Plant Molecular Biology **29**: 519–533, 1995.

Functional analysis of a recently originating, atypical presequence: mitochondrial import and processing of GUS fusion proteins in transgenic tobacco and yeast

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Received 14 November 1994; acccepted in revised form 5 July 1995

Key words: Cytochrome bc_1 complex, mitochondrial presequence, nonhomologous recombination, protein isoforms, Rieske iron-sulfur protein

Abstract

A gene family of at least five members encodes the tobacco mitochondrial Rieske Fe-S protein (RISP). To determine whether all five RISPs are translocated to mitochondria, fusion proteins containing the putative presequences of tobacco RISPs and *Escherichia coli* β-glucuronidase (GUS) were expressed in transgenic tobacco, and the resultant GUS proteins were localized by cell fractionation. The amino-terminal 75 and 71 residues of RISP2 and RISP3, respectively, directed GUS import into mitochondria, where fusion protein processing occurred. The amino-terminal sequence of RISP4, which contains an atypical mitochondrial presequence, can translocate the GUS protein specifically into tobacco mitochondria with apparently low efficiency.

Consistent with the proposal of a conserved mechanism for protein import in plants and fungi, the tobacco RISP3 and RISP4 presequences can direct import and processing of a GUS fusion protein in yeast mitochondria. Plant presequences, however, direct mitochondrial import in yeast less efficiently than the yeast presequence, indicating subtle differences between the plant and yeast mitochondrial import machineries. Our studies show that import of RISP4 may not require positively charged amino acid residues and an amphipathic secondary structure; however, these structural properties may improve the efficiency of mitochondrial import.

Introduction

The mitochondrion of a eukaryotic cell contains a limited amount of genetic information that encodes a small part of its constituent proteins. The majority of mitochondrial proteins are encoded by nuclear genes, translated as precursors in the cytoplasm, and imported into mitochondria. Most nuclear-encoded mitochondrial proteins contain an amino-terminal extension, termed a presequence, that is usually necessary and sufficient to target and translocate the protein to mitochondria [15, 25, 48]. During or after import, the presequence is removed by mitochondrial processing proteases, and the mature protein is folded and translocated to its functional submitochondrial compartment. Compared with the enormous amount of information on mitochon-

drial protein import in fungi, a few studies describe mitochondrial protein import in higher plants [10, 38]. Nevertheless, several studies indicate that eukaryotes, including mammals, fungi, and higher plants, have evolved a conserved mechanism for mitochondrial protein import [7, 11, 12, 29, 44].

The mitochondrial Rieske iron-sulfur protein (RISP) is an obligatory redox component of the cytochrome bc_1 complex of the mitochondrial respiratory electron transport chain [47]. In all eukaryotes this protein is encoded by the nuclear genome, synthesized in the cytosol as a precursor protein, imported and processed in the mitochondrion, and assembled into the bc_1 complex of the inner mitochondrial membrane. At some point on the import/assembly pathway, a 2Fe-2S cluster is inserted into the Rieske iron-sulfur apoprotein. Although it is uncertain exactly where and when the 2Fe-2S cluster is inserted, incorporation of the cluster may not be essential for the assembly of the Rieske protein because mutagenized forms of the Rieske proteins that have lost the ability to configure the 2Fe-2S cluster can still be assembled into the bc_1 complex [24].

The RISP is processed in two steps in fungi such as *Neurospora* [26] and yeast [21]. In the first step, a matrix processing protease cleaves the amino-terminal portion of the presequence, resulting in an intermediate form containing an octameric oligopeptide attached to the mature protein [26]. The octapeptide is then cleaved by a second protease that is thought to be different from the matrix processing enzyme involved in the first processing step [21]. The intermediate is copurified with yeast bc_1 complex under conventional conditions, suggesting that either cleavage of the octapeptide is not a prerequisite for the assembly or the assembly process cannot differentiate the intermediate and mature forms [23].

A conserved three-amino acid motif has been observed for most of the proteins processed in two steps via an octapeptide-attached intermediate form [27, 51]. The three-amino acid motif in the precursor mitochondrial protein does not always lead to a two-step processing. Studies on *in vitro* processing of bovine mitochondrial RISP by rat liver mitochondria showed that the bovine Rieske protein is processed in a single step, despite a cryptic two-step processing site [8]. A more recent study showed that the potato mitochondrial RISP is also processed in a single step [18]. The presequence of the bovine Rieske protein removed by this single-step cleavage probably functions as a constituent subunit of the bc_1 complex because its amino acid sequence is identical to that of subunit 9 of the bovine cytochrome bc_1 complex [8].

A single gene encodes the mitochondrial RISP in fungi and mammals; in contrast, this essential respiratory protein is encoded by a small gene family in higher plants [31]. The five Rieske protein genes cloned in tobacco can be grouped into three subfamilies based primarily on the characteristics of the amino-terminal region of the proteins [31]. The amino-terminal sequences of two subfamilies, including RISP1, RISP2, RISP3, and RISP5, feature positively charged, hydroxylated and hydrophobic residues, and they contain a region with the potential to form an amphipathic α-helix. These features are characteristic of mitochondrial presequences [25, 41, 50]. The amino-terminal sequence of RISP4, on the other hand, does not contain any charged residue and is not predicted by computer analysis to contain an amphipathic secondary structure, which is believed to be essential for mitochondrial presequence function [22, 41, 42]. A detailed sequence analysis has shown that *RISP4* arose by nonhomologous recombination between *RISP2* and an unknown sequence, and the *RISP2*-derived sequence in the *RISP4* gene is identical to the corresponding region in the native *RISP2* copy [31]. It is unclear where and when *RISP4* is expressed in tobacco. These data suggest that *RISP4* is a relatively new gene, which raises the question whether *RISP4* encodes a functional mitochondrial protein.

To determine whether the RISP isoforms are translocated to mitochondria, fusion proteins containing the putative presequences of tobacco RISPs and *E. coli* β-glucuronidase (GUS) were expressed in transgenic tobacco, and the resultant GUS proteins were localized by cell fractionation.

To investigate whether mitochondrial protein import is conserved in yeast and plants [7, 11], we studied the capacity of tobacco RISP presequences to direct GUS import and processing in yeast mitochondria.

Materials and methods

Analysis of presequences

The RISP presequences were analyzed using the HELWHEEL program of PC/GENE (IntelliGenetics, Mountain View, CA). The average hydrophobicity ($\langle H \rangle_{11}$) and average hydrophobic moment ($\langle \mu H \rangle_{11}$) were calculated [16] by moving an 11-amino acid window successively along the presequence. The highest $\langle \mu H \rangle_{11}$ of a potential helix-forming region was plotted versus its $\langle H \rangle_{11}$ to determine the possible functional classes of a presequence peptide [16, 17].

Construction of expression plasmids

pTG20. The tobacco expression construct pTG20 contains a fusion gene consisting of a 5′ sequence of *RISP2* coding for the amino-terminal 75 amino acids fused upstream to the *E. coli uidA* gene coding for the GUS protein. It was constructed by cloning the 375 bp *Xba* I-*Bgl* II fragment of pRISP2 and the *Bam* HI-*Sst* I fragment of pBI101.2 (Clontech Laboratories, Palo Alto, CA) into *Xba* I- and *Sst* I-digested binary vector pBI121 (Clontech Laboratories, Palo Alto, CA) in a three-fragment ligation. This procedure preserves the continuity of the fused open reading frames.

pTG30. The tobacco expression construct pTG30 is similar to pTG20. It was constructed by cloning the 328 bp *Xba* I-*Bgl* II fragment of pRISP3 and the *Bam* HI-*Sst* I fragment of pBI101.2 into *Xba* I- and *Sst* I-digested pBI121.

pTG40. The tobacco expression construct pTG40 was prepared by cloning the 435 bp *Xba* I-*Bgl* II fragment of pRISP4 and the *Bam* HI-*Sst* I fragment of pBI101.2 into *Xba* I- and *Sst* I-digested pBI121.

pYGUS. This yeast expression plasmid was constructed by cloning the *Hind* III-*Bgl* II fragment containing the promoter region of the *RIP1* gene coding for yeast Rieske Fe-S protein [3] from plasmid pRIPG [30] and the *Bam* HI-*Sst* I fragment of pBI121 into the yeast/*E. coli* shuttle vector YEp352 [28] in a three-fragment ligation. pYGUS is a control plasmid encoding a GUS protein without a presequence.

pRGUS. This yeast expression plasmid was constructed by replacing the *Bgl* II-*Sst* I fragment of pYT7 [30] with the *Bam* HI-*Sst* I fragment of pBI101.2. pRGUS is a positive control plasmid for yeast mitochondrial import.

pYG30. The yeast expression plasmid pYG30 was constructed by cloning the *Hind* III-*Bgl* II fragment of pRIPG and the *Bam* HI-*Sst* I fragment of pTG30 into *Hind* III-*Sst* I-digested YEp352 in a three-fragment ligation.

pYG40. This yeast expression plasmid was constructed by cloning the *Hind* III-*Bgl* II fragment of pRIPG and the *Bam* HI-*Sst* I fragment of pTG40 into *Hind* III-*Sst* I-digested YEp352 in a three-fragment ligation.

pYG40(Δ221). The yeast expression plasmid pYG40 (Δ221) is similar to pYG40, except that a 221 bp fragment was deleted in the 5′-untranslated region of *RISP4*. It was constructed by cloning the *Hind*III-*Spe* I fragment of pPrip (J. Huang, unpublished data) containing the yeast *RIP1* gene promoter and the *Spe* I-*Sst* I fragment of pTG40 into *Hind* III-*Sst* I-digested YEp352 in a three-fragment ligation.

pYG40(Δ286). This yeast expression plasmid is similar to pYG40(Δ221), except that a 286 bp fragment instead of a 221 bp fragment was deleted from the 5′-untranslated region of *RISP4*. This deletion eliminated a short open reading frame (ORF) upstream to the main ORF.

All plant and yeast fusion genes contain a linker region located between the presequence and the GUS gene, which encodes a short peptide of eight amino acids (DPRVGNSL). The fusion junction between the mitochondrial presequence and the GUS reporter was confirmed to be in frame in all expression plasmids by sequencing.

Plant transformation

Plant expression plasmids were mobilized into *Agrobacterium tumefaciens* strain LBA4404 (Clontech Laboratories, Palo Alto, CA) by electroporation [37] with Gene Pulsar (Bio-Rad). Transformants were selected, and the structural integrity of the transforming plasmids was confirmed by restriction analysis as described previously [29]. Transformation of tobacco (*Nicotiana tabacum* cv. Xanthi) by the cocultivation method [1], selection of transgenic calli, and regeneration of transgenic plants were as described previously [29]. Transgenic plants were identified by staining for GUS activity of leaf disks with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) [32].

Yeast transformation

Yeast expression plasmids were transformed into strain W303-1A (a kind gift of Dr K. Tatchell, Department of Microbiology, North Carolina State University) by electroporation [2] with Gene Pulsar. Selection of transformants and propagation of cells were as described previously [29]. Yeast mitochondria were isolated as described [14].

Protease accessibility assay

Purified mitochondria (1 mg/ml protein) were incubated with proteinase K (Boehringer Mannheim) at a final concentration of 65 μg/ml for 30 min at 37 °C in the presence or absence of 1% Triton X-100. The GUS protein was found to be resistant to proteinase K digestion at 0–4 °C. The incubation was terminated by adding phenylmethylsulfonyl fluoride in isopropanol to 1 mM. SDS-PAGE of protein gels was as described [34]. Prestained protein molecular weight standards were purchased from BRL.

Assays for GUS activity

GUS activities of purified chloroplasts or mitochondria were measured with the fluorometric substrate 4-methylumbelliferryl-β-glucuronide (4-MUG) with a fluorescence spectrophotometer (Perkin-Elmer, Model 650-10S). Mitochondria and chloroplasts were ruptured by incubation in a hypotonic assay buffer at 37 °C. A standard curve was prepared with known concentration of 4-methylumbelliferone (4-MU) and used for quantitation. SDS-PAGE of the denaturing activity gels (zymogram) and staining of the gel were done as described [36], except that the 2 h incubation step was omitted. Photographs of activity gels were taken by a Polaroid camera using a short-wave UV transilluminator and a Wratten 2B filter.

Results

Analysis of tobacco mitochondrial RISP presequences

The five tobacco RISPs all contain the highly conserved carboxyl domain encompassing the 2Fe-2S redox center that catalyzes electron transport in yeast [30]. Therefore, they are expected to be functional *in planta* if they are mitochondrially localized. Analyses of the amino-terminal sequences of tobacco RISP2 and RISP3 showed that they contain a high proportion of positively charged residues, particularly arginine, hydroxylated residues such as serine and threonine, and hydrophobic residues (Fig. 1A). Positively charged, hydroxylated, and hydrophobic residues are commonly found in mitochondrial presequences [25, 41]. These two presequences also contain regions that can potentially form an amphipathic α-helix (Fig. 1B), which is characteristic of most mitochondrial presequences [41, 50] and is believed to be essential for presequence function [22, 42].

The amino-terminal sequence of RISP4 does not contain positively or negatively charged residues (Fig. 1A). This feature is atypical of any known mitochondrial presequences. The average hydrophobicity ($\langle H \rangle_{11}$) and average hydrophobic moment ($\langle \mu H \rangle_{11}$) of the putative RISP4 presequence were calculated by moving an 11-amino

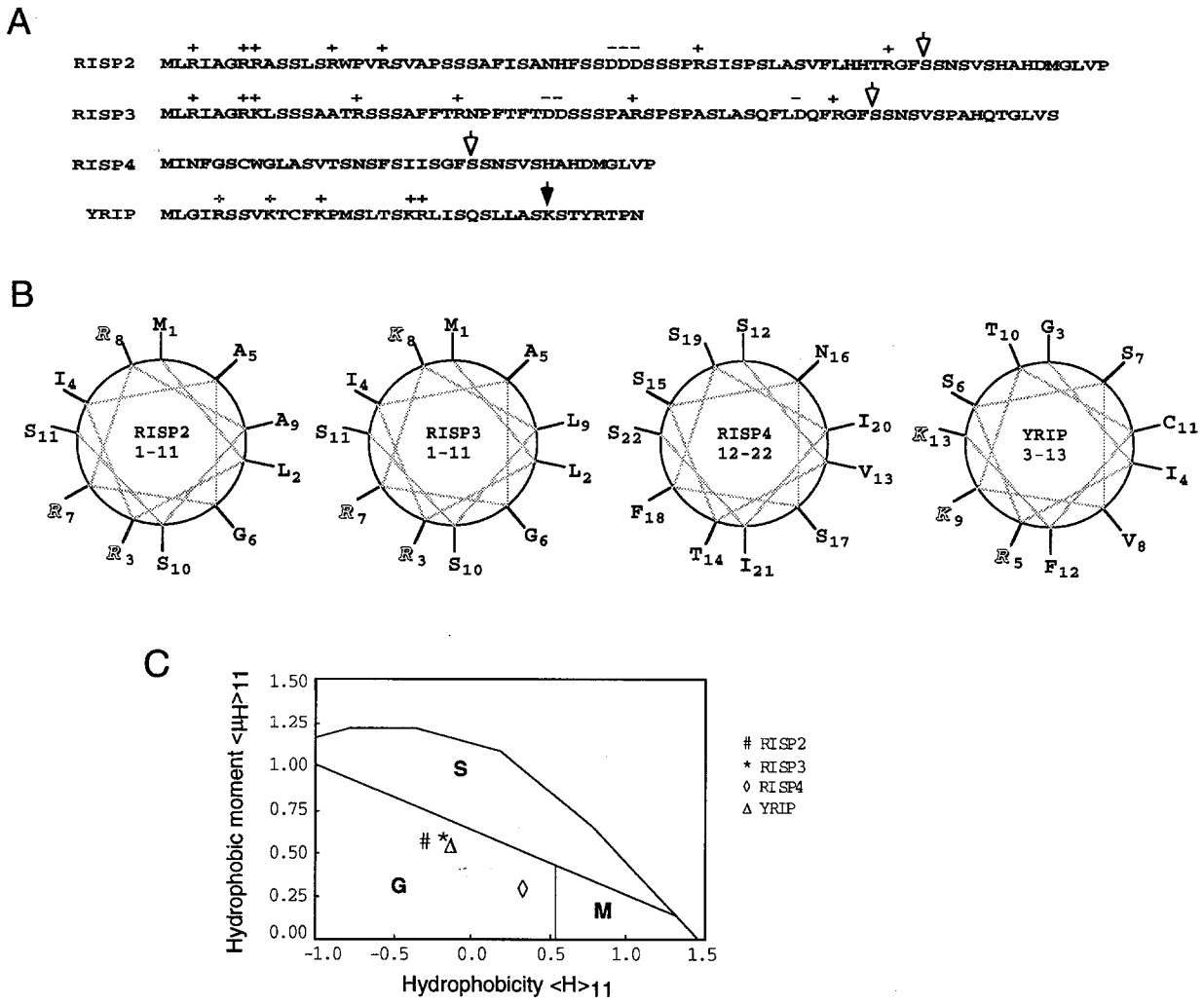

Fig. 1. Analysis of RISP presequences. A. Amino-terminal sequences of RISP2, RISP3, RISP4, and the yeast Rieske Fe-S protein (YRIP) used for making fusion gene constructs. Positively charged (+) and negatively charged (−) residues are designated above the amino acid shown in single-letter code. The open arrow designates the first amino acid of the predicted mature tobacco Rieske protein. The closed arrow indicates the first amino acid of the mature yeast Rieske protein. B. Helical-wheel projection of an 11 amino acid peptide from each of the four presequences shown in A. Positively charged residues, R and K, are outlined and italicized. Numbers indicate the position of the residue in the presequence, with the initiation methionine as number one. C. Localization of the 11 amino acid peptide on the Eisenberg [16] hydrophobic moment plot. The average hydrophobic moment ($\langle \mu H \rangle$) and hydrophobicity ($\langle H \rangle$) were calculated using the PC/GENE program. S, surface-seeking peptide; G, globular peptide; M, membrane peptide.

acid window successively along the presequence [16]. This window size was chosen because peptides of 9 to 14 residues are known to be sufficient to form an effective amphipathic targeting sequence [45]. The peptide encompassing residues 12 to 22 had the highest $\langle \mu H \rangle$. When this peptide was projected on an ideal helical wheel [43], it did not show any apparent amphipathicity (Fig. 1B). The $\langle \mu H \rangle_{11}$ of a potential helix-forming peptide was then plotted as a function of its $\langle H \rangle_{11}$ [16]. The peptides of RISP2, RISP3, and the yeast RIP are clustered near the area characteristic of a surface-seeking peptide as found for most mitochondrial presequences [10]

(Fig. 1C). In contrast, the peptide of RISP4 is separated from these three peptides and localized in a different region (Fig. 1C). The unusual features of the RISP4 presequence, therefore, raised the question whether all tobacco RISPs are mitochondrial proteins.

Construction of presequence-reporter gene fusions

To address the question of the subcellular localization of the tobacco RISPs, gene fusions coding for hybrid proteins containing various Rieske protein presequences fused in frame to the GUS reporter protein were constructed, and the subcellular location of the GUS reporter was analyzed *in vivo*. Because of the great similarity between presequences of members of the same subfamily, a single representative was chosen from each subfamily. Figure 2A shows the schematic structure of the gene fusions used for studies in transgenic plants. Plasmid pTG20 codes for a fusion protein containing the amino-terminal 75 residues of RISP2 fused to the amino terminus of GUS. Similarly, pTG30 and pTG40 code for fusion proteins containing the amino-terminal 71 and 39 residues of RISP3 and RISP4, respectively, fused to GUS. Fifteen amino acid residues of the mature RISP, based on sequence homology with the potato Rieske protein processing site [18], were included in each construct to ensure correct and efficient processing of the fusion proteins. A linker region is also present in the constructs, which encodes eight additional amino acid residues (DPRVGNSL). Consequently, the predicted mature fusion protein is 23 amino acids, or about 2.4 kDa, larger than the native GUS protein. Expression of all plant gene fusions was under control of the cauliflower mosaic virus 35S promoter.

To examine the similarity of mitochondrial protein import in plants and yeast, yeast expression plasmids were constructed to analyze the competence of tobacco RISP presequence in directing GUS import into yeast mitochondria (Fig. 2B). pYGUS served as a negative import control in which the GUS reporter was not fused

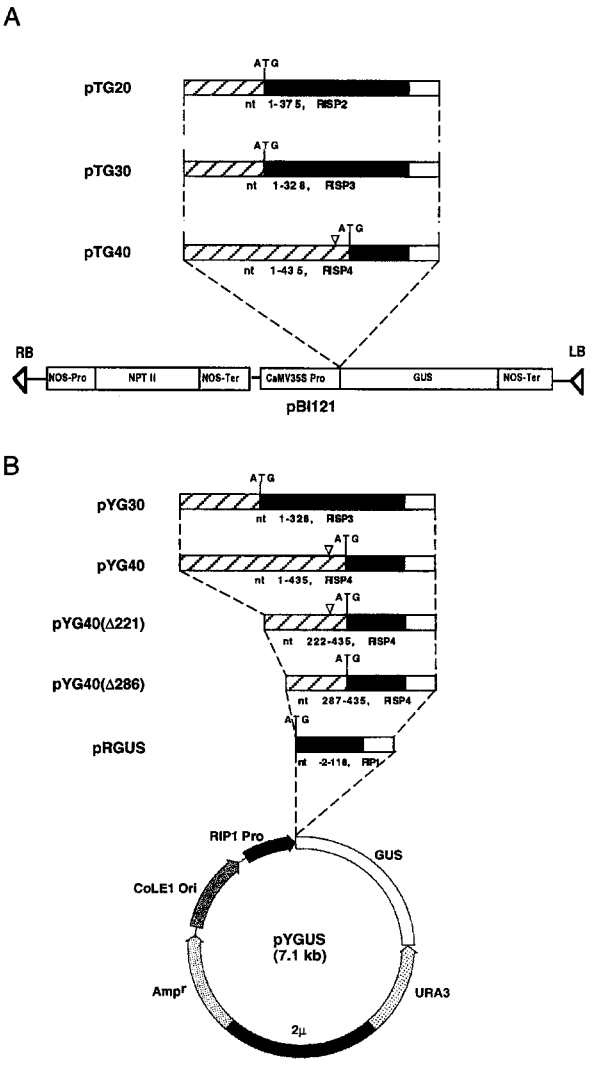

Fig. 2. Plant and yeast fusion gene constructs. A. Fusion genes between a tobacco RISP presequence and the *E. coli uidA* gene encoding GUS cloned into the plant expression binary vector pBI121. The hatched bar is the 5′-untranslated region of tobacco *RISP* genes. The solid bar is the presequence coding region. The open bar is the coding region for 15 amino acids of the tobacco RISP mature protein. The open arrowhead designates a short ORF upstream of the main coding region in the *RISP4* gene. Not drawn to scale. B. Yeast fusion genes. The 5′-untranslated region, the presequence coding region, the mature protein coding region, and the position of the upstream short ORF are labelled as in A. Expression of the fusion genes is under the control of the yeast *RIP1* promoter (RIP1 Pro). Not drawn to scale.

to a mitochondrial presequence. pRGUS codes for a fusion protein consisting of the amino-

terminal 38 residues of the yeast Rieske protein [3] fused to GUS, which was a positive control for import. The presequence/GUS gene fusions in pYG30 and pYG40 are identical to those in the plant expression plasmids pTG30 and pTG40, respectively. A 221 bp fragment was deleted from the 5′-untranslated region of the *RISP4* sequence in pYG40, resulting in pYG40(Δ221). This construct was made so that it has a shorter 5′-untranslated leader similar to the average yeast gene [13]. pYG40(Δ286) contains a longer deletion in the 5′ leader, which also eliminated a three-amino acid ORF (MLA) 27 bp upstream of the main coding frame. Expression of all yeast gene fusions is under control of the yeast *RIP1* gene promoter [3].

Subcellular distribution of GUS activity in transgenic tobacco

Transgenic plants expressing plasmids pTG20, pTG30, pTG40, and pBI121 were regenerated as described previously [29]. pBI121 encodes the native GUS protein; it does not contain a mitochondrial presequence and is a negative control for import. At least 10 plants were checked for GUS expression for each construct. Most of the regenerated plants showed GUS activity as revealed by staining leaf disks with chromogenic substrate X-gluc (data not shown). Untransformed, wild-type plants had no detectable GUS activity.

A representative plant was chosen for pTG20, pTG30, and pBI121 transformants and analyzed for subcellular distribution of GUS activities. Transformants expressing pTG40 consistently showed much lower GUS activity in the total cellular extract, probably because of the short ORF 27 bp upstream of the main ORF. A pTG40 transformant with the highest GUS activity was, therefore, chosen for the subcellular localization study. Mitochondria and chloroplasts were purified from leaves [29] and were assayed for GUS activity by fluorometry [32]. Table 1 shows that mitochondria and chloroplasts from a transgenic plant expressing the negative control plasmid pBI121 had only background-level GUS activity. Mitochondria purified from a pTG20 transformant, however, showed 273-fold higher GUS activity than the control without a presequence. Chloroplasts from the same plant had GUS activity that is not significantly different from the control (Table 1). The transformant expressing pTG30 also showed high GUS activity specifically in the mitochondrial fraction that was about 67-fold higher than the control. The reason(s) for the difference in mitochondrial GUS activity in the pTG20 and pTG30 transformants is unclear, although it might be partly due to lower expression in the pTG30 transformant. These results demonstrate that the first 75 and 71 amino acids of RISP2 and RISP3, respectively, can direct import of the GUS reporter protein specifically to mitochondria. The pTG40 transformant had lower GUS activity in the total cellular extracts. GUS activity was nevertheless enriched about 4.5-fold in the mitochondrial fraction compared with the control (Table 1). Although this enrichment was lower than in the pTG20 and pTG30 transformants, it was mitochondrion specific (Table 1). This result suggests that the first 39 amino acids of RISP4 can direct import of the GUS protein specifically into the mitochondrion with apparently low efficiency.

Table 1. Subcellular distribution of GUS-specific activity in tobacco.[a]

Plant line	Total extracts	Chloroplasts	Mitochondria
NT[b]	0.03 (0.01)	ND[c]	ND
pB1121	3.39 (0.06)	0.53 (0.02)	0.59 (0.04)
pTG20	5.97 (0.08)	0.61 (0.09)	160.93 (12.17)
pTG30	3.77 (0.15)	0.81 (0.07)	39.69 (1.22)
pTG40	0.89 (0.01)	0.07 (0.00)	2.66 (0.10)

[a] GUS-specific activity is given as nmol 4MU per μg protein per minute. The value is the mean of four measurements. SEM is given in parenthesis.
b NT, untransformed, wild-type plant.
[c] Not done.

Because enrichment of GUS activity in mitochondria of the pTG40 transformant was relatively low, protease accessibility assays were conducted to examine the possibility that this increase of activity was due to non-specific association of

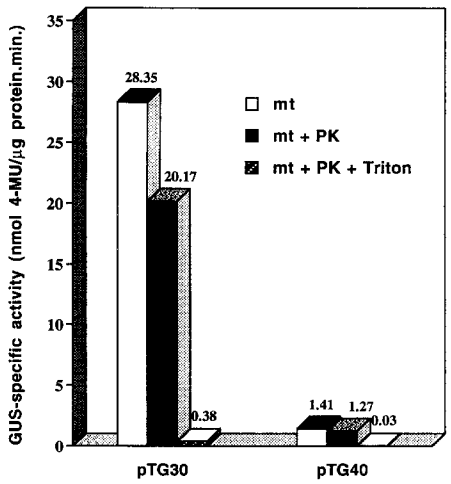

Fig. 3. Protease accessibility assay. Mitochondria (mt) from pTG30 and pTG40 transformants were treated with proteinase K in the absence (mt + PK) or presence (mt + PK + Triton) of Triton X-100 as noted. The GUS-specific activity was measured by a fluorometer. The number on top of each bar is its numeric value on the ordinate.

GUS protein with mitochondria. Figure 3 shows that the mitochondrial GUS activity of the pTG30 transformant was largely resistant to exogenous protease, indicating that the RISP3 presequence directed the GUS protein into the mitochondria. Although there was only a 4.5-fold increase of GUS activity in pTG40 mitochondria, this activity was resistant to protease treatment when mitochondrial membranes were not disrupted by detergent (Fig. 3). This result confirms that the first 39 amino acids of RISP4 can indeed function as a presequence, although with lower efficiency.

Processing of fusion proteins in tobacco mitochondria

The processing site has recently been determined for the potato RISP [18]. The tobacco RISP may be processed at the same site because a nine-amino acid peptide encompassing the cleavage site is identical between potato and tobacco RISP [18, 30]. The fusion proteins encoded by pTG20, pTG30, and pTG40 are likely to be processed after being translocated into mitochondria because the predicted cleavage site was included in each construct (Fig. 1). The precursor fusion proteins encoded by pTG20, pTG30, and pTG40 are ca. 76 kDa, 75.5 kDa, and 72 kDa, respectively, which after processing should give rise to mature proteins of about 70 kDa each. The mature fusion protein was expected to be about 2.4 kDa larger than the native GUS protein because of the mature RISP and linker coding regions in the constructs. In agreement with the results shown in Table 1, no GUS protein was detected in the non-transgenic plant (Fig. 4, lane 1). A protein of about 68 kDa was detected in the cellular extract of the transgenic plant expressing pBI121 (Fig. 4, lane 2). In contrast, a protein with an expected size of about 70 kDa was detected in both the cellular extract and the mitochondrial fraction of pTG20, pTG30, and pTG40 transformants (Fig. 4, lanes 3–8), suggesting that precursor proteins were transported into mitochondria and processed at or near the predicted cleavage site. The absence of the precursor protein in total cellular extracts indicated that the import was a rapid process and that the import machinery was not saturated. Alternatively, the precursor protein may be unstable in the cytosol and rapidly degraded.

Tobacco RISP presequence directs GUS fusion protein import and processing in yeast mitochondria

Although it has been proposed that yeast and higher plants have a conserved mechanism for

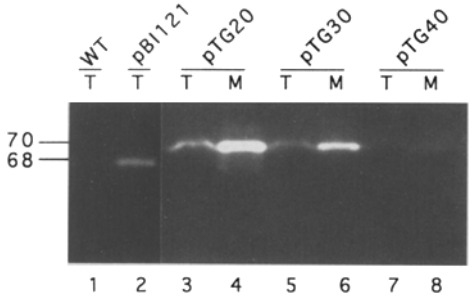

Fig. 4. Processing of GUS fusion proteins in tobacco. Thirty μg of total cellular protein (T) and mitochondrial protein (M) was loaded in each lane. The gel was stained for GUS activity as described [36]. The molecular mass of each band is given in kDa on the left. Lane 1 is the total cellular extracts from an untransformed, wild-type (WT) plant.

mitochondrial protein import [7, 11], recent studies indicate that yeast and higher-plant mitochondria have some intrinsic differences in their protease processing apparatus [9, 19, 20]. To examine the similarity in import, we analyzed the capacity of tobacco RISP3 and RISP4 presequences to direct GUS import into yeast mitochondria. When the GUS protein without a presequence (pYGUS) was expressed in yeast, high GUS activity was detected in total cellular extracts, but the mitochondrial fraction showed only minimal GUS activity (Table 2). When the presequence, plus eight amino acids of yeast RIP1 (Fig. 1A), was fused to GUS (pRGUS), it caused about a 660-fold enrichment of GUS activity in the yeast mitochondrial fraction as compared with the control without a presequence, suggesting that the GUS protein has been translocated into yeast mitochondria (Table 2). The tobacco RISP3 presequence fused to GUS (pYG30) enriched GUS activity about 534-fold in the yeast mitochondrial fraction, suggesting that it can also translocate the GUS reporter protein into yeast mitochondria (Table 2).

Yeast cells transformed with pYG40 had no detectable GUS activity (data not shown), probably because of a relatively long (318 bp), untranslated leader and a short ORF 27 bp 5′ to the main ORF. To test the hypothesis that a long 5′ leader sequence and/or an upstream short ORF is detrimental to RISP4/GUS fusion protein expression, two additional expression plasmids were constructed and expressed in yeast. When yeast cells were transformed with pYG40(Δ221), which contains a 97 bp, untranslated leader sequence, GUS activity was detected (Table 2). As found with transgenic plants, the expression of the RISP4/GUS fusion protein was low in yeast transformants (Table 2). Although deletion of the 5′ short ORF in *RISP4* dramatically increased translation in a cell-free translation system (data not shown), it caused only a marginal increase in expression in yeast (data not shown; Fig. 6). Nevertheless, the RISP4 presequence caused about a 1.8-fold increase in GUS activity in the mitochondrial fraction, although much less than that caused by either the RISP1 or RISP3 presequence (Table 2).

Protease accessibility assays were conducted to determine whether the tobacco RISP presequences can direct the GUS protein into yeast mitochondria. Two protein bands about 73 kDa and 70 kDa, respectively, were detected in isolated yeast mitochondria of pRGUS transformants in the absence of exogenous proteinase K (Fig. 5, lane 3). The 73 kDa band corresponds to the precursor protein, whereas the 70 kDa band is similar to that predicted for the processed mature protein. When mitochondria were treated with proteinase K, the 70 kDa processed mature

Table 2. Subcellular distribution of GUS-specific activity in yeast.[a]

Cell line	Total extracts	Mitochondria
NT[b]	0.01 (0.00)	ND[c]
pYGUS	90.21 (3.65)	0.58 (0.07)
pRGUS	373.22 (90.07)	383.12 (87.89)
pYGUS	220.0 (21.22)	309.95 (47.13)
pYGUS (Δ221)	4.56 (0.13)	1.07 (0.04)

[a] GUS-specific activity is expressed as nmol 4MU per μg protein per minute. The value is the mean of four measurements. SEM is given in parenthesis.
[b] NT, untransformed, wild-type cell.
[c] Not done.

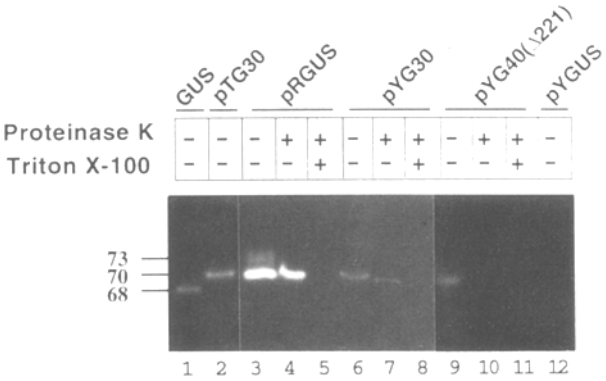

Fig. 5. Processing of GUS fusion proteins in yeast. Thirty μg of mitochondrial protein was treated with proteinase K in the absence or presence of Triton X-100 as noted and separated by SDS-PAGE. The gel was stained for GUS activity as described [36]. The molecular mass of each band is given in kDa on the left. Lane 1 is the GUS protein purified from *E. coli* (Clontech).

protein was protected by the mitochondrial membrane, but the 73 kDa precursor was completely digested (Fig. 5, lane 4). The protection was abolished when the membrane was disrupted by detergent (Fig. 5, lane 5). This result shows that the pRGUS-encoded fusion protein has been imported into and processed in yeast mitochondria.

Similar results were obtained for yeast transformed with pYG30. In this case, the precursor protein was not detected in the mitochondrion (Fig. 5, lane 6), suggesting that the import machinery is not saturated, probably because of the lower expression (Table 2). The mitochondrially associated protein was larger than the native GUS protein and had the size predicted if the processing site in tobacco mitochondria was utilized by the yeast mitochondrial processing protease (Fig. 5, cf. lanes 1, 2, and 6). Moreover, it was protected from proteinase K digestion when the membrane was not disrupted by detergent (Fig. 5, lanes 7 and 8). These results show that the tobacco RISP3 presequence directs the translocation of the GUS protein into yeast mitochondria and that yeast and plant processing enzymes probably recognize the same cleavage site. Interestingly, a significant decrease in GUS activity was consistently observed upon protease treatment of mitochondria isolated from yeast transformants expressing pYG30 (Fig. 5, cf. lanes 6 and 7). This reduction might be due to broken mitochondria in the sample. Alternatively, a significant portion of the GUS protein copurified with mitochondria was incompletely translocated across the mitochondrial membranes (i.e., the amino terminus of the fusion protein was within the mitochondria and accessible to the processing enzymes, whereas the catalytic domain of the GUS protein was still outside and accessible to the protease).

The GUS (pYGUS) without a presequence does not copurify with the mitochondrial fraction (Fig. 5, lane 12, and Table 2). In contrast, the RISP4 presequence resulted in the copurification of the GUS protein with yeast mitochondria (Fig. 5, lane 9). The fusion protein appeared to be processed because it had the same apparent size as the processed mature protein in the pYG30 transformant (Fig. 5, cf. lanes 6 and 9). Nevertheless, it did not seem to be protected from exogenous proteinase K in the absence of detergent (Fig. 5, lane 10). These results suggested that the RISP4 presequence targeted the GUS fusion protein to the mitochondrion but failed to translocate it completely across the yeast mitochondrial membranes. Because the GUS activity copurified with mitochondria of the pYG40(Δ221) transformant is low to begin with, it is possible that a small amount of GUS protein has been translocated into the mitochondrion, but the protease treatment digested the proteins transversing the membranes and rendered the remaining GUS activity in the matrix under the detection limit of the assay.

The following experiments were conducted to confirm that the RISP4/GUS fusion protein was targeted to the yeast mitochondrion by the RISP4 presequence and to investigate the possibility that part of the GUS-fusion protein copurified with mitochondria of the pYG30 and pYG40(Δ221) transformants is imported into mitochondria, whereas part of it still transverses the mitochondrial membranes. First, purified mitochondria were washed in a buffer containing 1 M NaCl and reisolated as a mitochondrial pellet and supernatant fractions [6]. Figure 6A shows that the RISP3/GUS fusion protein was recovered in the pellet as expected (Fig. 6A, lanes 1 and 2). Similarly, in a yeast transformant expressing pYG40(Δ286), the GUS protein was recovered only in the pellet fraction (Fig. 6A, lanes 3 and 4). Analysis of a pYG40(Δ221) transformant revealed that the mitochondrially associated GUS activity was also recovered only in the pellet after washing with high-salt buffer (data not shown). These results confirmed that the RISP4 presequence/GUS fusion protein is targeted to and tightly associated with mitochondrial membranes.

In the second experiment, purified mitochondria were sonicated in the presence of 1 M NaCl, reisolated as the soluble (matrix and intermembrane space) and pellet (membrane) fractions, and analyzed for distribution of the GUS activity in the two fractions. Because it has been confirmed

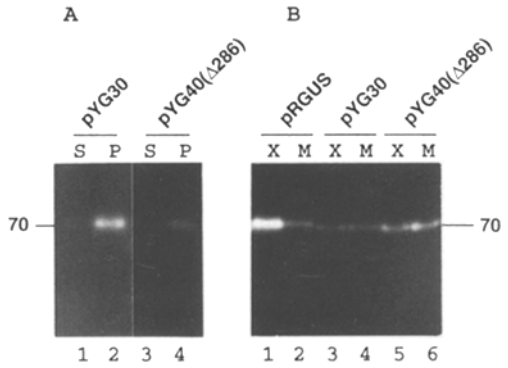

Fig. 6. Association of the RISP3/GUS and RISP4/GUS fusion proteins with mitochondrial membranes in yeast. A. Mitochondria (1 mg/ml) were washed in a washing buffer containing 1 M NaCl. The suspension was then separated into pellet (P) and supernatant (S) fractions. Each pellet contained 30 μg of mitochondrial proteins. The gel was stained for GUS activity as described [36]. The molecular mass of the protein band is given in kDa on the left. B. Mitochondria were sonicated three times (10 s each) in a buffer containing 1 M NaCl and separated into the matrix/intermembrane space (X) and membrane (M) fractions. Seven times more mitochondrial proteins were used for pYG40(Δ286) than for pYG30. The gel was stained for GUS activity. The molecular mass of the protein is given in kDa on the right.

that the RISP4 presequence can target the GUS protein to the yeast mitochondrion (Fig. 6A), seven times more mitochondrial proteins were used for the pYG40(Δ286) transformant than for the pYG30 transformant in this assay to facilitate the detection. When the mitochondria from pRGUS transformants were fractionated and analyzed, most of the GUS activity was found in the matrix (Fig. 6B, lanes 1 and 2), indicating that the yeast RIP1 presequence can translocate the GUS protein efficiently into yeast mitochondria. Similar analyses of mitochondria of the pYG30 (Fig. 6B, lanes 3 and 4) and pYG40(Δ286) (Fig. 6B, lanes 5 and 6) transformants revealed that about 50% of the GUS activity was in the matrix, whereas about 50% of the GUS activity remained in the membrane fractions. These results confirmed that the RISP4 presequence can translocate the GUS protein into yeast mitochondrial matrix and suggested that the RISP3 and RISP4 presequences have a lower efficiency in yeast.

Discussion

In tobacco there are at least five isoforms of the mitochondrial RISP that can be divided into three subfamilies largely based on the primary sequence and the length of their amino-terminal presequences [31]. Existence of multiple RISP genes in tobacco provides an opportunity for differential regulation of their expression. Indeed, expression of two subfamilies was significantly increased in tobacco flowers, most likely in response to high-energy demand during anther development [31]. Because the *in vivo* configuration of the cytochrome bc_1 complex is believed to be a dimer [35, 46], the presence of different isoforms within mitochondria might furnish further opportunity to regulate gene expression at the level of complex assembly and/or protein-protein interaction. To study regulation of expression and function of the mitochondrial RISP in higher plants, we investigated whether all five tobacco RISPs are localized to mitochondria, which is a prerequisite for function.

The five tobacco RISPS are all mitochondrial proteins

We have shown that the amino-terminal 75 and 71 residues of RISP2 and RISP3, respectively, can translocate the GUS reporter protein specifically into mitochondria *in vivo* (Table 1). These results strongly suggest that RISP2 and RISP3 are mitochondrial proteins *in planta*. Because the presequences of RISP2 and RISP1 are nearly identical with each other and the presequences of RISP3 and RISP5 are also nearly identical with each other, it is reasonable to surmise that RISP1 and RISP5 are also localized in mitochondria.

The GUS fusion proteins detected in mitochondria of pTG20, pTG30, and pTG40 transgenic plants have the same apparent molecular mass of about 70 kDa (Fig. 4), which is similar to the expected size if the predicted processing site (Fig. 1A) is utilized. Although the mitochondrial Rieske protein is processed in two steps in *Neurospora* [26] and yeast [21], recent studies show that this protein is processed in one step in bovine [8] and potato [18]. It is uncertain whether

the tobacco RISP is processed in one or two steps, although a two-step processing was postulated [30] based on the conserved three-amino acid motif commonly found in two-step processing proteins [27]. It is interesting that the −3 arginine with respect to the processing site of potato mitochondrial Rieske protein [18] is present in all characterized plant RISPs except RISP4 of tobacco (Fig. 1A), whereas the molecular mass of the processed final products of pTG20-, pTG30-, and pTG40-encoded fusion proteins were apparently the same (Fig. 4). This result implies that the −3 arginine is not essential for tobacco RISP4 processing. *In vitro* processing or import experiments are necessary to determine how the tobacco RISPs are processed.

It is not surprising that RISP1, RISP2, RISP3, and RISP5 are mitochondrially localized because their presequences contain all the characteristics of a mitochondrial presequence. The amino-terminal sequence of RISP4, on the other hand, does not contain any features common to typical mitochondrial presequences or chloroplast transit peptides, except that about 30% of the first 24 amino acids in the putative mitochondrial presequence are serine or threonine residues (Fig. 1A). Nevertheless, the first 39 residues of RISP4 were able to direct, with apparently low efficiency, import of the GUS protein specifically to mitochondria (Table 1; Fig. 3), suggesting that RISP4 is a mitochondrial protein in tobacco. Furthermore, the RISP4 presequence/GUS fusion protein was specifically processed (Fig. 4), which further supports the view that RISP4 is translocated into mitochondria. Our results do not exclude the possibilities that an internal targeting signal [52] in the mature RISP may participate in import *in vivo* and that the eight amino acids (DPRVGNSL) encoded by the linker region may contribute to the import of the RISP4 presequence/GUS fusion protein.

Tobacco RISP presequences function in yeast to direct mitochondrial protein import

Several studies have suggested that yeast and plants have evolved common mechanisms for mitochondrial protein import and processing [7, 11, 29, 44]. A recent study showed that the presequence to the ATPase δ-subunit of sweet potato failed to translocate the GUS reporter protein into yeast mitochondria [33], although this presequence may be peculiar because it could not translocate the GUS protein into tobacco and rice mitochondria. Several studies have shown that yeast and higher-plant mitochondria have some intrinsic differences in their protease-processing apparatus [9, 19, 20]. To compare the mitochondrial protein import mechanism in yeast and plants, we investigated the capacity of the tobacco RISP presequence to direct protein import in yeast. Consistent with most of the previous studies, RISP3 presequence translocated the GUS reporter protein into yeast mitochondria (Table 2) and directed the processing of the GUS fusion protein (Fig. 5). Because the processed products in plant and yeast mitochondria had the same apparent molecular size (Fig. 5, cf. lanes 2 and 6), it is likely that the same processing site was utilized in both organisms. These results support the notion that yeast and plants have a functionally conserved mechanism for mitochondrial protein import and/or processing [7, 11]. Because about 50% of the fusion proteins remained as translocation intermediates that transverse the mitochondrial membranes in the pYG30 and pYG40 transformants (Fig. 6B), the plant RISP presequences may not be as efficient in the yeast as the yeast RISP presequence, suggesting that subtle differences exist between plant and yeast import machineries.

Functional and evolutionary prospectives of the RISP4 mitochondrial presequence

Positively charged residues [4] and amphipathicity [22, 41, 42] are believed to be essential constituents of mitochondrial presequences. Surprisingly, even though the RISP4 presequence does not have these two important features, it can direct protein import into tobacco mitochondria. On the other hand, the enrichment of GUS activity in mitochondria by the RISP4 presequence

was relatively low compared with that conferred by presequences of RISP2 and RISP3, which are typical mitochondrial presequences. This low mitochondrial GUS activity is probably caused by the lower efficacy of RISP4 presequence. Other factors may also be involved; these include the reduced expression in the pTG40 transformants and the possibility that the RISP4 presequence directed mitochondrial import through a bypass pathway [39, 40] which is characterized by its low efficiency. It is interesting, however, that the RISP4 presequence could translocate the GUS reporter protein into yeast mitochondria with low efficiency (Fig. 6).

Sequence analysis has previously shown that the *RISP4* gene arose by nonhomologous recombination between *RISP2* and an unknown sequence [31]. The DNA sequence downstream of the recombination point in *RISP4*, including the protein-encoding and the 3′-untranslated regions, is identical to the corresponding sequence of *RISP2*. For these sequences to be identical, the recombinational event must have been recent, or a mechanism must exist for maintaining the identity. The *RISP4* gene has the necessary regulatory sequences to control its transcription and polyadenylation [31], and it encodes a mitochondrial presequence that can direct import, although weakly, of the GUS reporter protein into tobacco mitochondria (Table 1; Fig. 3). Mutational studies on truncated, nonfunctional precursor proteins of yeast ATPase β-subunit [49] and cytochrome oxidase subunit IV [5] have shown that a functional presequence can arise by point mutations which increase the overall amphipathicity of the newly formed presequence. If a selection pressure exists for the *RISP4* gene, its proximal sequence may evolve into a more efficient mitochondrial presequence. In a sense, the origination of the *RISP4* gene and its acquisition of a functional presequence coding region may be paralleled with the transfer of organellar genes to the nucleus and their subsequent activation during evolution. Our results are consistent with the idea that when mitochondrial genes transferred to the nucleus and gained a flanking sequence, the efficiency of the initial presequence does not have to be high. It is also interesting that the targeting specificity of the RISP4 presequence has been established, irrespective of its low efficiency (Table 1). Nevertheless, the current physiological role of *RISP4* may be trivial because its transcript is in low abundance [31], its processed protein product is expected to be identical to RISP2, and its mitochondrial import is inefficient.

Acknowledgements

The authors thank Jim Siedow and members of the Levings laboratory for critical reading of the manuscript; Long Nguyen for assistance with computer analysis; David Rhoads for discussion of presequence analysis; Mary Clark, Jane Suddith, and Carol Griffin for excellent technical assistance; and Suzanne Quick for editorial assistance. This work was supported by a grant from the National Science Foundation to C.S.L.

References

1. An G, Ebert PR, Mitra A, Ha SB: Binary vectors. In: Gelvin SB, Schilperoort RA, Verma DPS (eds) Plant Molecular Biology Manual, pp. A3/1–A3/19. Kluwer Academic Publishers, Dordrecht (1988).
2. Becker DM, Guarente L: High-efficiency transformation of yeast by electroporation. In: Guthrie C, Fink GR (eds) Guide to Yeast Genetics and Molecular Biology, pp. 182–187. Academic Press, San Diego (1991).
3. Beckmann JD, Ljungdahl PO, Lopez JL, Trumpower BL: Isolation and characterization of the nuclear gene encoding the Rieske iron-sulfur protein (RIP1) from *Saccharomyces cerevisiae*. J Biol Chem 262: 8901–8909 (1987).
4. Bedwell DM, Strobel SA, Yun K, Jongeward GD, Emr SD: Sequence and structural requirements of a mitochondrial protein import signal defined by saturation cassette mutagenesis. Mol Cell Biol 9: 1014–1025 (1989).
5. Bibus CR, Lemire BD, Suda K, Schatz G: Mutations restoring import of a yeast mitochondrial protein with a nonfunctional presequence. J Biol Chem 263: 13097–13102 (1988).
6. Boutry M, Nagy F, Poulsen C, Aoyagi K, Chua N-H: Targeting of bacterial chloramphenicol acetyltransferase to mitochondria in transgenic plants. Nature 328: 340–342 (1987).
7. Bowler C, Alliotte T, Van den Bulcke M, Bauw G, Vande-

kerckhove J, Van Montagu M, Inzé D: A plant manganese superoxide dismutase is efficiently imported and correctly processed by yeast mitochondria. Proc Natl Acad Sci USA 86: 3237–3241 (1989).

8. Brandt U, Yu L, Yu C-A, Trumpower BL: The mitochondrial targeting presequence of the Rieske iron-sulfur protein is processed in a single step after insertion into the cytochrome bc_1 complex in mammals and retained as a subunit in the complex. J Biol Chem 268: 8387–8390 (1993).
9. Braun H-P, Emmermann M, Kruft V, Schmitz UK: The general mitochondrial processing peptidase from potato is an integral part of cytochrome *c* reductase of the respiratory chain. EMBO J 11: 3219–3227 (1992).
10. Chaumont F, Boutry M: Protein import into plant mitochondria. In: Levings CS III, Vasil IK (eds) Molecular Biology of the Mitochondria. Kluwer Academic Publishers, Dordrecht (in press).
11. Chaumont F, O'Riordan V, Boutry M: Protein transport into mitochondria is conserved between plant and yeast species. J Biol Chem 265: 16856–16862 (1990).
12. Cheng MY, Pollock RA, Hendrick JP, Horwich AL: Import and processing of human ornithine transcarbamoylase precursor by mitochondria from *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 84: 4063–4067 (1987).
13. Cigan AM, Donahue TF: Sequence and structural features associated with translational initiator regions in yeast: a review. Gene 59: 1–18 (1987).
14. Daum G, Böhni PC, Schatz G: Import of proteins into mitochondria. Cytochrome b_2 and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. J Biol Chem 257: 13028–13033 (1982).
15. Dircks LK, Poyton RO: Overexpression of a leaderless form of yeast cytochrome *c* oxidase subunit V_a circumvents the requirement for a leader peptide in mitochondrial import. Mol Cell Biol 10: 4984–4986 (1990).
16. Eisenberg D, Schwarz E, Komaromy M, Wall R: Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J Mol Biol 179: 125–142 (1984).
17. Eisenberg D, Weiss RM, Terwilliger TC: The helical hydrophobic moment: a measure of the amphiphilicity of a helix. Nature 299: 371–374 (1982).
18. Emmermann M, Clericus M, Braun H-P, Mozo T, Heins L, Kruft V, Schmitz UK: Molecular features, processing and import of the Rieske iron-sulfur protein from potato mitochondria. Plant Mol Biol 25: 271–281 (1994).
19. Emmermann M, Schmitz UK: The cytochrome *c* reductase integrated processing peptidase from potato mitochondria belongs to a new class of metalloendoproteases. Plant Physiol 103: 615–620 (1993).
20. Eriksson AC, Glaser E: Mitochondrial processing proteinase: a general processing proteinase of spinach leaf mitochondria is a membrane-bound enzyme. Biochim Biophys Acta 1140: 208–214 (1992).
21. Fu W, Japa S, Beattie DS: Import of the iron-sulfur protein of the cytochrome $b.c_1$ complex into yeast mitochondria. J Biol Chem 265: 16541–16547 (1990).
22. Gavel Y, Nilsson L, von Heijne G: Mitochondrial targeting sequences. Why 'non-amphiphilic' peptides may still be amphiphilic. FEBS Lett 235: 173–177 (1988).
23. Graham LA, Brandt U, Sargent JS, Trumpower BL: Mutational analysis of assembly and function of the iron-sulfur protein of the cytochrome bc_1 complex in *Saccharomyces cerevisiae*. J Bioenerget Biomembr 25: 245–257 (1993).
24. Graham LA, Trumpower BL: Mutational analysis of the mitochondrial Rieske iron-sulfur protein of *Saccharomyces cerevisiae*. III. Import, protease processing, and assembly into the cytochrome bc_1 complex of iron-sulfur protein lacking the iron-sulfur cluster. J Biol Chem 266: 22485–22492 (1991).
25. Hartl F-U, Pfanner N, Nicholson DW, Neupert W: Mitochondrial protein import. Biochim Biophys Acta 988: 1–45 (1989).
26. Hartl F-U, Schmidt B, Wachter E, Weiss H, Neupert W: Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome *c* reductase. Cell 47: 939–951 (1986).
27. Hendrick JP, Hodges PE, Rosenberg LE: Survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins: leader peptides cleaved by two matrix proteases share a three-amino acid motif. Proc Natl Acad Sci USA 86: 4056–4060 (1989).
28. Hill JE, Myers AM, Koerner TJ, Tzagoloff A: Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast 2: 163–167 (1986).
29. Huang J, Hack E, Thornburg RW, Myers AM: A yeast mitochondrial leader peptide functions in vivo as a dual targeting signal for both chloroplasts and mitochondria. Plant Cell 2: 1249–1260 (1990).
30. Huang J, Struck F, Matzinger DF, Levings CS III: Functional analysis in yeast of cDNA coding for the mitochondrial Rieske iron-sulfur protein of higher plants. Proc Natl Acad Sci USA 88: 10716–10720 (1991).
31. Huang J, Struck F, Matzinger DF, Levings CS III: Flower-enhanced expression of a nuclear-encoded mitochondrial respiratory protein is associated with changes in mitochondrion number. Plant Cell 6: 439–448 (1994).
32. Jefferson RA: Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5: 387–405 (1987).
33. Kimura T, Takeda S, Kyozuka J, Asahi T, Shimamoto K, Nakamura K: The presequence of a precursor to the δ-subunit of sweet potato mitochondrial F_1ATPase is not sufficient for the transport of β-glucuronidase (GUS) into mitochondria of tobacco, rice and yeast cells. Plant Cell Physiol 34: 345–355 (1993).
34. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685 (1970).

35. Leonard K, Wingfield P, Arad T, Weiss H: Three-dimensional structure of ubiquinol:cytochrome *c* reductase from *Neurospora* mitochondria determined by electron microscopy of membrane crystals. J Mol Biol 149: 259–274 (1981).
36. Martin T, Wöhner R-V, Hummel S, Willmitzer L, Frommer WB: The GUS reporter system as a tool to study plant gene expression. In: Gallagher SR (ed), GUS Protocols: using the GUS Gene as a Reporter of Gene Expression, pp. 23–43. Academic Press, San Diego (1992).
37. Mersereau M, Pazour GJ, Das A: Efficient transformation of *Agrobacterium tumefaciens* by electroporation. Gene 90: 149–151 (1990).
38. Moore AL, Wood CK, Watts FZ: Protein import into plant mitochondria. Annu Rev Plant Physiol Plant Mol Biol 45: 545–575 (1994).
39. Pfaller R, Pfanner N, Neupert W: Mitochondrial protein import. Bypass of proteinaceous surface receptors can occur with low specificity and efficiency. J Biol Chem 264: 34–39 (1989).
40. Pfanner N, Pfaller R, Neupert W: How finicky is mitochondrial protein import? Trends Biochem Sci 13: 165–167 (1988).
41. Roise D, Schatz G: Mitochondrial presequences. J Biol Chem 263: 4509–4511 (1988).
42. Roise D, Theiler F, Horvath SJ, Tomich JM, Richards JH, Allison DS, Schatz G: Amphiphilicity is essential for mitochondrial presequence function. EMBO J 7: 649–653 (1988).
43. Schiffer M, Edmundson AB: Use of helical wheels to represent the structures of proteins and to identify segments with helical potential. Biophys J 7: 121–135 (1967).
44. Schmitz UK, Lonsdale DM: A yeast mitochondrial presequence functions as a signal for targeting to plant mitochondria in vivo. Plant Cell 1: 783–791 (1989).
45. Tamm LK: Membrane insertion and lateral mobility of synthetic amphiphilic signal peptides in lipid model membranes. Biochim Biophys Acta 1071: 123–148 (1991).
46. Trumpower BL: Cytochrome bc_1 complexes of microorganisms. Microbiol Rev 54: 101–129 (1990).
47. Trumpower BL: Function of the iron-sulfur protein of the cytochrome b–c_1 segment in electron-transfer and energy-conserving reactions of the mitochondrial respiratory chain. Biochim Biophys Acta 639: 129–155 (1981).
48. van Steeg H, Oudshoorn P, van Hell B, Polman JEM, Grivell LA: Targeting efficiency of a mitochondrial presequence is dependent on the passenger protein. EMBO J 5: 3643–3650 (1986).
49. Vassarotti A, Stroud R, Douglas M: Independent mutations at the amino terminus of a protein act as surrogate signals for mitochondrial import. EMBO J 6: 705–711 (1987).
50. von Heijne G: Mitochondrial targeting sequences may form amphiphilic helices. EMBO J 5: 1335–1342 (1986).
51. von Heijne G, Steppuhn J, Herrmann RG: Domain structure of mitochondrial and chloroplast targeting peptides. Eur J Biochem 180: 535–545 (1989).
52. Winning BM, Sarah CJ, Purdue PE, Day CD, Leaver CJ: The adenine nucleotide translocator of higher plants is synthesized as a large precursor that is processed upon import into mitochondria. Plant J 2: 763–773 (1992).