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Transfer of *rps19* to the nucleus involves the gain of an RNP-binding motif which may functionally replace RPS13 in *Arabidopsis* mitochondria

Hugo Sánchez, Thomas Fester, Sebastian Kloska, Werner Schröder[1] and Wolfgang Schuster[2]

Institut für Genbiologische Forschung Berlin GmbH, Ihnestrasse 63, D-14195 Berlin and [1]Freie Universität Berlin, Institut für Biochemie, Fabeckstrasse 36A, D-14195 Berlin, Germany

[2]Corresponding author

The discovery of disrupted *rps19* genes in *Arabidopsis* mitochondria prompted speculation about the transfer to the nuclear compartment. We here describe the functional gene transfer of *rps19* into the nucleus of *Arabidopsis*. Molecular cloning and sequence analysis of *rps19* show that the nuclear gene encodes a long N-terminal extension. Import studies of the precursor protein indicate that only a small part of this extension is cleaved off during import. The larger part of the extension, which shows high similarity to conserved RNA-binding domains of the RNP-CS type, became part of the S19 protein. In the *Escherichia coli* ribosome S19 forms an RNA-binding complex as heterodimer with S13. By using immuno-analysis and import studies we show that a eubacterial-like S13 protein is absent from *Arabidopsis* mitochondria, and is not substituted by either a chloroplastic or a cytosolic homologue of this ribosomal protein. We therefore propose that either a highly diverged or missing RPS13 has been functionally replaced by an RNP domain that most likely derived from a glycine-rich RNA-binding protein. These results represent the first case of a functional replacement of a ribosomal protein by a common RNA-binding domain and offer a new view on the flexibility of biological systems in using well-adapted functional domains for different jobs.

Keywords: gene transfer/plant mitochondria/RNP motif/ *rps13/rps19*

Introduction

Biogenesis of mitochondrial ribosomes requires components from both the nucleus and the mitochondrion, so that coordinated regulation is expected for the expression of the 60–80 ribosomal proteins distributed between different compartments. In *Marchantia polymorpha*, 16 genes coding for mitochondrial ribosomal proteins (MRPs) have been identified on the mitochondrial genome by homology to their eubacterial counterparts (Takemura *et al.*, 1992). All other MRPs are encoded by nuclear genes and must be imported post-translationally into the organelle. In the mitochondrial (mt) DNAs of higher plants a similar gene content is expected but the set of MRP genes differs between plant species with no apparent evolutionary pattern (reviewed by Grohmann *et al.*, 1993). Mitochondrial genes potentially coding for the ribosomal proteins RPS7 (Zhuo and Bonen, 1993), RPS10 (Zanlungo *et al.*, 1994), RPS12 (Gualberto *et al.*, 1988), RPS13 (Bland *et al.*, 1986), RPS14 (Wahleithner and Wolstenholme, 1988) and RPS19 (Conklin and Hanson, 1991) have so far been found in some plant mtDNAs, but cannot be identified in others (Nugent and Palmer, 1993). For example, while *rps7* is present in pea mitochondria, no homologous mitochondrial gene seems to be present in another legume, soybean (Zhuo and Bonen, 1993). Furthermore, some of the MRP genes identified are potentially silent or pseudogenes. In wheat mitochondria a complete gene for RPS13 has been found; however, this mitochondrial gene seems not to be expressed because *rps13* transcripts are undetectable (Bonen, 1987). In contrast, mitochondrial *rps14* of *Arabidopsis*, which is disrupted by a stop codon, is still transcribed and edited (Brandt *et al.*, 1993).

Because sequences of the MRPs are so similar to their eubacterial counterparts, a loss from the plant mitochondrial genome is attributed to a gene transfer into the nucleus rather than a complete loss from the plant. For two MRP genes, namely *rps12* in *Oenothera* (Grohmann *et al.*, 1992) and *rps10* in *Arabidopsis* (Wischmann and Schuster, 1995), transfer into the nucleus has been shown. Whereas *rps12* sequences are still present on the mtDNA in *Oenothera*, *rps10* sequences have been completely lost from the mitochondrial genome of *Arabidopsis*. In both gene transfers, the nuclear copy corresponds to the edited mitochondrial mRNA sequence as found in other species, implicating RNA as an intermediate of a functional mitochondrial gene transfer (Grohmann *et al.*, 1992; Wischmann and Schuster, 1995). This view is supported by the finding that *cox2*, a classical mitochondrial gene, is present as an edited version of the gene in the nucleus of several legumes (Nugent and Palmer, 1991; Covello and Gray, 1992). All recently translocated mitochondrial genes are longer than their organellar counterparts, due to extensions at the 5′ end. These upstream sequences encode mostly putative target signals, required in directing the protein, after synthesis on cytosolic ribosomes, into mitochondria (von Heijne *et al.*, 1989).

In yeast, where mitochondrial ribosomes have been most extensively analysed to date, all but one MRP have been translocated to the nucleus (Dujon, 1981). From the 60–80 MRPs, ~20 have been sequenced and characterized. Only about half of them show significant similarities to eubacterial and/or chloroplast ribosomal protein genes (Grohmann *et al.*, 1991). Other MRPs in yeast show no sequence similarity to any of the bacterial ribosomal proteins (Kang *et al.*, 1991) and may represent species-specific adaptations in replacing ribosomal proteins that have been lost during organellar evolution. Novel proteins,

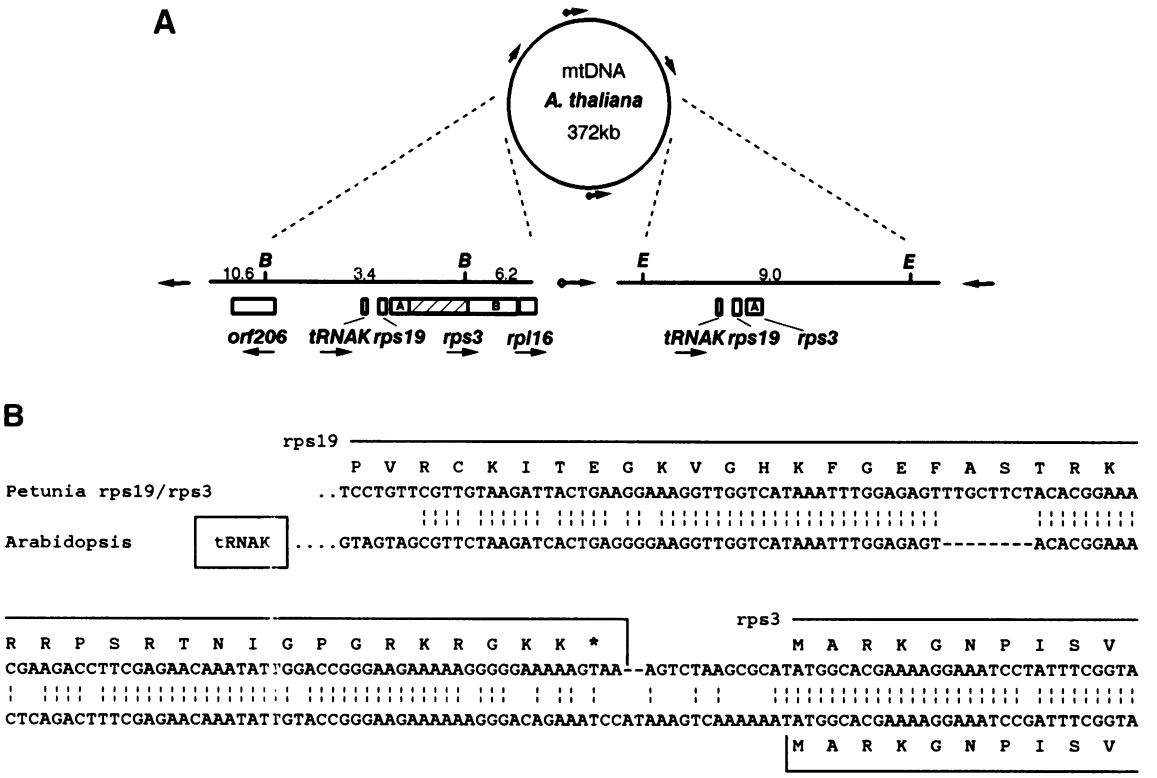

Fig. 1. A functional *rps19* gene is absent from the mitochondrial genome of *Arabidopsis thaliana*. (**A**) A schematic representation of the mtDNA of *A.thaliana* (Klein *et al.*, 1994) with the location of *rps19* sequences. Upstream of *rps19*, a tRNA gene for lysine is located showing the same orientation as the adjacent ribosomal protein genes *rps3* and *rpl16*. The *rps3* gene is separated by a group II intron (cross-hatched box) in two exons (boxes labelled with A and B). On the opposite strand a gene is present that encodes a subunit of a proposed transporter (*orf206*; Schuster, 1994). The second *rps19* locus represents a duplication of 600 nucleotides starting upstream of the tRNA gene and ending at the exon/intron border of the first exon of *rps3*. Restriction sites are shown for *Bam*HI (*B*) and *Eco*RI (*E*). (**B**) A sequence comparison of the *rps19* pseudogene from *Arabidopsis* with the intact mitochondrial *rps19* of *Petunia* (Conklin and Hanson, 1991).

which have no counterparts in the bacterial ribosome, are likewise present in chloroplast ribosomes (reviewed by Subramanian, 1993).

We have started to analyse recent transfers of mitochondrial ribosomal protein genes to the nucleus in higher plants, and report here the transfer of *rps19* into the nuclear compartment of *Arabidopis*. Surprisingly, an RNP motif was found linked to the organellar-derived S19 sequence which most likely functionally replaces another missing ribosomal protein in ribosomes of *Arabidopsis*, namely S13.

Results

rps19 is a pseudogene in Arabidopsis mitochondria

In the course of our studies on the gene content of mtDNA in higher plants, we found that an *rps19* gene is disrupted by a termination codon in *Oenothera* mitochondria. This pseudogene, however, is still transcribed and edited (Schuster and Brennicke, 1991), but editing has drifted away from conserved cytidine positions to other non-conserved editing sites, resulting in a less well conserved polypeptide after editing. To investigate this phenomenon further in a different plant, we used Southern blot hybridization to identify *rps19* in the mitochondrial genome of *Arabidopsis*. Two loci were identified that hybridized with the *Oenothera rps19* probe, which covered the entire coding region of the gene. DNA sequencing of both regions of the mitochondrial genome revealed that only pseudogenes of *rps19*, which cover only the C-terminal part of the gene where no editing sites are found, are present (Figure 1). Upstream of the *rps19* sequence a lysine tRNA gene is located, and the gene for RPS3 that normally flanks *rps19* (Bock *et al.*, 1994) is present downstream. One of the loci, however, encodes a pseudogene of *rps3*, because homology between the two copies immediately breaks down after the first exon of the *rps3* gene. The part upstream that covers *trnK* and *rps19* sequences is identical for ~600 nucleotides between both mtDNA locations.

rps19 is expressed as poly(A)$^+$ RNA

Based on the fact that an intact *rps19* gene is present in *Petunia* mitochondria (Conklin and Hanson, 1991), it seems unlikely that an active gene for this essential ribosomal protein is missing in *Arabidopsis* and more likely that the functional gene has been transferred to the nuclear compartment. To test this possibility, we used the *Oenothera* gene as probe to isolate clones from a cDNA library of *Arabidopsis* that could code for the mitochondrial RPS19 in the nucleus. Three polyadenylated cDNA clones were found to contain sequences homologous to the mitochondrial *rps19*. The clones showed identical 5′-termini whereas the 3′-ends differed due to varying polyadenylation sites and the length of the poly(A)-tails. Northern blot analysis of poly(A)$^+$ RNA from *Arabidopsis* using *rps19* as probe revealed that the gene is transcribed in a single transcript of 800 nucleotides (data not shown).

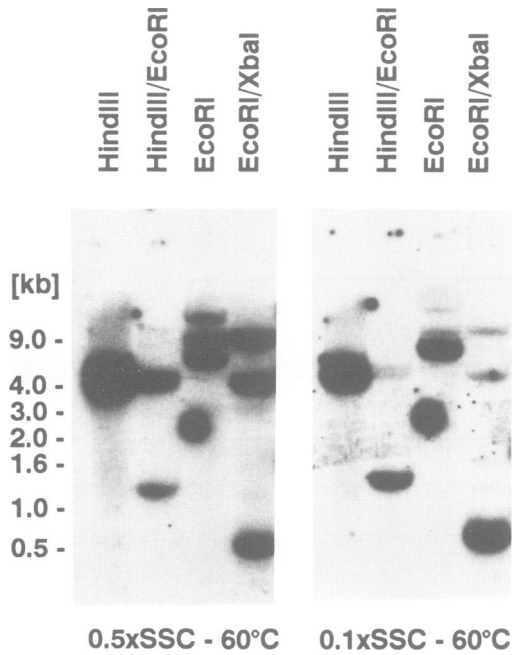

Fig. 2. *rps19* is present as a single copy gene in the nuclear genome of *Arabidopsis*. Genomic DNA of *Arabidopsis* (5 μg per lane) was digested with various restriction enzymes. DNA was size fractionated by agarose gel electrophoresis, blotted on nylon membrane and probed with the conserved *rps19* region (*Sph*I–*Hpa*I fragment with ~500 bp) of the cDNA sequence (only part of the blot is shown). The left panel shows hybridization conditions under low stringency, while the right panel was done under high stringency conditions. The hybridization signals disappearing under high stringency (0.1× SSC, –60°C) represent *rps19* pseudogene sequences of the mtDNA, the strong signal under these conditions indicates the nuclear *rps19* gene in *Arabidopsis*. The dot in the *Eco*RI lane at 2.5 kb represents an artifact and is not visible on other Southern blots. Migration of molecular mass standards is indicated on the left in kb.

Comparison of the cDNA sequences to edited mitochondrial *rps19* sequences indicated that the edited mitochondrial version seems to be present in the nuclear gene. In *Petunia*, four editing sites are present in the *rps19* gene (Conklin and Hanson, 1991); however, only two of the editing sites are at highly conserved positions. These conserved positions (amino acids 166 and 201) are present as edited codons in the *Arabidopsis* gene.

A single-copy gene of rps19 is present in the nucleus of Arabidopsis

Southern blotting using an internal part of *rps19* revealed that the gene is present as a single-copy gene in *Arabidopsis* (Figure 2, and data not shown). Homology to the mitochondrial pseudogenes is still high, as shown by hybridization at moderate stringency whereby both mitochondrial loci are detected. Two cosmid clones carrying *rps19* have been isolated from an *Arabidopsis* cosmid library using one of the cDNA clones as a probe. Comparison of the cDNA and genomic sequences revealed that the gene is split into four exons (Figure 3). Sequence comparison further revealed that the cDNA clones that were analysed were identical and contained a long uninterrupted reading frame of 212 codons, corresponding to a 24 kDa protein. Surprisingly, this reading frame could code for a protein of more than double the size of a typical RPS19 polypeptide. We attributed this observation

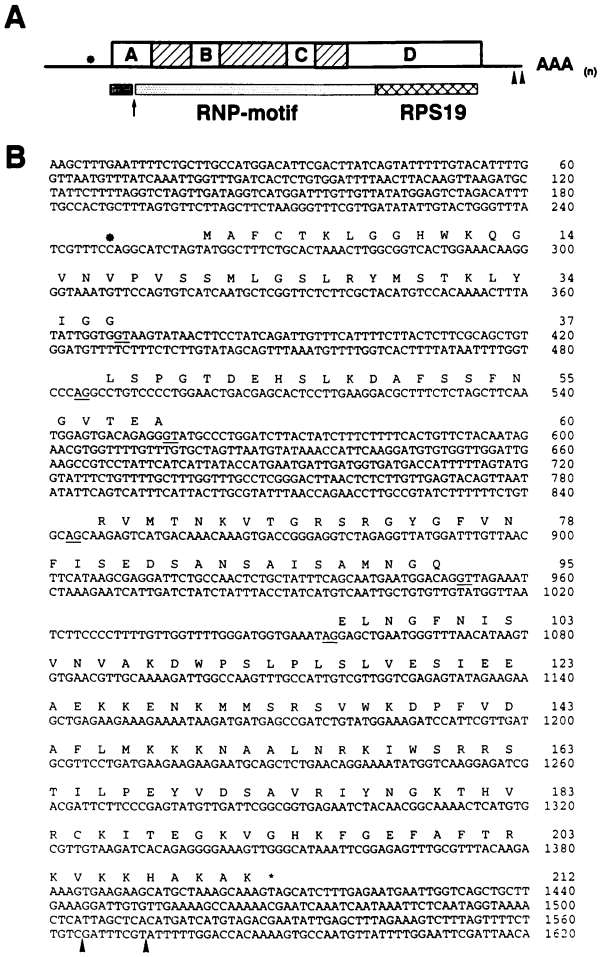

Fig. 3. Structure and DNA sequence of the *Arabidopsis rps19* gene. (**A**) The nuclear encoded *rps19* gene is separated by three introns (hatched boxes). All introns are located in the region that encodes the proposed RNA-binding domain, whereas no intron was found in the organellar-derived *rps19* part. The target signal sequence is encoded by the first half of exon A and is indicated by a vertical arrow. (**B**) DNA sequence of *rps19* containing the complete transcribed region. The deduced amino acids encoded by the open reading frame are given in the one-letter code. Intron borders are underlined. The arrowheads show two alternative poly(A) sites. The star indicates the 5′-termini of the cDNA clones.

to the fact that an N-terminal extension is needed for most of the nuclear encoded mitochondrial proteins to be targeted to mitochondria (von Heijne, 1986).

The 5′-terminal extension of rps19 encodes an RNP-CS motif

When the deduced amino acid sequence was used to search for homology in the sequence databases, significant similarity was found not only to mitochondrial and eubacterial S19 proteins but also for proposed RNA-binding proteins (Figure 4) carrying a common structural motif of ~90 amino acids, termed ribonucleoprotein consensus sequence (RNP-CS; Burd and Dreyfuss, 1994). Some of these RNA-binding proteins contain only a single copy of the RNP-CS motif, also known as RNA recognition motif (RRM), while others contain as many as four copies (reviewed by Bandzilius *et al.*, 1989). Two RNP-CS motifs have been found in tobacco chloroplast proteins that have been proposed to be involved in RNA splicing and/or

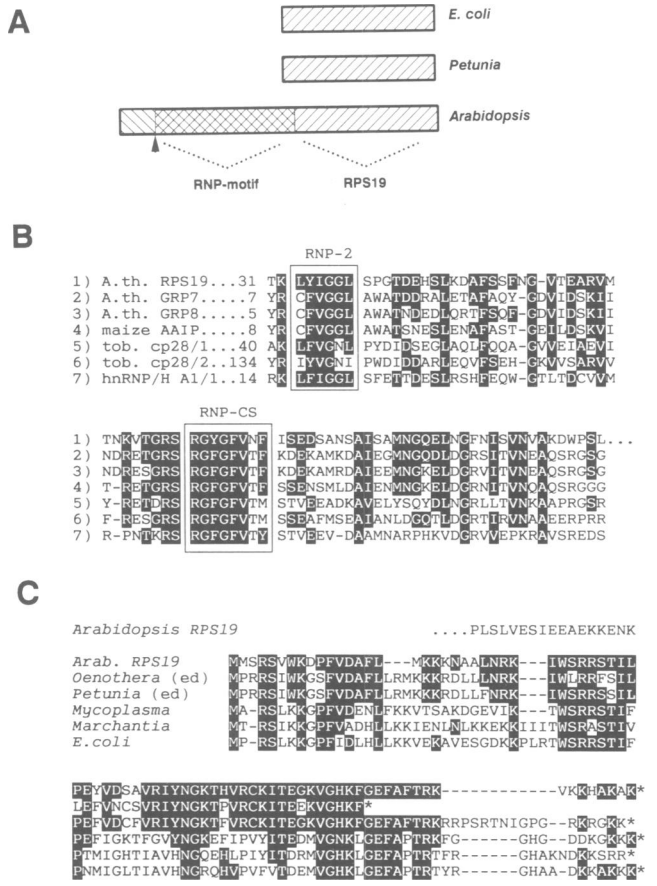

Fig. 4. The *Arabidopsis* RPS19 shows homology to proteins carrying conserved RNP motifs. (**A**) A schematic size comparison of the *Arabidopsis* RPS19 (212 amino acids) to the counterparts of *E.coli* (92 amino acids; Zurawski and Zurawski, 1985) and *Petunia* mitochondria (94 amino acids; Conklin and Hanson, 1991). The S19 homologous part is shown by hatched boxes and the amino acid comparison for this part of the polypeptide is given in part C of this figure. The upstream part of the *Arabidopsis rps19* shows high similarity to RNA-binding domains of the RNP-type (B). The putative mitochondrial target signal sequence is indicated by a vertical arrow. (**B**) The deduced amino acid sequence of the *Arabidopsis rps19* (A. th. RPS19; this paper) shows high similarity to conserved RNP motifs. Two highly conserved domains of the RNP motif are boxed (RNP-CS, RNP-2). The highest similarity was observed to glycine-rich proteins of *Arabidopsis* (A. th. GRP7; A. th. GRP8; van Nocker and Vierstra, 1993) and maize (maize AAIP; Gómez *et al.*, 1988). From the >150 proteins containing RNP motifs, two further examples are listed, one represents a plastid protein of tobacco that contains two RNP motifs (tob. cp28/1; tob. cp 28/2; Li and Sugiura, 1990) and the other the human hnRNP A1 (hnRNP/H A1/1; Riva *et al.* 1986) which is involved in splicing. Similar amino acid residues at identical postions are shown by inverse contrast. (**C**) A sequence alignment of RPS19 sequences from *Oenothera* (Schuster and Brennicke, 1991), *Petunia* (Conklin and Hanson, 1991) and *Marchantia* (Takemura *et al.*, 1992) mitochondria, and the S19 polypeptides from *Mycoplasma* (Ohkubo *et al.*, 1987) and *E.coli* (Zurawski and Zurawski, 1985).

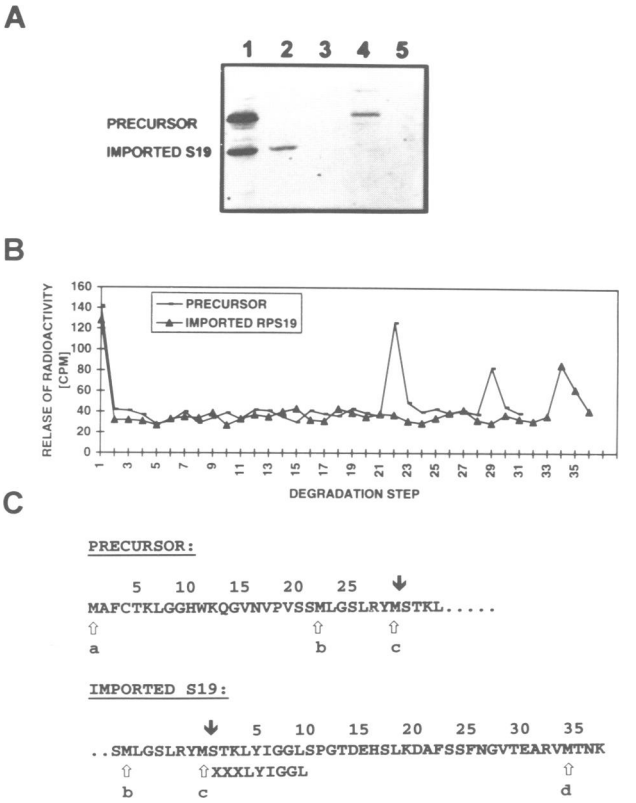

Fig. 5. The *in vitro*-translated RPS19 is imported into potato mitochondria. (**A**) The import of labelled RPS19 precursor into purified potato mitochondria. Lane 4 shows the *in vitro*-labelled precursor (1/10 of the amount used for import) which is completely digested after the addition of proteinase K (lane 5). After incubation of the RPS19 precursor with purified potato mitochondria (lane 1) the mature form of RPS19 appears. The mature form is resistant to proteinase K treatment of mitochondria (lane 2). After addition of Triton X-100 the mature form disappears after proteinase K treatment (lane 3). (**B**) The microsequencing of the labelled RPS19 precursor and the imported form. After blotting, the proteins were subjected to microsequencing. Radioactivity release at each sequencer cycle is shown for the precursor and the processed RPS19. Background radioactivity is not subtracted. (**C**) The N-terminal sequence of RPS19. Labelled amino acids are indicated by open arrows (a–d) below the polypeptide sequence. Numbers above the amino acid sequence refer to the degradation steps of (B). Significant release of radioactivity was observed in degradation steps 1 (a), 22 (b) and 29 (c) for the RPS19 precursor, which matches the positions of the labelled methionine residues of the polypeptide. For the imported RPS19, where the signal peptide has been cleaved off, degradation steps 1 (c) and 34/35 (d) showed significant release of radioactivity. From these results the putative processing site of the signal peptide is expected to be located upstream of amino acid position 29 (M) or 30 (S) of the RPS19 precursor. To confirm the results obtained by radiosequencing of the heterologous *in organello* import product, and to distinguish between the two possible processing sites, the genuine RPS19 from *Arabidopsis* mitochondria was immuno-purified and sequenced. The sequence obtained by Edman degradation is shown below the sequence-derived RPS19 amino acid sequence. The proposed cleavage site of the signal peptide is indicated by a solid arrow above the amino acid sequence.

processing (Li and Sugiura, 1990). The highest homology of the RNP domain of RPS19 was observed to putative RNA-binding proteins of the glycine-rich family of *Arabidopsis* (van Nocker and Vierstra, 1993) with ~54% identical amino acids, and to an abscisic acid (ABA)-induced transcript from maize (Gómez *et al.*, 1988), showing 52% homology.

Import of RPS19 into potato mitochondria

To demonstrate that RPS19 is targeted to mitochondria and to analyse the fate of the N-terminal extension, we imported the *in vitro*-translated RPS19 precursor into isolated potato mitochondria (Figure 5). This N-terminal extension with 130 amino acids could either code for a very large mitochondrial target signal that is removed during import, or, in a different scenario, could represent

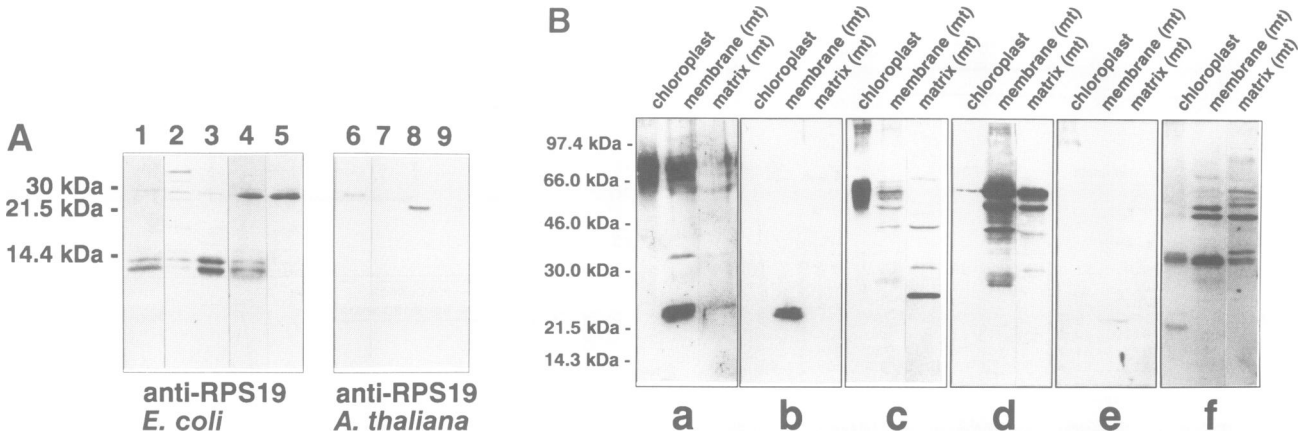

Fig. 6. RPS19 is present in *Arabidopsis* mitochondrial ribosomes. (**A**) An immunoblot analysis using antibodies directed against either RPS19 of *E.coli* (lanes 1–5) or RPS19 from *Arabidopsis* that has been overexpressed in *E.coli* (lanes 6–9). The samples are total protein of *E.coli* (lane 1), 50 S (lane 2) and 30 S (lane 3) subunits of *E.coli* ribosomes, total protein of *E.coli* overexpressing RPS19 from *Arabidopsis* (lane 4) and the purified RPS19 protein (lane 5), one-tenth of the amount of purified RPS19 that was loaded on lane 5 (lane 6), chloroplast (lane 7), mitochondrial (lane 8) and cytosolic (lane 9) ribosomal protein of *Arabidopsis*. *Escherichia coli* protein samples contained ~2 μg of protein, ribosomal protein fractions of *Arabidopsis* (lane 7–9) ~0.5 μg. (**B**) An immunoblot analysis of fractionated mitochondrial and total chloroplast protein from *Arabidopsis* using antibodies directed against specific marker proteins. Mitochondria were subfractionated into membrane (membrane mt) and matrix (matrix mt) by sonication and ultracentrifugation. Approximately 2 μg of plastid and 10 μg of mitochondrial protein were loaded. Identical immunoblots were incubated with *Arabidopsis* RPS19 antiserum (a), affinity-purified RPS19 antibodies (b), antibodies directed against the tobacco MnSOD (c; Bowler *et al.*, 1989), antibodies directed against the core proteins of the bc_1 complex from *Neurospora* (d; Schulte *et al.*, 1989), antibodies directed against a plastid-localized starch phosphorylase (e; Greve, 1992), and an anti-serum directed against two plastid ribosomal proteins from spinach (f; Bubunenko and Subramanian, 1994). The molecular weight marker sizes are indicated on the left in kDa.

a mitochondrial polypeptide of unknown function (e.g. comparable with the plastid RNA-binding proteins of the RNP-CS type) that was used by the organellar-transferred RPS19 as an import vehicle into mitochondria. Interestingly, during the *in organello* import into potato mitochondria, a signal peptide of only 3.5 kDa was removed from the 24 kDa RPS19 precursor. We therefore concluded that the mature ribosomal protein S19 was extended by an RNA-binding motif of the RNP-CS type. In order to determine the exact processing site of the signal peptide we radiosequenced the *in vitro*-imported RPS19 polypeptide that was labelled at the methionine positions (Figure 5). From the high release of radioactivity during Edman degradation at the 34th step we determined that the processing site is between a methionine at position 29 and a serine at position 30, which is in good agreement with the results obtained from the *in organello* import studies. However, significant release of radioactivity was also observed during the first degradation step (and to a lower degree at step 35); therefore it is likely that processing of the RPS19 precursor proceeds in two steps: whereby the first processing step, carried out by the general processing peptidase, leads to a cleavage between a tyrosine at position 28 and the methionine at position 29; and is followed by a second processing step by which the N-terminal methionine is removed. It has been shown by N-terminal sequencing that mitochondrial proteins of higher plants are missing methionine at the N-terminus, therefore the presence of a methionine aminopeptidase has been suggested (Braun and Schmitz, 1993). While in *Neurospora* mitochondrial precursors an arginine is found at position –1 upstream of the processing site, this conserved amino acid is located in plant precursors either at position –1 or –2. For that reason both processing sites are possible for RPS19. To confirm the results of the *in organello* import, we immuno-purified RPS19 from mitochondria of *Arabidopsis* in order to identify the N-terminus of the mature polypeptide. Microsequencing of the *Arabidopsis* RPS19 revealed the same processing site as expected from the import studies into potato mitochondria. From the location of the processing site we therefore conclude that the RNP motif is a fully functional domain.

rps19 is present in mitochondrial ribosomes of Arabidopsis

In order to show that RPS19 is present as a large protein of 20 kDa in mitochondrial ribosomes, we cloned RPS19 into a bacterial expression vector and overexpressed the protein in *Escherichia coli*. An antibody directed against the S19 ribosomal protein from *E.coli* (kindly provided by Dr R.Brimacombe) shows a cross-reaction with the highly overexpressed S19 product of the *Arabidopsis* reading frame (Figure 6A). However, in a mitochondrial ribosomal fraction, only a very faint signal was obtained with this *E.coli* antibody (data not shown). We therefore purified the overexpressed S19 protein and raised a polyclonal antibody against the S19 protein. The serum obtained was further affinity-purified using the overexpressed S19. This purified antibody was used in immunoblot analysis of mitochondrial, plastid and cytosolic ribosomal protein fractions from *Arabidopsis* (Figure 6A). While no signal was observed in the lanes carrying the plastid and cytosolic ribosomal fractions, a single distinct band of about 20 kDa was obtained in the lane with the mitochondrial ribosomal proteins. This band corresponds exactly to the imported RPS19 of 20 kDa carrying the organellar-transferred part of RPS19 as well as the RNP-CS domain linked at the N-terminus.

To ensure the purity of the ribosomal fractions, antibodies directed against specific marker proteins were used (Figure 6B). Manganese superoxide dismutase (MnSOD), a marker enzyme of the mitochondrial matrix, was identi-

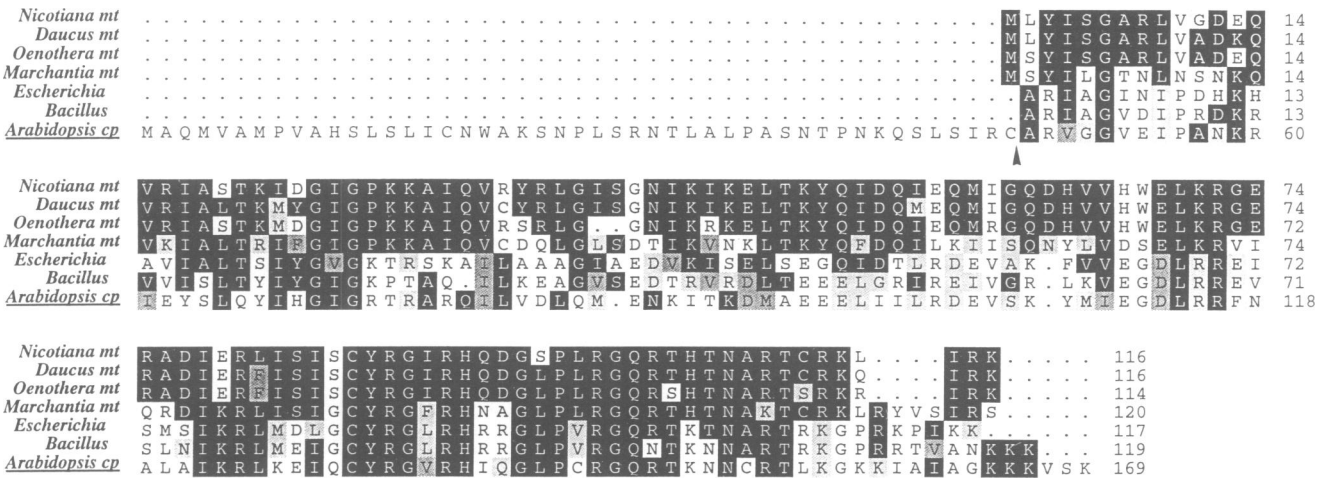

Fig. 7. Amino acid sequence comparison of the deduced *Arabidopsis* plastid ribosomal protein S13 (*Arabidopsis cp*) with the respective polypeptides from *Nicotiana* (Bland *et al.*, 1986), *Daucus* (Wissinger *et al.*, 1990), *Oenothera* (Schuster and Brennicke, 1987) and *Marchantia* (Oda *et al.*, 1992) mitochondria (mt), and the bacterial counterparts of *E.coli* (Lindemann and Wittmann-Liebold, 1977) and *Bacillus* (Brockmöller and Kamp, 1988). Identical amino acids are highlighted by inverse contrast, similar residues by grey shading. The proposed cleavage site between the transit peptide and the mature RPS13 is indicated by an arrowhead.

fied by immunoblotting in the soluble mitochondrial fraction as 28 kDa polypeptide; whereas in the lanes with the membrane and chloroplast fractions no signal of similar size was obtained. Antiserum directed against the core proteins of the bc_1 complex from *Neurospora* (kindly provided by Professor H.Weiss) identified cross-reacting proteins exclusively in the mitochondrial fractions; we therefore conclude that contamination with mitochondrial protein is low in the chloroplast fractions. An antibody directed against a plastid-specific starch phosphorylase (kindly provided by Dr B.Greve) was used to prove that the mitochondrial fractions are free of chloroplast contamination. A polypeptide with the expected size of 105 kDa was identified by this antibody exclusively in the lane with plastid protein, although five times more mitochondrial protein was loaded, indicating that the mitochondrial protein fractions are free of plastid protein. Moreover, an antiserum raised against two plastid ribosomal proteins (PSRP1 and L21) from spinach (kindly provided by Dr A.R.Subramanian) was also used to ensure that the mitochondrial fractions are free of plastid ribosomes. Two polypeptides of 23 and 34 kDa showed a specific cross-reaction in the plastid fraction with this antiserum. This finding indicates that these ribosomal proteins are also present in *Arabidopsis* chloroplasts. In the mitochondrial fractions the 23 kDa polypeptide is absent; however, several larger proteins were detected by this antiserum, in both the matrix and the membrane protein fraction. These signals may be due to cross-reactions of mitochondrial ribosomal proteins to its plastid counterparts, or may be unspecific. The immunopurified RPS19 antibody identified the protein quantitatively in the membrane fraction, whereas no soluble RPS19 could be found in the matrix, suggesting that the function of this polypeptide may be restricted to mitochondrial ribosomes.

Nuclear-encoded RPS13 is imported into chloroplasts

In the *E.coli* ribosome, RPS19 has been located on the head of the 30S subunit in close contact to ribosomal protein S13 (reviewed by Wower *et al.*, 1993). It has been shown that a heterodimer of RPS19 and RPS13 can be formed that binds tightly and specifically to 16S rRNA (Dijk *et al.*, 1977). The N-terminal region of the S13 protein is important for the interaction with S19 while the C-terminal part is involved in RNA binding (Schwarzbauer and Craven, 1985).

To unravel the possible function of the RNP motif that is linked to RPS19 in *Arabidopsis* we concentrated on the possible interaction with the S13 protein that we also expected for mitochondrial ribosomes. As an *rps13* gene is also absent from the mtDNA of *Arabidopsis* (Klein *et al.*, 1994), we investigated the presence of this MRP gene in the nucleus. Using the mitochondrial gene of *Daucus* (Wissinger *et al.*, 1990) as probe we screened a cDNA library of *Arabidopsis* for the presence of a mitochondrial *rps13*; however, no positive signal was observed. We attributed this finding to a highly diverged nuclear gene encoding the mitochondrial S13 that had drifted away in sequence after translocation to the nuclear compartment. By a different approach, three cDNA clones with sequences homologous to *rps13* were isolated, using degenerate oligonucleotides deduced from a conserved amino acid sequence at the C-terminal part of mitochondrial S13 polypeptides. All cDNA clones showed identical sequences and coded for a reading frame of 169 codons. The 3′-ends of the cDNA clones differed, however, due to alternative poly(A) sites. *rps13* is present as single-copy gene in the nuclear genome of *Arabidopsis* (data not shown). The deduced amino acid sequence showed high similarity to bacterial (65% similarity) but much lower similarity to mitochondrial (37% similarity) S13 polypeptides (Figure 7). At the N-terminus an extension of 47 residues was found which we expected to function as mitochondrial target signal. *In organello* import of the *in vitro*-translated RPS13 polypeptide into mitochondria failed, however (Figure 8A, lower part). As an *rps13* gene is likewise absent from the plastid genome of all higher plants it was considered much more likely that the cDNA clones that we isolated encoded the chloroplast counterpart

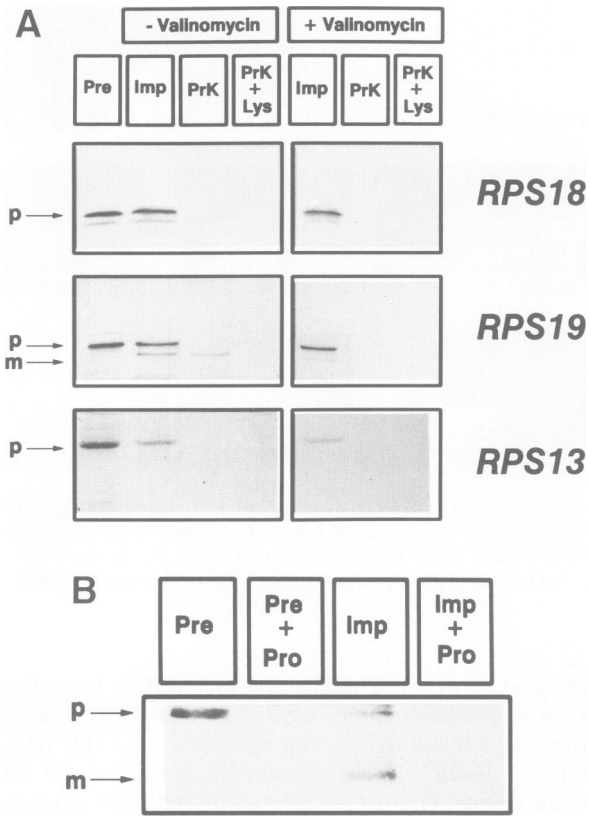

Fig. 8. *In organello* import of *in vitro*-translated ribosomal precursor proteins into potato mitochondria and pea chloroplasts. (**A**) *In organello* import of the cytosolic RPS18, the mitochondrial RPS19, and the plastid RPS13 of *Arabidopsis*. *In vitro*-translated ribosomal precursors (p) are shown in the first lane (Pre), incubation with purified potato mitochondria in the second lane (Imp). Proteinase K treatment (PrK) after the import is shown in lane 3. When mitochondria are lysed by the addition of Triton X-100 (Lys) the protected mature protein (m) is digested by proteinase K (PrK + Lys). Import of the polypeptides into mitochondria is completely abolished in the presence of valinomycin (+valinomycin). (**B**) The import into pea chloroplasts of the plastid RPS13 of *Arabidopsis*. The first lane (Pre) shows the RPS13 precursor protein with 19 kDa that is completely digested after addition of the protease thermolysin (Pre + Pro). After incubation of the S13 precursor polypeptide with pea chloroplasts (Imp), a mature product of ~14 kDa appears that is protected from protease degradation of chloroplasts (Imp + Pro).

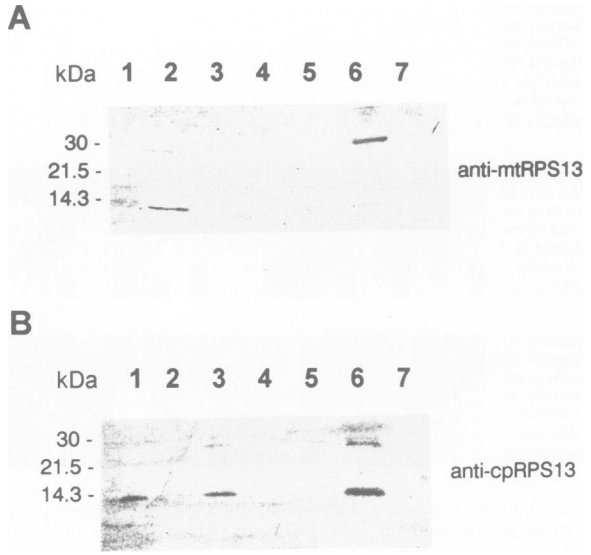

Fig. 9. Western blot analysis using antibodies directed against either the mitochondrial RPS13 (anti-mtRPS13) of maize (**A**) or the plastid RPS13 (anti-cpRPS13) of *Arabidopsis* overexpressed in *E.coli* (**B**). The samples are ribosomal proteins of pea (lane 1) and potato (lane 2) mitochondria, plastid ribosomal proteins of potato (lane 3), and total mitochondrial (lane 4), mitochondrial ribosomal (lane 5), plastid ribosomal (lane 6) and cytosolic ribosomal (lane 7) proteins of *Arabidopsis*. Approximately 1 μg of proteins was loaded on lanes 1, 2, 3, 5 and 7. Lanes 4 (total mt proteins) and 6 (plastid ribosomal proteins of *Arabidopsis*) contained ~5 μg. The molecular weight marker sizes are indicated on the left in kDa.

of S13 rather than the mitochondrial protein. To prove this possibility, we investigated the *in organello* import of RPS13 into pea chloroplasts (Figure 8B). After incubation of the *in vitro*-translated S13 precursor with isolated pea chloroplasts we observed a protease-protected polypeptide with a molecular weight of ~14 kDa. During import, a peptide of ~5 kDa was removed from the S13 precursor molecule. We conclude, therefore, that the first 47 amino acids at the N-terminus encode a transit peptide for chloroplast import.

RPS13 is absent from mitochondrial ribosomes of Arabidopsis

Genes for RPS13 have been described in mtDNAs of plants for *Oenothera* (Schuster and Brennicke, 1987), *Daucus* (Wissinger *et al.*, 1990), maize, tobacco (Bland *et al.*, 1986), wheat (Bonen, 1987) and *Marchantia* (Takemura *et al.*, 1992), and expression has been confirmed by an antibody directed against the maize mitochondrial S13 polypeptide at least in some of the species (Kumar *et al.*, 1995). We used this antibody (kindly provided by Professor C.S.Levings III), in order to investigate the presence of a mitochondrial-derived RPS13 in mitochondrial, chloroplast or cytosolic ribosomes of *Arabidopsis* (Figure 9). A strong signal in the Western blot at 14 kDa was obtained only for ribosomal proteins from potato mitochondria. Weak signals have been detected for the ribosomal proteins of pea mitochondria and *Arabidopsis* chloroplasts, however, no signal appeared in the lanes with the mitochondrial and cytosolic fractions. From these results we conclude that a mitochondrial-derived RPS13 is absent from ribosomes of *Arabidopsis* mitochondria. To verify the results of the S13 import studies we used an antibody directed against the plastid RPS13 (kindly provided by Dr I.Small) to probe an identical Western blot. Distinct signals for plastid ribosomal protein S13 appeared in the lanes for *Arabidopsis* and potato chloroplasts at the expected molecular weight, corroborating the plastid localization of this protein. Surprisingly, in pea mitochondria a strong signal was obtained with the plastid S13 antibody. We therefore conclude that the missing mitochondrial gene for RPS13 in pea was replaced by the plastid homologue rather than by a mitochondrial S13 that was transferred to the nucleus. In the lanes carrying the mitochondrial fractions of *Arabidopsis*, however, no signal was observed, indicating that neither a mitochondrial-derived nor a plastid-derived S13 protein is present.

RPS18, the cytosolic homologue of RPS13, is not imported into mitochondria

In *Arabidopsis*, the cytosolic counterpart of the eubacterial RPS13, namely RPS18, has been identified by investiga-

ting the *PFL* mutant that causes pointed first leaves (van Lijsebettens *et al.*, 1994). Three copies of this gene are present in the nuclear genome of *Arabidopsis*, all of them encoding identical polypeptides. One of the genes, however, is only expressed in meristems and affects plant development. It has been suggested that an extra copy of this ribosomal protein is needed in tissues with high expression to increase translational efficiency. In spinach chloroplasts it has been shown that the plastid RPL23 has been functionally replaced by a cytosolic counterpart (Bubunenko *et al.*, 1994); we therefore investigated the putative replacement of RPS13 in *Arabidopsis* mitochondria by an RPS18 homologue. By *in organello* import and immunoblot analyses we examined the presence of a cytosolic RPS18 in mitochondria of *Arabidopsis*. To test whether RPS18 polypeptides could be imported into plant mitochondria, we cloned the complete reading frame of one of the S18 copies for *in vitro* translation and incubated the labelled protein with potato mitochondria to see if import occurs (Figure 8A). While in the control experiment RPS19 was imported, no protease-protected polypeptide was detectable in the S18 import.

Based on the earlier finding that an antibody against the *E.coli* RPS13 shows a weak cross-reaction with RPS18, we investigated the presence of the cytosolic RPS18 in mitochondria of *Arabidopsis* by immunoblot analysis. A protein of 17.5 kDa was identified by this antibody in the cytosolic ribosomal fraction (Figure 10A). This polypeptide corresponds exactly in size to the cytosolic RPS18. A protein of similar size could not be observed in the mitochondrial fractions. To verify that the cross-reaction of the S13 antibody with RPS18 is specific, we performed immunoprecipitiation experiments where we show that the *in vitro*-translated RPS18 protein could be immunoprecipitated by the *E.coli* antibody (Figure 10B). Control experiments, with either *in vitro*-translated RPS19 protein and S13 antibody or S19 antiserum and *in vitro*-translated RPS18, showed no precipitation product. Therefore we conclude that cross-reaction of the *E.coli* S13 antibody with RPS18 is specific, most likely due to a highly conserved protein domain at the C-terminus that is present in all members of the S13 protein family (Figure 10C). A weak cross-reaction of this antibody was likewise observed with the S13 polypeptides from potato mitochondria and *Arabidopsis* chloroplasts. From the results of both the *in vitro* import experiments and the immunoblotting, we conclude that a functional replacement of the missing RPS13 in *Arabidopsis* by a cytosolic counterpart is unlikely.

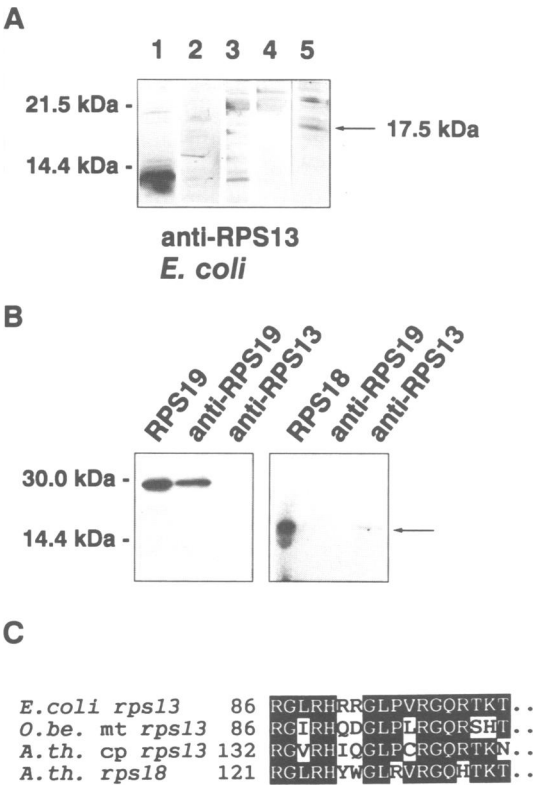

Fig. 10. Western-blot analysis and immunoprecipitation of the cytosolic RPS18 from *Arabidopsis* using the *E.coli* S13 antibody. (**A**) Western-blot using an antibody directed against RPS13 from *E.coli*. The samples are total *E.coli* protein (lane 1), plastid ribosomal protein of *Arabidopsis* (lane 2), mitochondrial ribosomal protein of potato (lane 3), mitochondrial ribosomal protein of *Arabidopsis* (lane 4) and cytosolic ribosomal protein of *Arabidopsis* (lane 5). The position of the putative cytosolic RPS18 at 17.5 kDa is indicated by an arrow. (**B**) Immunoprecipitation experiments using antibodies directed against either RPS13 from *E.coli* or RPS19 from *Arabidopsis*. To prove that the cross-reaction of the RPS13 antibody with the cytosolic S18 was specific, *in vitro*-translated RPS18 (right panel; RPS18) or, as control, *in vitro*-translated RPS19 (left panel; RPS19) was immunoprecipitated with the S13 (anti-RPS13) and S19 (anti-RPS19) antibodies. The faint band of precipitated RPS18 is indicated by an arrow. (**C**) An amino acid sequence comparison of the protein domain that is conserved in all members of the S13 family. The sequences of the bacterial RPS13 from *E.coli* (*E.coli rps13*), the mitochondrial RPS13 from *Oenothera berteriana* (*O.be.* mt *rps13*), the plastid RPS13 from *Arabidopsis thaliana* (*A.th.* cp *rps13*) and the cytosolic RPS18 from *Arabidopsis thaliana* (*A.th. rps18*) are shown. Identical amino acids are highlighted by inverse contrast.

Discussion

We have shown that a complete gene for RPS19 is absent from the mitochondrial genome of *Arabidopsis*, but that a functional *rps19* is present as a single-copy gene in the nuclear compartment. Comparison of the deduced S19 amino acid sequence with its mitochondrial counterparts revealed that the nuclear gene is highly similar to the edited organellar copy, and that it encodes a long N-terminal extension showing high similarity to RNA-binding domains of the RNP-CS type.

This RNP-CS domain is the most common RNA-binding motif and is found in a number of proteins involved in processing, translation and transport of mRNA precursors (Burd and Dreyfuss, 1994). Some of these proteins contain only a single copy of the RNP motif, while others contain as many as four copies (Bandzilius *et al.*, 1989). A typical RNP domain is composed of ~80 amino acids showing two highly conserved short sequences of eight (RNP1 or RNP-CS) and six (RNP2) residues respectively (Dreyfuss *et al.*, 1988). Such motifs are also present in the RNP domain of RPS19 from *Arabidopsis*. In plants, several cDNAs encoding putative RNA-binding proteins of the RNP-CS type have been isolated. Three proteins, each containing two RNP-CS domains, have been isolated from tobacco chloroplasts (Li and Sugiura, 1990). These polypeptides are proposed to be involved in splicing and/or processing of chloroplast RNAs. The three-dimensional structures of two RNP-containing proteins

[*sn RNP U1A* (Nagai *et al.*, 1990) and *hnRNP C* (Görlach *et al.*, 1992)] have been analysed by X-ray crystallographic and/or NMR solution studies. Despite low overall amino acid sequence identity, these proteins assume similar globular structures, formed by a four-stranded anti-parallel β-sheet packed against two perpendicularly oriented α-helices. Recently, the crystal structure of ribosomal protein S6 from *Thermus thermophilus* has been determined (Lindahl *et al.*, 1994) and this shows a similar folding pattern to the RNP-CS-type motifs. It has been suggested, therefore, that this structure was established early in molecular evolution and that the RNA-binding domains of the RNP family may have originated from an ancestral RNA-interacting motif of ribosomal proteins. The putative RNA-binding domain of RPS19 shows the highest similarity to glycine-rich proteins that have also been described from *Arabidopsis*, and that most likely resulted by a gene duplication or inactivation of a member of this protein family. Glycine-rich proteins can be separated into two distinct groups, one showing no RNA-binding motifs at all (de Oliveira *et al.*, 1990), the other consisting of a C-terminal glycine-rich domain appended to a single RNP-CS motif (van Nocker and Vierstra, 1993). It has been shown that proteins of this family are induced either by wounding (Sturm, 1992) or abscisic acid (ABA; Gómez *et al.*, 1988) and play a vital role during stress. The RNA-binding domains of these polypeptides may therefore be involved in hormonal regulation; however, the real function of the RNP domain in these proteins is unclear.

In RPS19, the gain of the RNP motif could either be tolerated by the mitochondrial ribosome of *Arabidopsis*, without having a real meaning for the translation apparatus, or it may supply the ribosome with an additional function. It has been shown that an extended plastid ribosomal protein is assembled into bacterial ribosomes, and is functional in protein synthesis (Giese and Subramanian, 1991); however, it is unclear whether the extension is needed for the chloroplast translation system. Similarly, large size polymorphisms have been found between eubacterial aminoacyl-tRNA synthetases and their mitochondrial and cytosolic counterparts. These extra sequence domains are dispensable for synthetase activity *per se*; rather, they provide new functional and/or structural characteristics for the enzymes (Akins and Lambowitz, 1987, 1991; Schimmel, 1987). In yeast it has been shown that MRPs are, in general, larger than their eubacterial equivalents (Li *et al.*, 1993) and that these additional domains provide new functions for ribosome assembly and respiratory growth (Huff *et al.*, 1993). These new functional domains, of unknown origin, are highly specific adaptations in yeast and are not found elsewhere. However, in the case of *Arabidopsis* mitochondrial RPS19, the gain of the additional protein domain can be followed back to the glycine-rich protein family, due to the high similarity found between the RNP motif sequences.

The assumption that the RNP domain of RPS19 is functionally important for *Arabidopsis* mitochondrial ribosomes is based on two observations, one relating to analyses of ribosome structure and function in *E.coli*, the other to analysis of a possible ribosomal gene transfer of *rps13* into the nucleus of *Arabidopsis*. Earlier in *E.coli* it had been shown that, individually, neither ribosomal protein S13 nor S19 binds significantly to 16S rRNA, but

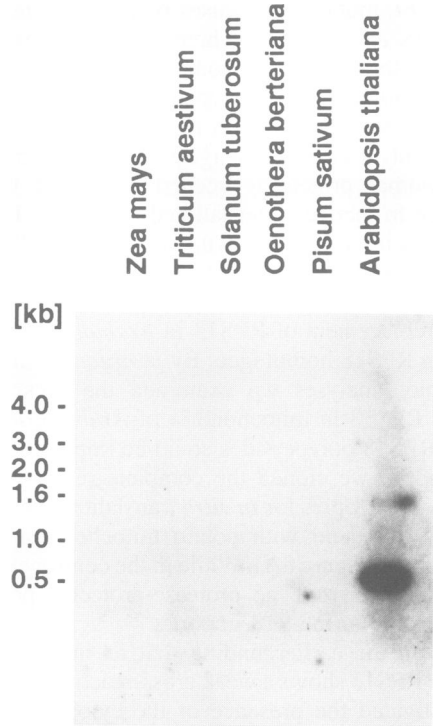

Fig. 11. Hybridization of the *rps19* RNP motif sequence from *Arabidopsis* to nuclear DNAs of higher plant species. Genomic DNA (~5 μg) of maize, wheat, potato, *Oenothera*, pea and *Arabidopsis* was hydrolysed with *Xba*I. DNA was size fractionated by agarose gel electrophoresis, blotted to nylon membrane and probed with an *Xba*I–*Xba*I fragment of 710 bp covering the RNP motif sequence (see Figure 3B, nucleotide position 170–880) under high stringency conditions. Migration of molecular mass standards is indicated on the left in kb.

when they are both present, a rather strong binding is observed (Dijk *et al.*, 1977). RPS13 has two separable functional domains: whereas the N-terminal part is important for the association with RPS19 and forms a protein-binding domain for RPS19, the C-terminal part is primarily responsible for RNA recognition (Schwarzbauer and Craven, 1985). Similar results have been obtained by cross-linking studies of *Bacillus* ribosomes, where these proteins are found to be neighbours (Brockmöller and Kamp, 1988). Cross-linking of initiation factor IF-3 to the S13/S19 protein pair has also been observed (Pon *et al.*, 1982), which indicates that both proteins are involved in initiation and have an important functional role. This view is supported by experiments where it has been shown that the initiator tRNA (but also other tRNAs) can be cross-linked to these two ribosomal proteins (reviewed in Wower *et al.*, 1993).

Because we determined that a functional *rps13* gene was absent from *Arabidopsis* mitochondria, a transfer to the nuclear compartment was expected. However, the only nuclear *rps13* copy showing homology to eubacterial S13 sequences encodes the plastid equivalent of RPS13. While the *rps13* gene product is easily imported into chloroplasts, import into mitochondria was not found. We therefore conclude that a functional replacement of the missing mitochondrial counterpart by the plastid S13 protein is unlikely. The absence of RPS13 from *Arabidopsis* mitochondrial ribosomes is further indicated by Western blot analysis where no eubacterial-like S13 protein in

mitochondrial ribosomes was detected, either by using the mitochondrial RPS13 antiserum or the plastid RPS13 antibody. Similar results have also been obtained by others (Kumar *et al.*, 1995). This result could be due to a highly diverged *rps13* sequence in the nucleus, coding for a mitochondrial RPS13 which for that reason is undetectable by both antisera.

Based on the knowledge of the interaction of the bacterial RPS19 and RPS13 proteins, we propose that the S19 RNP domain either works as suppressor of a highly diverged RPS13 protein, or it replaces the probably missing S13 in *Arabidopsis* mitochondrial ribosomes. The gain of an additional RNA-binding motif by the S19 polypeptide may compensate (or also induce) a putative sequence drift (or loss) of the *rps13* gene, and furthermore may allow a stronger binding of S19 to ribosomal RNA and/or initiation factors. A mutant in *E.coli* that lacks ribosomal protein S13 does not show a clear phenotype (Dabbs, 1991); however, omission of RPS13 from a reconstituted 30S particle results in ~25% loss of ribosome activity (Schwarzbauer and Craven, 1985). These observations indicate that RPS13 is not absolutely essential for ribosome function and may functionally be replaced by different suppressor factors. Functional replacement by a cytosolic counterpart, as has already been observed for a plastid ribosomal protein (Bubunenko *et al.*, 1994), also seems unlikely, because the cytosolic RPS18 is not imported into mitochondria and cannot be identified by immunodetection in this organelle. Degenerate oligonucleotides, derived from the most highly conserved C-terminal part that is conserved between S13 polypeptides of all compartments, failed to detect additional eubacterial-like S13 sequences. If an active cytosolic homologue of S18 exists in the nucleus of *Arabidopsis*, one that is able to replace the mitochondrial S13, its sequence at both the nucleotide and amino acid level is expected to have drifted far away from that of the eubacteria-like ribosomal S13 protein. Furthermore, the time available for such an enormous sequence drift is not long enough because functional *rps13* genes are still present in several mitochondrial genomes of higher plants; therefore, gene transfer of *rps13* from the organelle to the nuclear compartment is expected to have happened relatively recently.

A more general function of the RNP motif in mitochondria, e.g. as import factor of tRNAs into mitochondria or as a possible processing/splicing factor, cannot absolutely be excluded; however, such functions seem unlikely because this proposed RNA-binding domain sequence is only conserved in the *Arabidopsis* genome (Figure 11). Southern blot analysis of different higher plants revealed that the sequence encoding the *S19* RNP motif is not present in others and so may fulfil a very specific function in *Arabidopsis*. In legumes the missing *rps13* gene may be replaced in different ways, either by a chloroplast or cytosolic counterpart, or by a functional mitochondrial S13 gene transfer as proposed for bean (Kumar *et al.*, 1995).

The possibility that the S19 RNP domain acts as a simple import signal for S19 is unlikely, because import studies show that only a small part of the N-terminal extension is cleaved off as signal peptide, while the RNP domain remains fully intact. From our results we conclude instead that the gain of this RNP domain is involved in the functional replacement of ribosomal protein S13 in mitochondria of *Arabidopsis*, and therefore provides an example of how the nucleus can replace organellar functions by analogous functional domains and thereby gain control over their regulation. Many of the observed extensions of other ribosomal proteins may have arisen by a similar process, where shuffling of otherwise well-proven functional units can suppress or replace ancient functions. The origin for most of these additional sequences is unclear; in the case of the RNP domain described here, however, it can be traced back to its ribosomal roots.

Materials and methods

Isolation of mitochondria

Mitochondria were isolated from potato tubers (*Solanum tuberosum* var. *Bintje*) and suspension cell cultures of *Arabidopsis thaliana* by four 5 s pulses in a high speed blender in extraction buffer containing 400 mM mannitol, 1 mM EGTA, 25 mM tricine (pH 7.2), 10 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% BSA and subsequent filtering through two layers of miracloth. The filtrate was spun at 2000 *g* for 15 min at 4°C to remove cellular debris, then at 15 000 *g* for 20 min at 4°C to pellet mitochondria. After several washes in a buffer containing 400 mM mannitol, 10 mM tricine (pH 7.2), 1 mM EGTA, 0.2 mM PMSF, mitochondria were layered onto a 14%–28%–45% Percoll step gradient and spun at 70 000 *g* for 45 min in a Beckman SW-28 rotor at 4°C. The interface band between 28% and 45% was collected and washed several times.

Protein import into mitochondria and radiosequencing

For *in vitro* import studies the purified mitochondria were washed and resuspended in a buffer containing 400 mM mannitol, 10 mM KH_2PO_4 (pH 7.2) and 0.1% BSA. The precursors of RPS19 and RPS13 were synthesized from full-length cDNAs of *Arabidopsis* in the vector pBluescript by coupled transcription–translation in the presence of [^{35}S]methionine according to the supplier's instructions (Promega, Madison, WI). The precursor of RPS18 was synthesized from a PCR fragment, covering a full-length *rps18* cDNA, that was cloned behind the T7 promoter of the vector pCR II. Import assays contained 40 µl purified mitochondria (10 mg protein/ml), 160 µl import buffer and 10 µl of the reticulocyte lysate translation mix. Import buffer consisted of 250 mM mannitol, 20 mM HEPES (pH 7.5), 80 mM KCl, 1 mM K_2HPO_4, 1 mM ATP, 1 mM malate, 2 mM NADH and 1 mM DTT. Import was allowed to proceed for 20 min at 10°C or 20°C. For protease treatment after the import reaction 200 µg/ml proteinase K was added and samples were incubated for 20 min at 20°C, then 1 mM PMSF was added and samples were incubated for a further 15 min. For detergent solubilization, 1% (w/v) Triton X-100 was added together with proteinase K (200 µg/ml). Inhibition of mitochondrial import was tested by the addition of 1 µM valinomycin. After the various import reactions were completed, mitochondria were repurified by centrifugation through a 25% sucrose cushion. The repurified mitochondria were resuspended in loading buffer and heated for 2 min at 100°C prior to fractionation on SDS–polyacrylamide gels. After separation the radiolabelled products were analysed by fluorography. For microsequencing, proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes after SDS–PAGE and sequenced by automatic Edman degradation on an Applied Biosystems gas-phase sequenator (model 473A). Radiolabelled protein bands were visualized on the PVDF membranes by autoradiography, and then subjected to sequencing. To minimize non-specific release of radioactivity, especially at step 1 of the Edman degradation, the membranes were washed by omitting delivery of the coupling reagent.

Protein import into chloroplasts

Intact pea chloroplasts were isolated from pea leaves essentially as described by Waegemann and Soll (1991). Import reactions were performed in the light for 30 min at 25°C in a total volume of 200 µl containing 10 µl of the RPS13 translation assay, 3 mM ATP and chloroplasts corresponding to 50 µg of chlorophyll. The chloroplasts were pelleted, resuspended in 500 µl of 50 mM HEPES, 0.33 M sorbitol (pH 8.0), and treated with thermolysin (100 µg/ml). Unbroken chloroplasts were reisolated by centrifugation through a 45% Percoll cushion at 4500 *g* for 10 min, and analysed by electrophoresis, followed by fluorography.

Isolation of nucleic acids, DNA sequence analysis

Isolation of nucleic acids and Southern blot hybridization has been described previously (Schuster *et al.*, 1988). Sequences were determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using T7 DNA polymerase. Both strands of the DNA were sequenced. DNA manipulations were carried out by standard techniques (Sambrook *et al.*, 1989). Computer analysis was performed on a VAX/VMS system using the GCG package, version 8.1 (Madison, WI). The sequence data of *rps19* and *rps13* have been deposited under accession numbers X77989 and X86734 in the EMBL Nucleotide Sequence database.

Screening for rps19 and rps13

Approximately 5×10^5 clones of a cDNA library (Uni-ZAP XR; Stratagene, La Jolla, CA, USA) made from poly(A)$^+$ mRNA of *Arabidopsis* were screened with the *Oenothera* probes for *rps19* and *rps13* from *Oenothera*. Two degenerate oligonucleotides (5′-TGY TAY CGN GGN ATN CGN CAY-3′, 5′-TGY TAY AGR GGN ATN AGR CAY-3′), derived from the highly conserved C-terminal part of RPS13, were used to screen for S13 cDNA clones. Cloning of cDNAs and excision of cDNA clones from positive phages was done according to the manufacturer's conditions. A genomic library of *Arabidopsis*, cloned into the cosmid vector lorist X, has been screened for the *rps19* gene. Fragments have been further subcloned for detailed analysis into the vector pBluescript.

PCR analysis

For amplification of *rps18* cDNA sequences for *in vitro* translation the following primers were used: 5′-CTG TGG TGG CGC CTC CAG A-3′ and 5′-AGA GAC TAG TTT TTG GAT TCA-3′. PCR was carried out as already described (Schuster, 1994). PCR products obtained were cloned into the vector pCR II using a TA cloning kit (Invitrogen) and sequenced.

Ribosome isolation, overexpression of RPS19 and immunological procedures

Ribosomes of *E.coli* were isolated by a method described by Spedding (1990). Ribosome-enriched fractions of mitochondria and chloroplasts were prepared by a method published by Mache *et al.* (1980) Preparation of cytosolic ribosomes of *Arabidopsis* followed the protocol of Capel and Bourque (1982).

The full-size of *rps19* was cloned in-frame into the expression vector pET-15b and expressed according to the suppliers' instructions (Novagen Inc., Madision, WI). The overexpressed RPS19 was purified on Ni-NTA resin (Quiagen Inc., CA) as described by the manufacturers, and immunization of a rabbit was done according to a standard protocol (Eurogentec, Belgium). Antibodies were affinity-purified on an RPS19-Ni-NTA resin and used further for immunopurification of the *Arabidopsis* mitochondrial S19 protein.

Immunoprecipitation of the *in vitro*-translated RPS18 and RPS19 polypeptides with antibodies directed against *Arabidopsis* RPS19 and *E.coli* RPS13 followed the protocol of Friedrich *et al.* (1989). The immunoprecipitated polypeptides were loaded on 15% SDS–polyacrylamide gels and electrophoresed. After electrophoresis, the gels were fixed and treated with Amplify (Amersham). For detection of the radiolabelled proteins, the gels were dried and fluorographed.

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References

Akins,R.A. and Lambowitz,A.M. (1987) A protein required for splicing group I introns in *Neurospora* mitochondria is mitochondrial tyrosyl-tRNA synthetase or a derivative thereof. *Cell*, **50**, 331–345.

Bandzilius,R.J., Swanson,M.S. and Dreyfuss,G. (1989) RNA-binding proteins as developmental regulators. *Genes Dev.*, **3**, 431–437.

Bland,M., Levings III,C.S. and Matzinger,D.F. (1986) The tobacco mitochondrial ATPase subunit 9 gene is closely linked to an open reading frame for a ribosomal protein. *Mol. Gen. Genet.*, **204**, 8–16.

Bock,H., Brennicke,A. and Schuster,W. (1993) *Rps3* and *rpl16* do not overlap in *Oenothera* mitochondria: GTG as a potential translation initiation codon in plant mitochondria? *Plant Mol. Biol.*, **24**, 811–818.

Bonen,L. (1987) The mitochondrial S13 ribosomal protein gene is silent in wheat embryos and seedlings. *Nucleic Acids Res.*, **15**, 10393–10404.

Bowler,C., Alliotte,T., De Loose,M., van Montagu,M. and Inze,D. (1989) The induction of manganese superoxide dismutase in response to stress in *Nicotiana plumbaginifolia*. *EMBO J.*, **8**, 31–38.

Brandt,P., Unseld,M., Eckert-Ossenkopp,U. and Brennicke,A. (1993) An *rps14* pseudogene is transcribed and edited in *Arabidopsis* mitochondria. *Curr. Genet.*, **24**, 330–336.

Braun,H.-P. and Schmitz,U.K. (1993) Purification and sequencing of cytochrome *b* from potato reveals methionine cleavage of a mitochondrially encoded protein. *FEBS Lett.*, **316**, 128–132.

Brockmöller,J. and Kamp,R.M. (1988) Cross-linking amino acids in the protein pair S13-S19 and sequence analysis of protein S13 of *Bacillus stearothermophilus* ribosomes. *Biochemistry*, **27**, 3372–3381.

Bubunenko,M.G. and Subramanian,A.R. (1994) Recognition of novel and divergent higher plant chloroplast ribosomal proteins by *Escherichia coli* ribosome during *in vivo* assembly. *J. Biol. Chem.*, **269**, 18223–18231.

Bubunenko,M.G., Schmidt,J. and Subramanian,A.R. (1994) Protein substitution in chloroplast ribosome evolution: a eukaryotic cytosolic protein has replaced its organelle homologue (L23) in spinach. *J. Mol. Biol.*, **240**, 28–41.

Burd,C.G. and Dreyfuss,G. (1994) Conserved structures and diversity of functions of RNA-binding proteins. *Science*, **265**, 615–621.

Capel,M.S. and Bourque,D.P. (1982) Characterization of *Nicotiana tabacum* chloroplast and cytoplasmic ribosomal proteins. *J. Biol. Chem.*, **257**, 7746–7755.

Conklin,P.L. and Hanson,M.R. (1991) Ribosomal protein S19 is encoded by the mitochondrial genome in *Petunia hybrida*. *Nucleic Acids Res.*, **19**, 2701–2705.

Covello,P.S. and Gray,M.W. (1992) Silent mitochondrial and active nuclear genes for subunit 2 of cytochrome *c* oxidase (*cox2*) in soybean: evidence for RNA-mediated gene transfer. *EMBO J.*, **11**, 3815–3820.

Dabbs,E.R. (1991) Mutants lacking individual ribosomal proteins as a tool to investigate ribosomal properties. *Biochimie*, **73**, 639–645.

de Oliveira,D.E., Seurinck,J., Inzé,D., van Montagu,M. and Botterman,J. (1990) Differential expression of five *Arabidopsis* genes encoding glycine-rich proteins. *Plant Cell*, **2**, 427–436.

Dijk,J., Littlechild,J. and Garrett,R.A. (1977) The RNA binding properties of 'native' protein–protein complexes isolated from the *Escherichia coli* ribosome. *FEBS Lett.*, **77**, 295–300.

Dreyfuss,G., Swanson,M.S. and Piñol-Roma,S. (1988) Heterogeneous nuclear ribonucleoprotein particles and the pathway of mRNA formation. *Trends Biochem. Sci.*, **13**, 86–91.

Dujon,B. (1981) Mitochondrial genetics and functions. In Strathern,J.N., Jones,E.W. and Broach,J.R. (eds), *Molecular Biology of the Yeast* Saccharomyces*: Life Cycle and Inheritance*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 505–635.

Friedrich,T., Hofhaus,G., Ise,W., Nehls,U., Schmitz,B. and Weiss,H. (1989) A small isoform of NADH:ubiquinone oxidoreductase (complex I) without mitochondrially encoded subunits is made in chloramphenicol-treated *Neurospora crassa*. *Eur. J. Biochem.*, **180**, 173–180.

Giese,K. and Subramanian,A.R. (1991) Expression and functional assembly into bacterial ribosomes of a nuclear-encoded chloroplast ribosomal protein with a long NH_2-terminal extension. *FEBS Lett.*, **288**, 72–76.

Gómez,J., Sánchez-Martínez,D., Stiefel,V., Rigau,J., Puigdomènech,P. and Pagès.M. (1988) A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. *Nature*, **334**, 262–264.

Görlach,M., Wittekind,M., Beckman,R.A., Mueller,L. and Dreyfuss,G. (1992) Interaction of the RNA-binding domain of the hnRNP C proteins with RNA. *EMBO J.*, **11**, 3289–3295.

Greve,B. (1992) Immunochemische Analyse und submolekulare Charakterisierung pflanzlicher Heteropolymere mit Hilfe von mono- und polyklonaler Antikörper. Dissertation, Universität Münster.

Grohmann.L., Graack,H.-R., Kruft,V., Choli,T., Goldschmidt-Reisin,S. and Kitakawa,M. (1991) Extended N-terminal sequencing of proteins of the large ribosomal subunit from yeast mitochondria. *FEBS Lett.*, **284**, 51–56.

Grohmann,L., Brennicke,A. and Schuster,W. (1992) The gene for mitochondrial ribosomal protein *S12* has been transferred to the nuclear genome in *Oenothera*. *Nucleic Acids Res.*, **20**, 5641–5646.

Grohmann,L., Brennicke,A. and Schuster,W. (1993) Genes for mitochondrial ribosomal proteins in plants. In Nierhaus,K., Subramanian,A.R., ErdmannV., Franceschi,F. and Wittmann-Liebold,B. (eds), *The Translational Apparatus*. Plenum Publishing, New York, pp. 599–607.

Gualberto,J.M., Wintz,H., Weil,J.-H. and Grienenberger,J.-M. (1988) The genes coding for subunit 3 of NADH dehydrogenase and for ribosomal protein S12 are present in the wheat and maize mitochondrial genomes and are co-transcribed. *Mol. Gen. Genet.*, **215**, 118–127.

Huff,M.O., Hanic-Joyce,P.J., Dang,H., Rodrigues,L.-A. and Ellis,S.R. (1993) Two inactive fragments derived from the yeast mitochondrial ribosomal protein MrpS28 function *in trans* to support ribosome assembly and respiratory growth. *J. Mol. Biol.*, **233**, 597–605.

Kang,W., Matsushita,Y., Grohmann,L., Graack,H.-R., Kitakawa,M. and Isono,K. (1991) Cloning and analysis of the nuclear gene for YmL33, a protein of the large subunit of the mitochondrial ribosome in *Saccharomyces cerevisiae*. *J. Bacteriol.*, **173**, 4013–4020.

Klein,M., Eckert-Ossenkopp,U., Schmiedeberg,I., Brandt,P., Unseld,M., Brennicke,A. and Schuster,W. (1994) Physical mapping of the mitochondrial genome of *Arabidopsis thaliana* by cosmid and YAC clones. *Plant J.*, **6**, 447–455.

Kumar,R., Drouaud,J., Raynal,M. and Small,I. (1995) Characterization of the nuclear gene encoding chloroplast ribosomal protein S13 from *Arabidopsis thaliana*. *Curr. Genet.*, **28**, 346–352.

Li,Y. and Sugiura,M. (1990) Three distinct ribonucleoproteins from tobacco chloroplasts: each contains a unique amino terminal acidic domain and two ribonucleoprotein consensus motifs. *EMBO J.*, **9**, 3059–3066.

Li,Y., Huff,M.O., Hanic-Joyce,P.J. and Ellis,S.R. (1993) Derivatives of the yeast mitochondrial ribosomal protein MrpS28 replace ribosomal protein S15 as functional components of the *Escherichia coli* ribosome. *J. Mol. Biol.*, **233**, 606–614.

Lindahl,M. *et al.* (1994) Crystal structure of the ribosomal protein S6 from *Thermus thermophilus*. *EMBO J.*, **13**, 1249–1254.

Lindemann,H. and Wittmann-Liebold,B. (1977) Primary structure of protein S13 from the small subunit of *Escherichia coli* ribosomes. *Hoppe-Seyler's Z. Physiol. Chem.*, **358**, 843–863.

Mache,R., Dorne,A.M. and Marti-Battle,R.M. (1980) Characterization of spinach plastid ribosomal proteins by two-dimensional gel electrophoresis. *Mol. Gen. Genet.*, **177**, 333–338.

Nagai,K., Oubridge,C., Jessen,T.H., Li,J. and Evans,P.R. (1990) Crystal structure of the RNA-binding domain of the U1 small nuclear ribonucleoprotein A. *Nature*, **348**, 515–520.

Nugent,J.M. and Palmer,J.D. (1991) RNA-mediated transfer of the gene *coxII* from mitochondrion to the nucleus during flowering plant evolution. *Cell*, **66**, 473–481.

Nugent,J.M. and Palmer,J.D. (1993) Evolution of gene content and gene organization in flowering plant mitochondrial DNA: a general survey and further studies on *coxII* gene transfer to the nucleus. In Brennicke,A. and Kück,U. (eds), *Plant Mitochondria with Emphasis on RNA Editing and Cytoplasmic Male Sterility*. VCH, Germany, pp. 163–170.

Oda,K. *et al.* (1992) Gene organization deduced from the complete sequence of the liverwort *Marchantia polymorpha* mitochondrial DNA. *J. Mol. Biol.*, **223**, 1–7.

Ohkubo,S.A., Muto,Y., Kawauchi,F., Yamao,F. and Osawa,S. (1987) The ribosomal protein gene cluster of *Mycoplasma capricollum*. *Mol. Gen. Genet.*, **210**, 314–322.

Pon,C., Cannistraro,S., Giovane,A. and Gualerzi,C. (1982) Structure–function relationship in *Escherichia coli* initiation factors. Environment of the Cys residue and evidence for a hydrophobic region in initiation factor *IF3* by fluorescence and ESR spectroscopy. *Arch. Biochem. Biophys.*, **217**, 47–57.

Riva,S. *et al.* (1986) Mammalian single-stranded DNA binding protein UP I is derived from the hnRNP core protein A1. *EMBO J.*, **5**, 2267–2273.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger,F., Nicklen,S. and Coulson,A. (1977) DNA sequencing with chain-termination inhibitors *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.

Schimmel,P. (1987) Aminoacyl tRNA synthetases: general scheme of structure–function relationship in the polypeptides and recognition of transfer RNAs. *Annu. Rev. Biochem.*, **56**, 125–158.

Schulte,U., Arretz,M., Schneider,H., Tropschug,M., Wachter,E., Neupert,W. and Weiss,H. (1989) A family of mitochondrial proteins involved in bioenergetics and biogenesis. *Nature*, **339**, 147–149.

Schuster,W. (1994) The highly edited *orf206* in *Oenothera* mitochondria may encode a component of a heme transporter involved in cytochrome *c* biogenesis. *Plant Mol. Biol.*, **25**, 33–42.

Schuster,W. and Brennicke,A. (1987) Plastid DNA in the mitochondrial genome of *Oenothera*: inter- and intraorganellar rearrangements involving part of the plastid ribosomal cistron. *Mol. Gen. Genet.*, **210**, 44–51.

Schuster,W. and Brennicke,A. (1991) RNA editing makes mistakes in plant mitochondria: editing loses sense in transcripts of a *rps19* pseudogene and in creating stop codons in *coxI* and *rps3* mRNAs of *Oenothera*. *Nucleic Acids Res.*, **19**, 6923–6928.

Schuster,W., Hiesel,R., Wissinger,B., Schobel,W. and Brennicke,A. (1988) Isolation and analysis of plant mitochondria and their genomes. In Shaw,C. (ed.), *Plant Molecular Biology. A Practical Approach*. IRL Press, Oxford, UK, pp. 79–102.

Schwarzbauer,J. and Craven,G.R. (1985) Evidence that *E.coli* ribosomal protein S13 has two separable functional domains involved in 16S RNA recognition and protein S19 binding. *Nucleic Acids Res.*, **13**, 6767–6786.

Spedding,G. (1990) Isolation and analysis of ribosomes from prokaryotes, eukaryotes, and organelles. In Spedding,G. (ed.), *Ribosomes and Protein Synthesis: A Practical Approach*. IRL Press at Oxford University Press, Oxford, UK, pp. 1–29.

Sturm,A. (1992) A wound-inducible glycine-rich protein from *Daucus carota* with homology to single-stranded nucleic acid-binding proteins. *Plant Physiol.*, **99**, 1689–1692.

Subramanian,A.R. (1993) Molecular genetics of chloroplast ribosomal proteins. *Trends Biochem. Sci.*, **18**, 177–181.

Takemura,M., Oda,K., Yamato,K., Ohta,E., Nakamura,Y., Nozato,N., Akashi,K. and Ohyama,K. (1992) Gene clusters for ribosomal proteins in the mitochondrial genome of a liverwort, *Marchantia polymorpha*. *Nucleic Acids Res.*, **20**, 3199–3205.

van Lijsebettens,M., Vanderhaeghen,R., de Block,M., Bauw,G., Villarroel,R. and van Montagu,M. (1994) An S18 ribosomal protein gene copy at the *Arabidopsis PFL* locus affects plant development by its specific expression in meristems. *EMBO J.*, **13**, 3378–3388.

van Nocker,S. and Vierstra,R.D. (1993) Two cDNAs from *Arabidopsis thaliana* encode putative RNA binding proteins containing glycine-rich domains. *Plant Mol. Biol.*, **21**, 695–699.

von Heijne,G., Steppuhn,G. and Herrmann,R.G. (1989) Domain structure of mitochondrial and chloroplast peptides. *Eur. J. Biochem.*, **180**, 535–545.

Waegemann,K. and Soll,J. (1991) Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. *Plant J.*, **1**, 149–158.

Wahleithner,J.A. and Wolstenholme,D.R. (1988) Ribosomal protein S14 genes in broad bean mitochondrial DNA. *Nucleic Acids Res.*, **16**, 6897–6913.

Wischmann,C. and Schuster,W. (1995) Transfer of *rps10* from the mitochondrion to the nucleus in *Arabidopsis thaliana*: evidence for RNA-mediated transfer and exon shuffling at the integration site. *FEBS Lett.*, **374**, 152–156.

Wissinger,B., Schuster,W. and Brennicke,A. (1990) Species-specific RNA editing patterns in the mitochondrial *rps13* transcripts of *Oenothera* and *Daucus*. *Mol. Gen. Genet.*, **224**, 389–395.

Wower,J., Sylvers,L.A., Rosen,K.V., Hixson,S.S. and Zimmermann,R.A. (1993) A model of the tRNA binding sites on the *Escherichia coli* ribosome. In Nierhaus,K.H., Franceschi,F., Subramanian,A.R., Erdmann,V.A. and Wittmann-Liebold,B. (eds), *The Translational Apparatus*. Plenum Press, New York, pp. 599–607.

Zanlungo,S., Quiñones,V., Moenne,A., Holuigue,L. and Jordana,X. (1994) A ribosomal protein S10 gene is found in the mitochondrial genome in *Solanum tuberosum*. *Plant Mol. Biol.*, **25**, 743–749.

Zhuo,D. and Bonen,L. (1993) Characterization of the S7 ribosomal protein gene in wheat mitochondria. *Mol. Gen. Genet.*, **236**, 395–401.

Zurawski,G. and Zurawski,S.M. (1985) Structure of the *Escherichia coli* S10 ribosomal protein operon. *Nucleic Acids Res.*, **13**, 4521–4526.

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