiosperm genera, the *rps13* gene was inferred to have been lost from the mitochondrial genome 30 times among the surveyed angiosperm DNAs (Adams et al., 2002). Most of the losses (24) are in the rosids (one of the major groups of eudicots). These losses encompass 32 of the 69 examined genera of core rosids, including *Gossypium*, *Glycine*, and *Medicago*, and the previously reported loss in Arabidopsis (Unseld et al., 1997). The high concentration of *rps13* gene losses among rosids contrasts with the more uniform distribution of losses across angiosperms of 13 other ribosomal protein genes and of two *sdh* genes (Adams et al., 2002). In Arabidopsis, the missing mitochondrial *rps13* does not appear to have been transferred to the nucleus and instead was hypothesized to have been substituted functionally by a ribonucleoprotein (RNP) domain that was acquired by the transferred *rps19* gene (Sánchez et al., 1996). In contrast, the transferred *rps19* genes in cotton and legumes do not contain the RNP domain (Adams et al., 2002), suggesting either gene transfer to the nucleus or substitution by another protein to account for the missing *rps13*. Although not essential for ribosomal function in bacteria, ribosomal protein S13 nonetheless is necessary for normal translational efficiency and cell growth rate (reviewed by Faxén et al., 1994), indicating that loss of RPS13 function from the cell is unlikely to account for the missing *rps13* in the mitochondria of cotton and legumes.

To identify transferred *rps13* genes in the nucleus of cotton and legumes, tBLASTn searches of the NCBI EST databases were performed. An *rps13* gene of eubacterial origin was identified in cotton, *Medicago trunculata*, and *Lotus japonicus* (see Methods for accession numbers) that was highly divergent from both the mitochondrially located *rps13* in other angiosperms and the nuclear gene for chloroplast RPS13 from Arabidopsis (Kumar et al., 1995), cotton, and soybean. Considering that the last common ancestor of cotton and legumes also was an ancestor of Arabidopsis, we predicted that the Arabidopsis nucleus also might contain a homologous *rps13* gene. Searches of the Arabidopsis genome sequence revealed an annotated 30S *rps13* on chromosome 1 (Theologis et al., 2000). The products of these newly identified *rps13* genes (Figure 1A) were predicted by three mitochondrial protein prediction programs, Mitoprot (Claros and Vincens, 1996), TargetP (Emanuelsson et al., 2000), and Predotar version 0.5 (www.inra.fr/Internet/Produits/Predotar), to be mitochondrial proteins. Mitoprot and TargetP predict that all three gene products contain a cleavable mitochondrial targeting presequence (Predotar does not predict cleavage sites). Furthermore, the predicted presequences are readily alignable (Figure 1B) and hence homologous. These nuclear *rps13* genes for putative mitochondrial proteins are referred to herein as "numit *rps13*" to distinguish them from other *rps13* genes. There are 69 conserved amino acid positions in the numit and mitochondrial RPS13 sequences (Figure 1A), with 17 amino acids being con-

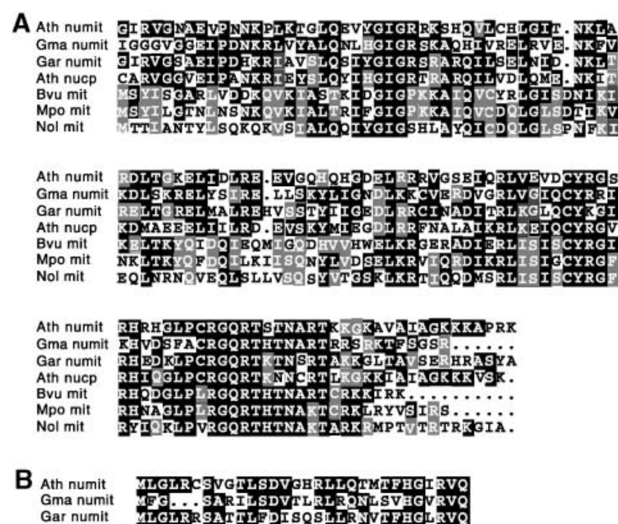

Figure 1. RPS13 Sequence Alignments.

(A) Alignment of RPS13 sequences. Identical amino acids are shown on black and gray backgrounds.

(B) Predicted targeting presequences of numit RPS13 sequences. Identical amino acids are shown in white on a black background. Dots indicate gaps inserted to improve alignment.

Ath, Arabidopsis; Gma, *Glycine max*; Gar, *Gossypium arboreum*; Bvu, *Beta vulgaris*; Mpo, liverwort *Marchantia polymorpha*; Nol, green alga *Nephroselmis olivacea*.

served completely among all six numit and mitochondrial genes.

Numit *rps13* from Arabidopsis contains two introns. The genomic sequence of numit *rps13* from soybean was determined by polymerase chain reaction amplification and sequencing, revealing two introns in the same positions as those in Arabidopsis. Because the *rps13* genes from legumes and Arabidopsis have homologous putative mitochondrial targeting sequences (Figure 1B) and also share two intron positions, they are clearly homologous genes. Surprisingly, though, the numit *rps13* genes are rather dissimilar, with only 36 to 45% amino acid identity to each other. However, mitochondrial RPS13 amino acid sequences from the liverwort Marchantia (Takemura et al., 1992) and the green alga *Prototheca wickerhamii* (Wolff et al., 1994) are only 42% identical, indicating the sequence flexibility of this protein. Thus, it is not at all surprising that numit RPS13A from Arabidopsis, a gene of chloroplast ancestry (see below), is 31% identical to RPS13 synthesized in the mitochondria of *P. wickerhamii*.

To determine if homologs of numit *rps13* are present in other angiosperms, the NCBI EST databases were searched for homologous sequences in tomato and several grasses (maize, rice, barley, wheat, and sorghum). The searches revealed the nucleus-encoded chloroplast *rps13* gene, referred to herein as nucp *rps13*, and the cytosolic counterpart of *rps13* (*rps18*), but no numit *rps13* genes. Considering the large number of ESTs that are available publicly for tomato (>116,000) and the five grasses (>321,000), numit *rps13* may be restricted to rosids.

Numit *rps13* Genes Are Derived from the Nucleus-Encoded Chloroplast *rps13*, and Their Products Are Imported into Mitochondria but Not Chloroplasts

The BLAST similarity scores of the numit *rps13* genes generally were higher in relation to nucp *rps13* genes than to mitochondrial *rps13* genes of other angiosperms. Also, the percent amino acid identities were higher between the numit *rps13* genes and nucp *rps13* from Arabidopsis (43 to 51%) than between the numit *rps13* genes and the angiosperm mitochondrial *rps13* sequences (35 to 37%). To determine if the numit *rps13* genes were derived from a mitochondrial gene transfer to the nucleus or from the nucp *rps13*, phylogenetic analyses were performed with a variety of genes for mitochondrial and chloroplast RPS13 proteins. Analyses were performed using maximum parsimony and maximum likelihood methods, and bootstrapping was performed to assess the support at each node (see Methods for details).

Essentially identical results were obtained with both methods; the likelihood results are shown in Figure 2. The numit *rps13* genes from cotton, Arabidopsis, and legumes all branched together, as expected for orthologous genes, and their sequences have diverged rapidly, as indicated by their long branch lengths compared with the branch lengths of nucp *rps13* genes from a similar set of angiosperms (Figure 2). The numit *rps13* genes from angiosperms branched with the set of angiosperm nucp *rps13* genes, and these two sets of angiosperm genes branched with the *Chlamydomonas* nucp *rps13*. The *rps13* genes still resident in angiosperm mitochondrial genomes (bottom) branched within the larger clade of mitochondrially located *rps13* genes. Together, these results clearly indicate that the numit *rps13* genes of angiosperms are derived via duplication and substitution from the nucp *rps13* rather than from a recent transfer of mitochondrial *rps13* to the nucleus. The predicted targeting sequences of numit *rps13* and nucp *rps13* do not have any sequence similarity.

To determine if the numit *rps13* genes actually encode mitochondrial proteins, and to determine if any sequence is cleaved from the precursor protein upon import, their gene products were tested for the ability to be imported in vitro into isolated mitochondria. When incubated with isolated soybean mitochondria under conditions that support mitochondrial import, each of the RPS13 proteins from cotton, soybean, and Arabidopsis was imported into soybean mitochondria (Figure

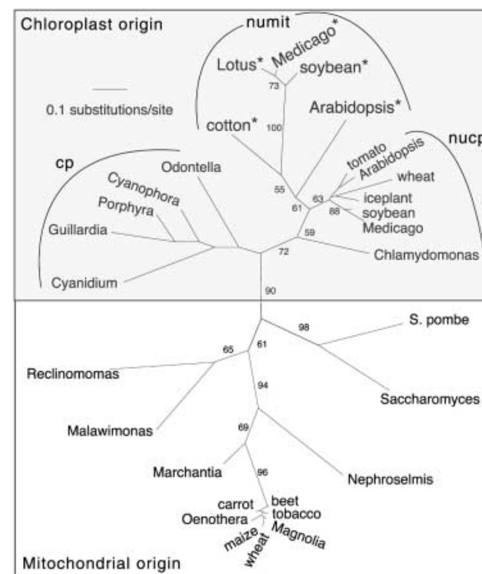

Figure 2. Phylogenetic Analysis of Mitochondrial and Chloroplast *rps13* Genes.

Unrooted phylogram depicting the results of maximum likelihood analyses of the first and second nucleotide positions. The genes for numit *rps13* from Arabidopsis, cotton, and legumes are indicated by asterisks; note the long branch lengths among these sequences compared with those for the nucp *rps13* genes (genes for chloroplast *rps13*, located in the nucleus) from angiosperms. Genes located in the chloroplast genome are indicated by cp. The *rps13* genes from the yeasts are located in the nucleus; other genes in the mitochondrial origin box are in the mitochondrial genome. Numbers indicate bootstrap values. See Methods for details of the analysis.

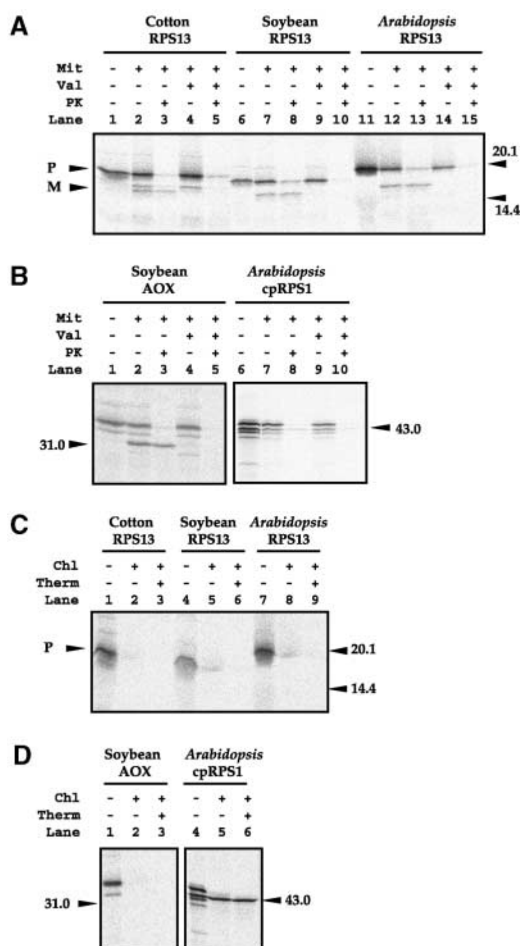

Figure 3. RPS13 Protein Import Experiments.

(A) Import of RPS13 into isolated mitochondria. Lane 1, ^{35}S-labeled precursor protein; lane 2, precursor protein incubated with soybean mitochondria; lane 3, as in lane 2 except that proteinase K was added after mitochondrial incubation to degrade unimported proteins; lanes 4 and 5, as in lanes 2 and 3, respectively, except that valinomycin was added before import to dissipate the membrane potential. Lanes 6 to 10 and 11 to 15 are equivalent to lanes 1 to 5. Markers are given in relative molecular mass (kD). Precursor (P) and mature (M) forms are indicated at left.

(B) Import of the mitochondrial AOX protein (positive control) and the chloroplast protein RPS1 (cpRPS1; negative control) into isolated soybean mitochondria. Lanes are as in **(A)**.

(C) Import of RPS13 into isolated chloroplasts. Lane 1, ^{35}S-labeled RPS13 precursor protein; lane 2, RPS13 precursor protein incubated with pea chloroplasts; lane 3, as in lane 2 except that thermolysin was added after chloroplast incubation to degrade unimported proteins. Lanes 4 to 6 and 7 to 9 are equivalent to lanes 1 to 3.

(D) Import of the mitochondrial protein AOX (negative control) and the chloroplast protein RPS1 (positive control) into isolated pea chloroplasts. Lanes are as in **(C)**.

Chl, chloroplast; Mit, mitochondria; PK, proteinase K; Therm, thermolysin; Val, valinomycin; (+), presence; (−), absence.

3A; note the proteinase K–insensitive bands in lanes 3, 8, and 13) and processed into a smaller form, most likely by cleavage of the mitochondrial targeting presequence. In contrast, when incubated with isolated pea chloroplasts under conditions that support chloroplast protein import, none of the three RPS13 proteins was imported (note that there were no thermolysin-insensitive proteins detected; Figure 3C, lanes 3, 6, and 9).

To examine further the specificity of protein import into mitochondria and chloroplasts using the in vitro import assays, the mitochondrial protein alternative oxidase (AOX) and the chloroplast protein RPS1 were tested in the assays. As expected, there was strong import of AOX into mitochondria (Figure 3B, lane 3) but no import into chloroplasts (Figure 3D, lane 3). Conversely, there was essentially no import of RPS1 into mitochondria (Figure 3B, lane 8) but strong import into chloroplasts (Figure 3D, lane 6). (When chloroplast RPS1 was tested in the mitochondrial import assay, a very weak band was seen in this figure but not in Figure 6B; most likely, this is caused by a process known as bypass import [Pfaller et al., 1989] rather than authentic import, because the intensity of the signal is extremely weak compared with the signal normally seen in the mitochondrial import assay and compared with the amount of RPS1 imported into chloroplasts.) Overall, these results indicate that the RPS13 proteins from cotton, soybean, and Arabidopsis function in the mitochondrion but not in the chloroplast.

A Eubacteria-Like *rps8* Gene Is Missing from the Mitochondrion and Nucleus of Angiosperms

rps8 is present in the mitochondrial genome of the liverwort Marchantia (Oda et al., 1992), and DNA gel blot hybridization indicated that it is present also in the mitochondria of several other bryophytes and lycopods (data not shown). The *rps8* gene has not been discovered in the mitochondrion of any angiosperm; one hypothesis is that *rps8* was transferred to the nucleus before the emergence of angiosperms. However, our searches of the Arabidopsis genome and the NCBI EST databases have not revealed a transferred *rps8* gene of mitochondrial origin in the nucleus of any angiosperm. Considering that the Arabidopsis genome is 92% sequenced, including all of the gene-rich regions (Arabidopsis Genome Initiative, 2000), and that >65,000 EST sequences are available publicly from each of nine flowering plants (soybean, *M. trunculata*, Arabidopsis, tomato, maize, wheat, barley, rice, and sorghum), for a total of >895,000 ESTs, the absence of a detectable transferred *rps8* gene in the NCBI databases suggests that this gene was not transferred to the nucleus in angiosperms.

Instead, RPS8 probably was replaced by another protein. Note that RPS8 is thought to be an essential ribosomal protein in bacteria, because *rps8* mutants cannot assemble the large and small ribosomal subunits (Wower et al., 1992). Might a divergent copy of the nucleus-encoded chloroplast

RPS8 be functioning in the mitochondrion, as appears to be the case for RPS13? The gene for chloroplast RPS8 is located in the chloroplast genome in all of the plants examined, and we found no evidence in any sequence databases for transferred copies of chloroplast *rps8* in the nucleus of angiosperms. If chloroplast RPS8 itself has taken the place of mitochondrial RPS8 in the mitochondrion, then the protein would have to be exported from the chloroplast and imported into the mitochondrion, an unprecedented and highly unlikely scenario.

A Divergent Copy of Cytosolic RPS15A Is Imported into Mitochondria

We next considered the possibility that a copy of the cytosolic counterpart of *rps8*, *rps15A* (also named *rps22* and *rps24* in some eukaryotes), might provide a product to the mitochondrion. Cytosolic *rps15A* has been characterized from Arabidopsis (Bonham-Smith and Moloney, 1994), and we used this sequence as a query to the Arabidopsis genome sequence to identify all *rps15A*-like genes. Two genes with 53 to 54% identity to Arabidopsis cytosolic *rps15A* were discovered on chromosomes 2 and 4 (Figure 4) (Lin et al., 1999; Mayer et al., 1999; see Methods for accession numbers); these genes were identified concurrently as *rps15A* genes (RPS15aB and RPS15aE) by Barakat et al. (2001). The two genes are equal in length and are closely related to

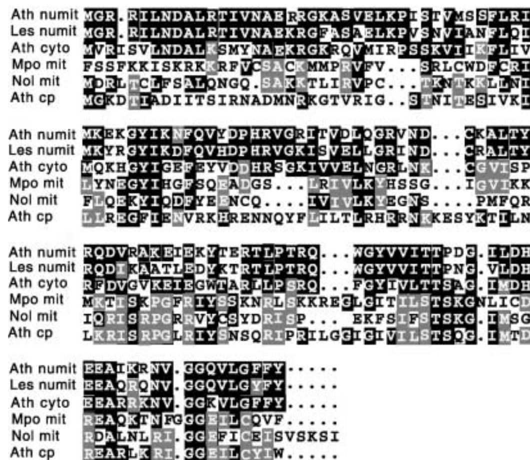

Figure 4. Alignment of RPS15A and RPS8 Sequences.

Identical amino acids are shown in white on a black or gray background. Dots indicate gaps inserted to improve alignment. The N-terminal extension of Marchantia RPS8 is not shown. Ath cp, chloroplast RPS8 from Arabidopsis; Ath cyto, cytosolic RPS15A from Arabidopsis; Ath numit, numit RPS15A from Arabidopsis; Les numit, numit RPS15A from tomato; Mpo mit, mitochondrial RPS8 from Marchantia; Nol mit, mitochondrial RPS8 from *N. olivacea*.

each other (90% amino acid identity), probably reflecting a recent gene duplication; the copy on chromosome 4 was used for further experiments and analyses. Although the product of this *rps15A*-like gene product has no N-terminal extension relative to cytosolic *RPS15A*, it is predicted to be a mitochondrial protein by three prediction programs (Mitoprot, TargetP, and Predotar), with part of the putative mature coding sequence as the targeting domain. Thus, the *rps15A*-like gene, referred to herein as numit *rps15A*, is a good candidate as a replacement for the missing *rps8* gene product in Arabidopsis mitochondria.

To identify homologs of the numit *rps15A* gene in other angiosperms, we used tBLASTn searches of the NCBI EST databases; complete sequences of homologs were identified in tomato (Figure 4), barley, and *M. truncatulata* (see Methods for accession numbers), representing a diversity of angiosperms. Each of the numit RPS15A amino acid sequences is ∼75 to 80% identical to the others. To verify that each of the numit *rps15A* genes is derived from a common ancestor and to assess the amount of divergence among the numit *rps15A* genes compared with each other and compared with the cytosolic *rps15A* genes, phylogenetic analyses were performed using a variety of *rps15A* genes from eukaryotes. The four numit *rps15A* genes branch together in a well-supported clade (Figure 5), and this group branches with the plant cytosolic *rps15A* sequences. The exact placement of the numit *rps15A* genes, as sister to the Marchantia gene, probably is a phylogenetic artifact caused by the short length of the gene and the very long branch leading to the mitochondrial sequences relative to the short-branched cytosolic sequences. This long branch, together with the much longer branches within the clade of angiosperm numit *rps15A* sequences compared with those of the angiosperm cytosolic *rps15A* genes, indicates much higher rates of diversification of the mitochondrial ribosomal proteins than the cytosolic proteins after the gene duplication event that gave rise to these two lineages of genes. We also identified in the NCBI EST databases numit *rps15A* ESTs from *Pinus taeda* (see Methods for accession numbers); although incomplete at the 5′ end, this gene branches with numit *rps15A* genes in phylogenetic trees (data not shown).

The rather low sequence identity of numit *rps15A* to its mitochondrial counterparts in other plants (*rps8*) is to be expected, because it is derived from the counterpart cytosolic ribosomal protein gene (Figure 4). Mitochondrial *rps8* is not highly conserved in general. For example, mitochondrial RPS8 amino acid sequences from Marchantia and the green alga *Nephroselmis olivacea* (i.e., from genes that still reside in the mitochondrion in both organisms) are only 27% identical. Considering that these are both mitochondrially encoded proteins from relatively closely related organisms (land plants having arisen from green algae), it is not surprising that the Marchantia mitochondrial RPS8 would show only 18% identity to a ribosomal protein of far greater evolutionary distance (i.e., numit RPS15A) that is of cytosolic/nuclear

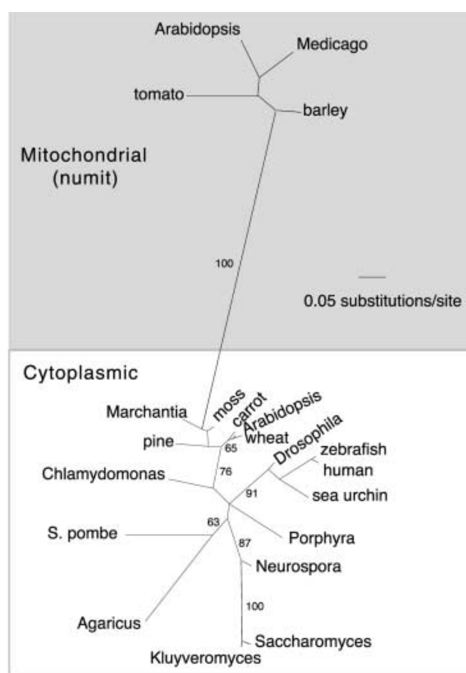

Figure 5. Phylogenetic Analysis of *rps15A* Genes.

Unrooted phylogram of maximum likelihood analyses of the first and second nucleotide positions of an *rps15A* nucleotide alignment. Numbers indicate bootstrap values. See Methods for details of the analyses.

ancestry. Nonetheless, there are 34 conserved amino acids in the numit and mitochondrial genes shown in Figure 4, with 11 being conserved completely.

To determine if the numit *rps15A* genes encode mitochondrial proteins and if they contain a cleavable presequence, numit RPS15A from Arabidopsis and tomato were tested for importability into isolated soybean mitochondria. When incubated with mitochondria under conditions that support protein import into mitochondria, both numit RPS15A proteins clearly were imported into mitochondria, and the imported proteins appear to be the same size as the precursor proteins (Figure 6A, lanes 3 and 8). To determine if targeting was specific for the numit RPS15A but not for the cytosolic RPS15A, the import experiments were repeated with the cytosolic RPS15A from Arabidopsis. When incubated with mitochondria under conditions that support protein import into mitochondria, it was evident that that cytosolic RPS15A did not import into mitochondria (Figure 6, lane 13). Again, the specificity of the in vitro import assay was verified with the mitochondrial protein AOX and the chloroplast protein RPS1. Thus, the numit RPS15A gained mitochondrial targeting ability as a result of sequence divergence from the cytosolic RPS15A.

Expression Patterns of Numit *rps13* and Numit *rps15A* in Arabidopsis

The number of mitochondria per cell increases in anthers in response to the high energy demands of the process of pollen development (Huang et al., 1994). Several studies have shown an increased level of steady state transcripts in floral versus vegetative organs for nuclear genes encoding subunits of respiratory complex I (Grohmann et al., 1996; Heiser et al., 1996; Zabaleta et al., 1998), complex II (Figueroa et al., 2001), complex III (Huang et al., 1994), and cytochrome *c* (Felitti et al., 1997), along with a few metabolic enzymes (reviewed by Mackenzie and McIntosh, 1999). Therefore, increased expression in flowers appears to be a hallmark of nuclear genes for mitochondrial respiratory and metabolic proteins. In contrast, much less is known about the tissue-specific expression patterns of genes for mitochondrial ribosomal proteins; to our knowledge, only one such study has been reported. Handa et al. (2001) found comparable levels of *rpl11* transcripts in flowers, shoots, and roots of wheat, but the transcript levels in flowers and shoots decreased upon cold exposure.

To determine if steady state transcript levels of numit *rps13* and numit *rps15A* are increased in flowers of Arabidopsis relative to other organs, we performed RNA gel blot hybridizations using probes for these genes along with the

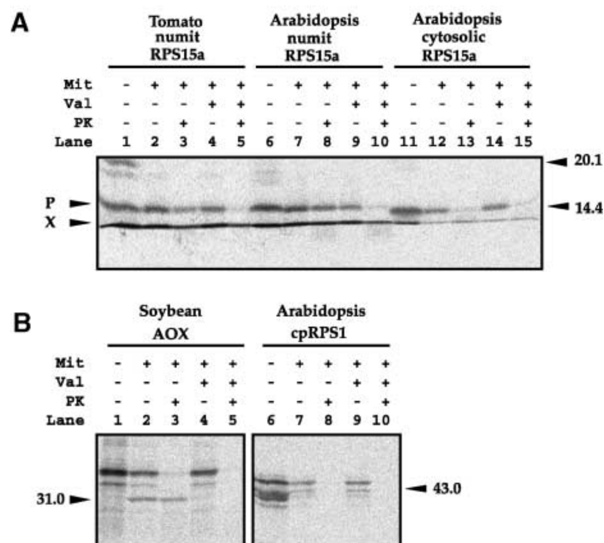

Figure 6. Import of RPS15A into Mitochondria.

(A) Import of RPS15A into soybean mitochondria. Lanes are the same as in Figure 3A. X denotes the ion front of the gel.

(B) Import of the mitochondrial protein AOX and the chloroplast protein RPS1 (cpRPS1) into isolated soybean mitochondria. Lanes 1 to 5 and 6 to 10 are equivalent to lanes 1 to 5 of **(A)**, except that the AOX and cpRPS1 precursor proteins were used.

Mit, mitochondria; P, precursor; PK, proteinase K; Val, valinomycin.

recently transferred nuclear genes for mitochondrial *rps10* (Wischmann and Schuster, 1995) and 3′ *rpl2* (Adams et al., 2001a). Figure 7 shows that, for all four ribosomal protein genes tested, transcript accumulation is comparable in whole flowers, leaves, and stems. (Note that the flower lanes are underloaded, as indicated by comparison with the rRNA bands on the ethidium bromide–stained gels.) These data and those of Handa et al. (2001) suggest that steady state transcript levels of genes for mitochondrial ribosomal proteins are not increased in flowers, in contrast to genes for other mitochondrial proteins.

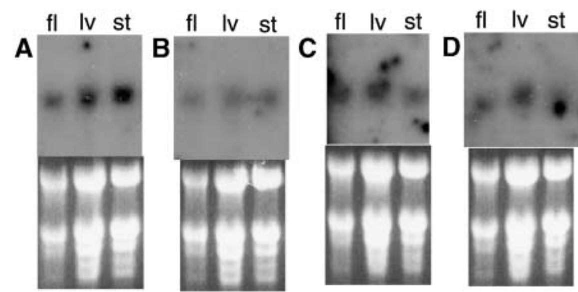

Figure 7. Expression Patterns of Four Nuclear Genes for Mitochondrial Ribosomal Proteins.

(A) 3′ *rpl2*.
(B) *rps10*.
(C) Numit *rps15A*.
(D) Numit *rps13*.
Top panels show hybridizations, and bottom panels show the corresponding ethidium bromide–stained gels with prominent rRNA bands. The *rps10* and 3′ *rpl2* transcripts are ∼1.8 kb, the *rps15A* transcripts are ∼1.0 kb, and the *rps13* transcripts have an approximate range of 900 to 1000 bp (with smaller transcripts present in flowers). fl, whole flowers; lv, rosette leaves; st, stems.

DISCUSSION

Substitution of Two Mitochondrial Ribosomal Proteins by Highly Derived Copies of Their Chloroplast or Cytosolic Counterparts

The results of this study indicate that a divergent copy of the cytosolic *rps15A* gene, termed numit *rps15A*, is present in the nucleus of diverse angiosperms and a gymnosperm and that the gene products from tomato and Arabidopsis are imported into mitochondria. We also have shown that a divergent copy of the chloroplast-derived *rps13* gene (numit *rps13*) is present in the nucleus of cotton, Arabidopsis, and legumes and that its product is imported into mitochondria but not chloroplasts.

Three lines of evidence indicate that the gene products of numit *rps13* and *rps15A* are mitochondrial ribosomal proteins. First, *rps13* and *rps8* (the organellar homolog of *rps15A*) clearly are absent from the mitochondrial genomes of the plants in question, and recently transferred copies are absent from the Arabidopsis genome and from the NCBI EST databases containing extensive cotton and legume ESTs. Second, feasible alternative ribosomal proteins are encoded by nuclear genes (numit *rps13* and numit *rps15A*), and their products are predicted to be mitochondrial proteins by all three mitochondrial protein prediction programs used. Third, the products of numit *rps13* and numit *rps15A* were shown to be imported by isolated plant mitochondria. Numit RPS13 was not imported by chloroplasts, and cytosolic RPS15A was not imported by mitochondria; thus, numit *rps13* and *rps15A* have evolved specific signals for mitochondrial targeting. Most likely, therefore, numit RPS13 and RPS15A have partially or completely replaced the function of the missing ribosomal proteins RPS8 and RPS13 of mitochondrial origin. To convincingly prove that numit RPS13 and RPS15A function in mitochondrial ribosomes in the plants in question would require a substantial undertaking, one that is well beyond the scope of the current study. Immunoblot analyses of mitochondrial ribosomes would not suffice, because homologous ribosomal proteins of different compartmental origins will sometimes cross-react. Instead, it probably would be necessary to purify and microsequence the candidate proteins from mitochondrial ribosomes.

A model of the numit *rps15A* gene substitution process is shown in Figure 8A. The region used to target the protein to the mitochondria probably is within the mature coding region, given the apparent lack of processing of the protein upon import into mitochondria. A small number of mitochondrial proteins in plants have been shown to be targeted and imported without a mitochondrial targeting presequence (Adams et al., 2000, and references therein). The numit *rps15A* gene has diverged considerably from the very highly conserved cytosolic *rps15A* genes (Figure 5), consistent with functional divergence in the new ribosomal environment. This gene substitution event is accompanied by, and presumably led to, loss of the *rps8* gene from the mitochondrion. This seldom-used pathway of mitochondrial gene content evolution is in contrast to the many examples of mitochondrial gene transfer to the nucleus that seem to account for most mitochondrial gene losses (see Introduction).

A model for the complex history of the organellar *rps13* genes is shown in Figure 8B. Chloroplast *rps13* was transferred to the nucleus (Kumar et al., 1995), and this nuclear gene was duplicated, followed by sequence and functional divergence of one copy to allow replacement of the mitochondrial *rps13*. Numit *rps13* has a mitochondrial targeting sequence that has no similarity to the targeting sequence of nucp *rps13*. Thus, either there has been extensive sequence divergence in the numit *rps13* presequence, even more than for the mature coding region (Figure 2), so that the sequence is unrecognizable, or the numit *rps13* gene acquired

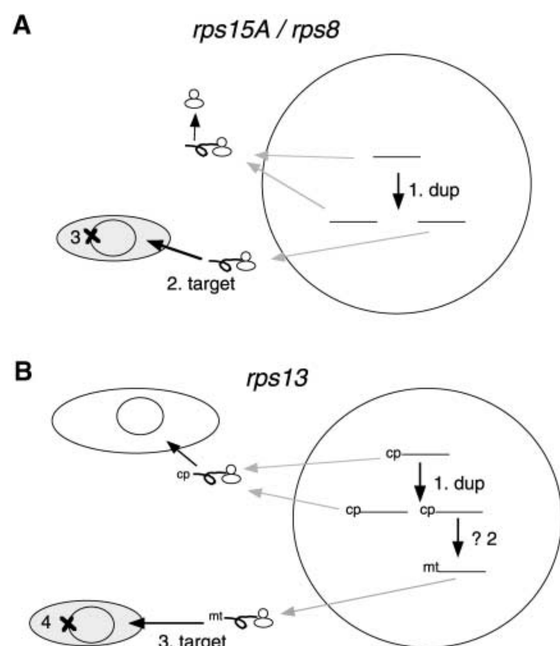

Figure 8. Models of Gene Substitution Events in the Evolution of Mitochondrial Ribosomal Proteins.

Large circles indicate nuclei, gray ovals indicate mitochondria, and white ovals indicate chloroplasts. Black arrows indicate gene duplication (dup) or protein targeting to mitochondria (target); gray arrows indicate movement of gene transcripts to the cytosol. Bars indicate the mature coding regions of genes; targeting presequences are indicated by cp (chloroplast) and mt (mitochondrial). Proteins are indicated by loops attached to the ribosomes. Gene deletion (or pseudogene formation) is indicated by ×.

(A) *rps8*/*rps15A* substitution. Step 1, cytosolic *rps15A* duplication; step 2, targeting of the duplicate to mitochondria; step 3, loss of *rps8* from mitochondrial DNA.

(B) *rps13* substitution. Step 1, duplication of nucp *rps13*; step 2, possible acquisition of mitochondrial targeting presequence; step 3, targeting of the duplicate to mitochondria; step 4, loss of *rps13* from mitochondrial DNA.

a mitochondrial presequence that replaced the original chloroplast presequence. Eventually, the mitochondrial *rps13* gene was lost multiple times in different descendant lineages (Figure 8B). Numit RPS13 represents an example of the substitution of a chloroplast-derived ribosomal protein for a mitochondrial ribosomal protein and illustrates another pathway by which the nuclear genome can usurp mitochondrial genes.

Our results differ from, but in a sense are consistent with, those of two previous studies on *rps13*. A maize mitochondrial RPS13 antibody and a nucp RPS13 antibody used by Kumar et al. (1995) and Sánchez et al. (1996), respectively, did not react with Arabidopsis mitochondrial ribosomal proteins, leading these authors to hypothesize that a eubacteria-like ribosomal protein, S13, is absent from Arabidopsis mitochondria. The extensive sequence divergence of the numit *rps13* from both the nucp *rps13* and the mitochondrial *rps13* of other angiosperms (Figure 2) might explain why neither antibody recognized the numit *rps13* gene product reported here. The mitochondrial RPS13 antibody did react with mitochondrial ribosomal proteins from bean (Kumar et al., 1995), and the nucp RPS13 antibody reacted with mitochondrial ribosomal proteins from pea (Sánchez et al., 1996), leading the authors to hypothesize that the gene was transferred to the nucleus or that the nucp RPS13 might have replaced the mitochondrial RPS13 in pea. Sánchez et al. (1996) proposed that the supposedly missing eubacteria-derived RPS13 protein in Arabidopsis mitochondria was functionally replaced by the RNP domain on the transferred RPS19. In light of the identification of numit *rps13* in Arabidopsis, one must reconsider this proposal. It now seems entirely possible, perhaps even likely, that this RNP domain carries no RPS13 function. Alternatively, the function of RPS13 in Arabidopsis mitochondria might have been partitioned between the numit RPS13 and the RNP domain of RPS19 after the divergence of Arabidopsis and cotton from a common ancestor.

Evolutionary Timing of the Gene Substitutions

The losses of *rps13* from angiosperm mitochondria are highly clustered in core rosids (Adams et al., 2002), and numit *rps13* genes have been found from three diverse groups of core rosids (cotton, legumes, and Arabidopsis), the common ancestor of which is at the base of core rosids (Soltis et al., 1999, 2000). Conversely, there have been very few losses of *rps13* from the mitochondria of nonrosid angiosperms, and numit *rps13* genes have not been found in any nonrosid species (including tomato and the grasses, with many EST sequences available; see Results). From these observations, we infer that the gene substitution event occurred more or less at the base of the core rosid lineage.

The results of *rps13* phylogenetic analyses (Figure 2) provide little insight into the timing of the gene substitution, probably because *rps13* is a short gene and has a small number of informative characteristics. The chloroplast *rps13* sequences from angiosperms do not branch according to their expected phylogenetic relationships. For example, Arabidopsis and legumes (both rosids) do not branch together but instead are interspersed with other eudicots and even monocots. Thus, it is completely unsurprising that the divergent numit *rps13* genes from rosids do not branch as sister to a clade of rosid nucp *rps13* sequences, as would be expected for a gene substitution at the base of core rosids.

rps13 has been sequenced from the mitochondrial genomes of several nonrosid angiosperms, including maize, wheat, *Magnolia*, carrot, tobacco, and beet. Interestingly, an intact, transcribed, and RNA-edited *rps13* gene also is present in the mitochondrial genome of the rosid *Oenothera*

(Wissinger et al., 1990). It is possible, therefore, that *Oenothera* mitochondria might contain RPS13 proteins produced by both nuclear (numit *rps13*) and mitochondrial *rps13* genes. Alternatively, numit *rps13* might have been lost or become a pseudogene in *Oenothera*, or the transcribed and edited mitochondrial *rps13* mRNA might not be translated.

rps8 has not been found in the mitochondria of any angiosperm, but it is present in the mitochondrial genome of the liverwort Marchantia (Oda et al., 1992). DNA gel blot hybridizations of ~30 nonseed plants using a Marchantia *rps8* probe revealed good hybridization to the DNAs of many liverworts, mosses, and lycopods (data not shown), suggesting the widespread presence of mitochondrial *rps8* in these basal land plant lineages. The substitution of *rps8* by the numit *rps15A* occurred minimally in a common ancestor of angiosperms and gymnosperms, as shown by the presence of an orthologous numit *rps15A* gene in both groups. Together, these observations indicate that the gene substitution occurred after the divergence of lycopods (the first branch of vascular plant evolution) and before the diversification of angiosperms and gymnosperms from a common ancestor. (The *rps15A* phylogenetic tree [Figure 5] shows little meaningful resolution among the land plant *rps15A* sequences and thus is uninformative regarding the timing of gene substitution.)

Gene Substitution in the Evolution of Organellar Proteins

As with the gene losses from mitochondria, most investigated chloroplast gene losses in plants also appear to reflect gene transfer to the nucleus (Gantt et al., 1991; Martin et al., 1998; Millen et al., 2001). We are aware of only two described cases of gene substitution in the evolution of chloroplast ribosomal proteins in land plants. A long-standing controversy regarding the origin of the angiosperm nuclear gene for chloroplast RPL21 (Smooker et al., 1990; Martin et al., 1990) was settled recently by Gallois et al. (2001), who showed that this gene was derived by substitution from a nuclear *rpl21* gene of mitochondrial origin. The chloroplast-derived mitochondrial RPS13 (numit *rps13*) and the mitochondrially derived chloroplast RPL21 essentially represent the same type of substitution but in opposite directions: a chloroplast protein for a mitochondrial protein in one case, and a mitochondrial protein for a chloroplast protein in the other.

The second case of chloroplast gene substitution parallels the cytosol-to-mitochondria substitution described in this study for *rps8* and *rps15A*. Spinach chloroplast DNA contains only a pseudogene for *rpl23*, and the nuclear gene that supplies the spinach chloroplast ribosome with RPL23 evidently is derived by substitution from a duplicated copy of cytosolic *rpl23* (Bubunenko et al., 1994). (Although there was biochemical evidence for this conclusion, no cytosolic *rpl23* gene had been isolated from plants at the time. The sequence of cytosolic *rpl23* from spinach has since been determined [X92367], and homologous sequences from Arabidopsis and other angiosperms also are available in the NCBI sequence databases. The nucleus-encoded chloroplast *rpl23* from angiosperms is much more similar to the angiosperm cytosolic *rpl23* [~51%] than to the angiosperm chloroplast-encoded *rpl23* [~20%]. Thus, the inference of Bubunenko et al. [1994] about the origin of the spinach chloroplast *rpl23* from the mitochondrial *rpl23* seems to be correct.) An interesting difference between the *rps15A* and *rpl23* substitutions is that *rpl23* gained an N-terminal chloroplast targeting presequence but *rps15A* did not gain a mitochondrial presequence and relies on an internal signal that has evolved as a result of sequence divergence. These four cases of substituted organellar ribosomal protein genes in land plants provide clear and dramatic examples of the gain of new function by one gene copy after gene duplication, one of the major possible fates of duplicated genes (Lynch and Conery, 2000, and references therein).

Although gene substitution appears to be relatively rare in the evolution of organellar ribosomal proteins in plants, and gene transfer appears to be the predominant mode, in the case of metabolic and other enzymes, gene substitution is rather common (reviewed by Martin and Schnarrenberger, 1997; Small et al., 1998). To a significant extent, this probably reflects differences in the degree to which the different classes of proteins are coadapted to interact over large portions of their length with other molecules. Ribosomal proteins often interact with multiple other ribosomal proteins and the rRNA within the ribosome, and it may be relatively difficult for a ribosomal protein that is adapted to the ribosome of the chloroplast or cytosol to substitute effectively for a missing mitochondrial ribosomal protein. In contrast, many metabolic enzymes interact with the same few, relatively simple substrates and cofactors regardless of their disparate evolutionary histories, so functional substitution may be achieved more easily. Fundamentally, this is the same argument—the "complexity hypothesis" of Jain et al. (1999)—used to explain the observation that lateral gene transfer is much more common in the evolution of metabolic enzymes in bacteria than for components of the genetic apparatus.

The considerable divergence of the substituted copies of *rps13* and *rps15A* relative to those that still function in their original compartments (Figures 2 and 5) may be related to the apparent difficulty of substitution of ribosomal proteins. That is, it may reflect selection for a highly altered form of the protein, one that functions better in its new ribosome. In contrast, in a few cases, a single nuclear gene (of either chloroplast or mitochondrial origin) encodes a tRNA synthetase (Akashi et al., 1998; Menand et al., 1998; Small et al., 1998) or a metabolic enzyme (Creissen et al., 1995) that is targeted to and functions in both the chloroplast and the mitochondrion, highlighting the ready interchangeability of these enzymes.

Further indication of the interchangeability of some metabolic enzymes and tRNA synthetases is shown by cytosolic proteins that are derived from their organellar counterparts (e.g., Flechner et al., 1996; Mireau et al., 1996; Peeters et

al., 2000; Krepinsky et al., 2001; reviewed by Martin and Schnarrenberger, 1997). Perhaps the most intriguing cases of gene substitution in organellar evolution involve the single-subunit, phage-type RNA polymerase (nucleus encoded but of uncertain origin) that has completely or partly substituted for the multisubunit eubacteria-type polymerase that was encoded originally by the mitochondrial or the chloroplast genome, respectively (Hedtke et al., 1997, 2000; Gray and Lang, 1998; Cahoon and Stern, 2001). These cases are intriguing both because they involve an enzyme (RNA polymerase) that interacts with so many other players (i.e., promoters) and because they include at least one case of dual targeting of a single enzyme to both organelles.

METHODS

Gene Amplification, Cloning, and Sequencing

A soybean (*Glycine max*) mitochondrial *rps13* cDNA clone (BG047149) was purchased from Incyte Genomics (Palo Alto, CA) and sequenced fully using M13 forward and reverse primers, along with the internal forward primer 5′-AGATGTGGGAAGGTTGGTGG-3′. The genomic sequence of soybean mitochondrial *rps13* was isolated by polymerase chain reaction (PCR) using primers 5′-CAACTCCACCATGTTCGGTTC-3′ and 5′-TCACATCATCATAGTTTTCCGGCTCC-3′.

To construct cDNA clones for protein import experiments, complete cDNAs were isolated by reverse transcriptase–mediated RT-PCR (Adams et al., 1999), and additional Met codons were added to the 3′ end of each cDNA using the following primers: *Arabidopsis thaliana* *rps13* (F1, 5′-ATGGGTATCTCTCGTGATTCA-3′; R2, 5′-TTCGGGGAGCGTACTACTACTACACT-3′; and R3, 5′-TCACATCATCATCATCATCATCATCATG-3′), cotton (*Gossypium arboreum*) *rps13* (F1, 5′-TCAGCGTCTAAGAATGTTGGG-3′; R1, 5′-TCACATCATCATCATAGCATAAGAAGC-3′; and R2, 5′-TCACATCATCATCATCATCATCATAGCA-3′), soybean *rps13* (F1, 5′-CAACTCCACCATGTTCGGTTC-3′; R1, 5′-TCACATCATCATAGTTTTCCGGCTCC-3′; and R2, 5′-TCACATCATCATCATCATCATCATCATCATAGT-3′), tomato (*Lycopersicon esculentum*) *rps15A* (F1, 5′-AATGGGGAGGAGAATATTGAAC-3′; R1, 5′-TCACATATAAAAGTAGCCAAGAAC-3′; and R2, 5′-TCACATCATCATCATCATCATATAAAAGT-3′), Arabidopsis mitochondrial *rps15A* (F1, 5′-ATGGGGAGGAGGATTTTGAAC-3′; R1, 5′-TCACATCATGTAAAAGAAGCCAAGAAC-3′; and R2, 5′-TCACATCATCATCATCATGTAAAAGAAG-3′), and Arabidopsis cytosolic *rps15A* (F1, 5′-AAGGCAAGATGGTAAGAATCAG-3′; and R1, 5′-TCACATATAGAAGAAGCCGAGAACCT-3′).

All RT-PCR products were cloned into vector pCR2.1 TOPO (Invitrogen, Carlsbad, CA), and some inserts were subcloned into pBluescript KS− (Stratagene). All clones were sequenced using an ABI 3700 DNA sequencer (Foster City, CA) to verify the orientation of the insert relative to the T7 site and to detect Taq polymerase misincorporation errors.

Sequence and Phylogenetic Analyses

Sequence alignments were made using the xPileUp program from the Genetics Computer Group (Madison, WI) and refined by eye. Pairwise distance comparisons were performed using PAUP* version 4.0b8 (Swofford, 2001) and represent uncorrected pairwise distances.

Parsimony analyses were performed with PAUP* version 4.0b8 (Swofford, 2001) using heuristic searches with random taxon addition (10 replicates) and tree bisection reconnection (TBR) branch swapping. Bootstrapping was performed as described above, with stepwise addition and 100 replicates. Maximum likelihood analyses were conducted with PAUP* version 4.0b8. The HKY85 model was used, assuming a discrete gamma distribution with four categories of site-to-site rate variability. For each analysis, the transition/transversion ratio and base frequencies were estimated using Tree-PUZZLE version 4.02 (Strimmer and von Haeseler, 1996) under the HKY model of evolution with gamma-distributed rates and parameter estimation set to “approximate.” Analyses used heuristic searches with random taxon addition (10 replicates) and TBR branch swapping. Bootstrapping was performed using PAUP* version 4.0b8 as described above, with stepwise addition and 100 replicates.

RNA Isolation and RNA Gel Blot Hybridization

Total RNA was isolated from Arabidopsis ecotype Columbia using the Qiagen Plant RNAeasy kit (Valencia, CA) according to the manufacturer’s instructions. The following tissues and organs were used: whole flowers at various stages, rosette leaves, and stems. Approximately 10 μg of total RNA per lane was electrophoresed, blotted, and hybridized with ^{32}P-labeled probes as described by Adams et al. (1999).

Organelle Isolation and in Vitro Import Assays

Mitochondria were prepared from 7-day-old soybean cotyledons (cv Stevens) as described by Day et al. (1985). ^{35}S-labeled precursor proteins were synthesized from cDNA clones as described by Whelan et al. (1995). In vitro import assays were performed as described by Whelan et al. (1996). Proteins were separated by 16% SDS-PAGE, and gels were dried and exposed to a BAS TR2040S plate for 24 hr. Detection was performed on a BAS 2500 phosphorimager according to the manufacturer’s instructions (Fuji, Tokyo, Japan).

Chloroplasts were prepared from 10-day-old pea leaves (*Pisum sativum* cv Greenfeast) as described by Bruce et al. (1994). In vitro import assays were performed as described by Bruce et al. (1994) and Waegemann and Soll (1995). Protein separation and detection were performed as for mitochondrial in vitro import assays.

In some cases, smaller precursor proteins were denatured before in vitro import into mitochondria or chloroplasts. ^{35}S-labeled precursor proteins were centrifuged at 128,000*g* for 15 min at 4°C. The supernatant was precipitated by the addition of 4 volumes of water-saturated $(NH_4)_2SO_4$, incubated on ice for 30 min, and centrifuged at 14,000*g* for 15 min at 4°C. The protein pellet containing the ^{35}S-labeled precursor protein was resuspended in half of its original volume with 8 M urea, incubated on ice for 30 min, and then diluted with an equal volume of 20 mM Hepes, pH 7.7.

Accession Numbers

The soybean mitochondrial *rps13* genomic sequence has been assigned GenBank accession number AY044157. Accession numbers for EST and Arabidopsis genomic sequences used in this study are

as follows. Numit *rps13*: Arabidopsis (AC012193, AV440855, AV442527, and AV552576), cotton (BF272717), soybean cDNAs (BG047149 and BG237013), *M. truncatula* (BG449364, AL388944, and AL388196), and *L. japonicus* (AV423337). Numit *rps15A*: Arabidopsis (AC005169 and AL161575), tomato (AW220956 and BG135881), *M. truncatula* (AW776943, AW559986, and BF519104), and *P. taeda* (BE607125, BE607249, and BE758611).

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NOTE ADDED IN PROOF

Another report of numit *rps13* from Arabidopsis was recently published online: **Mollier, P., Hoffmann, B., Debast, C., and Small, I.** (2002). The gene encoding *Arabidopsis thaliana* mitochondrial ribosomal protein S13 is a recent duplication of the gene encoding plastid S13. Curr. Genet. DOI 10.1007/s00294-002-0271-5 (http://link.springer.de/link/service/journals/00294/contents/02/00271). These authors also concluded that the gene is derived from nucp *rps13* and that its product functions in mitochondria.