Identification, Expression, and Import of Components 17 and 23 of the Inner Mitochondrial Membrane Translocase from Arabidopsis[1][w]

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Characterization of components 17 and 23 of the inner mitochondrial membrane translocase (TIM17:23) from Arabidopsis indicated that there were three genes present for *TIM17* and *TIM23* and two for *TIM44*. *AtTIM17* differed from the yeast (*Saccharomyces cerevisiae*) and mammalian homologs in that two genes encoded proteins that were longer and one gene encoded a shorter protein. All Arabidopsis TIM23 predicted proteins appeared to lack the first 34 amino acids compared with yeast TIM23. All *AtTIM17* and *AtTIM23* genes were expressed but displayed different tissue and developmental profiles. Complementation of deletion mutants in yeast indicated that for *AtTIM17*, the extension at the C terminus not present in yeast had to be removed to achieve complementation, whereas for *TIM23*, a preprotein and amino acid transporter domain had to be present for complementation. Import assays with *At*TIM17 and *At*TIM23 indicated that they both contained internal signals for integration into the inner mitochondrial membrane in a membrane potential-dependent manner. The C terminus of imported *At*TIM17-2 was susceptible to degradation by externally added protease with intact mitochondria. Removal of the 85 C-terminal amino acids resulted in import and full protection of the truncated protein. This suggests that the novel extension at the C terminus of *At*TIM17-2 links the outer and inner membrane in a manner analogous to yeast TIM23.

Mitochondria import several hundred nuclear encoded cytosolically synthesized proteins via the combined action of multisubunit protein complexes present in the outer and inner mitochondrial membranes (Bauer et al., 2000; Pfanner and Geissler, 2001). The translocase of the outer mitochondrial membrane (TOM) contains the receptor(s) for recognizing mitochondrial proteins and forms a pore in the outer membrane to pass the imported protein to one of the two translocase complexes of the inner membrane (TIM), which form pores in the inner mitochondrial membrane (Moro et al., 1999; Donzeau et al., 2000; Stan et al., 2000; Truscott et al., 2001; Kovermann et al., 2002; Model et al., 2002). The proposed uniform nomenclature will be used for components of the mitochondrial import apparatus (Pfanner et al., 1996). A number of chaperone proteins in the cytosol, small TIM proteins in the inter membrane space, chaperone proteins, and peptidases in the matrix act with the membrane bound translocases to import the hundreds of different proteins necessary to maintain mitochondrial function (Hartl et al., 1989; Koehler, 2000; Matouschek et al., 2000; George et al., 2002).

The TOM and TIM complexes were first characterized in yeast (*Saccharomyces cerevisiae*) and subsequently identified to different extents in *Neurospora crassa*, mammalian, and plant systems. The primary components of the TOM complex, the receptor subunits of 20 and 70 kD, the central organizer subunit of 22 kD, and pore-forming subunit of 40 kD appear well conserved in structural organization between yeast and mammalian systems (Hill et al., 1998; Rapaport et al., 1998; Brix et al., 1999; van Wilpe et al., 1999; Kanaji et al., 2000; Saeki et al., 2000; Stan et al., 2000; Suzuki et al., 2000, 2002; Yano et al., 2000; Model et al., 2002). The plant TOM complex differs from the characterized yeast and mammalian complexes in that it migrates as a smaller complex on Blue-Native-PAGE, 250 kD compared with 400 kD for yeast and mammalian complexes (Pfanner and Geissler, 2001; Werhahn et al., 2001; Suzuki et al., 2002). This plant TOM complex on BN-PAGE contains the TOM20 receptor in contrast to the 400-kD core complex of yeast, which does not contain TOM20 or TOM70 (Pfanner and Geissler, 2001; Werhahn et al., 2001). A noteworthy difference with the plant Tom complex is the absence of a 22-kD subunit. A subunit with a molecular mass of 9 kD, which is structurally similar to TOM22 but lacks the cytosolic receptor domain, is the likely replacement for TOM22 (Mascasev et al., 2000). Furthermore plant TOM20

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may be anchored in the outer membrane by the C terminus in contrast to the N terminus evident in yeast and mammalian systems (Werhahn et al., 2001).

Genetic rather than biochemical approaches have been used to characterize the two TIM complexes in yeast (Rehling et al., 2001). The TIM17:23 complex was the first identified and is responsible for the import of proteins that generally contain cleavable targeting amino acid extensions. The TIM22 translocase is responsible for the import of proteins with internal targeting sequences (Rehling et al., 2001). TIM23 and TIM22 form voltage sensitive channels in the inner membrane that are activated by mitochondrial targeting signals (Truscott et al., 2001; Kovermann et al., 2002). TIM17, TIM22, and TIM23 all contain four transmembrane regions with the N and C termini on the intermembrane space side of the mitochondrial inner membrane (Rassow et al., 1999). Structural and genetic characterization of human TIM17:23 led to the conclusion that this translocase is highly conserved in all eukaryotes (Rassow et al., 1999). Gene sequences from a variety of organisms indicate that TIM22 and associated small TIM proteins 8, 9, 10, and 13 are well conserved (Bauer et al., 1999). Only one report exists concerning functional characterization of TIM components in plants (Lister et al., 2002), where it was shown that TIM9 and TIM10 are necessary for import of carrier proteins into the inner membrane using a biochemical reconstitution assay with potato (*Solanum tuberosum*) mitochondria.

Although the protein import process has been studied in plants for more than 10 years, progress on the identification and role of various import components is still at an early stage. The availability of the Arabidopsis and yeast genome sequences with the detailed characterization of the components of the import apparatus in yeast opens the way for a comparative analysis of the plant import apparatus (Goffeau, 1987; Arabidopsis Genome Initiative, 2000). It is well established that plant mitochondria differ from their yeast and mammalian counterparts in several respects, including biochemistry, genome structure, expression, use of genetic code, coding capacity, and the process of programmed cell death (Douce and Neuberger, 1989; Schuster et al., 1991; Schuster and Brennicke, 1994; Neuburger et al., 1996; Lam et al., 2001). The limited analysis of plant mitochondrial components to date indicates major differences in targeting requirements and specificity (Tanudji et al., 1999; Mascasev et al., 2000) and in the location of processing peptidases (Glaser and Dessi, 1999). Plant mitochondria import tRNA (Marechal-Drouard et al., 1993), and dual targeting of several proteins to mitochondria and plastids takes place (Small et al., 1998; Peeters et al., 2000). Thus homologous components in plants may carry out different or additional functions compared with other organisms.

Biochemical methods can be used to identify and characterize the TOM complex of plant mitochondria because it is the only major complex visible on BN-PAGE analysis (Werhahn and Braun, 2002). This direct approach cannot be used to characterize TIMs due to the presence of several multisubunit respiratory chain complexes in excess to the TIM complexes. We used genome information available from Arabidopsis to characterize homologs of TIM17 and TIM23 because they appeared to differ substantially in nature from their well-characterized yeast counterparts. We characterized the expression, function, import signals, and topology of the Arabidopsis homologs to TIM17 and TIM23 to gain a better understanding of these components compared with other eukaryotes.

RESULTS

Identification of Arabidopsis Homologs of TIM17:23

We searched the Arabidopsis genome for homologs of *TIM17:23*. Three homologs for *TIM17* on chromosomes 1, 2 and 5 were found and called *AtTIM17-1*, *AtTIM17-2*, and *AtTIM17-3*, respectively (*At*1g20350, *At*2g37410, and *At*5g11690). The predicted proteins display 52% similarity and 44%, 47%, and 42% identity, respectively, with *Sc*TIM17. Three homologs for *TIM23* were found, two on chromosome 1 (*AtTIM23-1* [*At*1g17530] and *AtTIM23-2* [At1g72750]) and one on chromosome 3 (*AtTIM23-3* [*At*3g04800]), predicted proteins displaying 35% similarity and 27%, 26%, and 22% identity, respectively, to *Sc*TIM23. Two homologs to *TIM44* (*AtTIM44-1* [*At*2g20510] and *AtTIM44-2* [*At*2g36070]) were detected on chromosome 2, predicted proteins both displayed 23% identity and 34% similarity to *Sc*-TIM44. Analysis of the predicted proteins for these homologs indicated that there were significant differences in the structures of the predicted TIM17 and TIM23 proteins (Fig. 1; Supplementary Fig. 1; supplementary data can be viewed at www.plantphysiol.org). In comparison with *Sc*TIM17 with 158 amino acids, the plant homologs *At*TIM17-1 and *At*TIM17-2 contain a 60 and 85 amino acid extension at the C

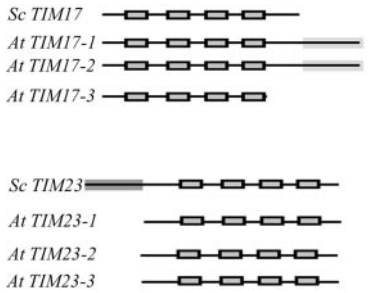

Figure 1. Diagrammatic representation of the *TIM17* and *TIM23* genes in Arabidopsis. The predicted proteins encoded by Arabidopsis homologs to *TIM17* and *TIM23* are indicated with the yeast proteins as comparison. Predicted transmembrane regions are indicated by a black box.

terminus of the protein, respectively (Supplementary Fig. 1). In contrast, *At*TIM17-3 was 25 amino acids shorter at the C terminus (Supplementary Fig. 1). All three were predicted to have four transmembrane regions. However, with *At*TIM17-3, the fourth predicted transmembrane region was at the very end of the protein (Supplementary Fig. 1). All three of the predicted TIM23 proteins were very similar, containing 187 or 188 amino acids, 34 and 35 amino acids shorter than *Sc*TIM23. Four transmembrane regions were predicted, similar to the yeast protein. The Arabidopsis predicted TIM44 proteins were 42 and 69 amino acids longer than *Sc*TIM44 at the N terminus of the protein and were not predicted to contain any transmembrane regions. The N-terminal extension of the Arabidopsis proteins are predicted by TargetP to represent a mitochondrial targeting sequence and contain a potential −3R processing signal of RRF*S between amino acids 113 and 114 (Emanuelsson et al., 2000; Zhang et al., 2001). There is no evidence of N-terminal cleavable targeting signals for the TIM17 and TIM23 proteins. None of the Arabidopsis *TIM17* or *TIM23* genes contains any introns. The two genes encoding *TOM9* in Arabidopsis are the only other import components that do not appear to contain introns (Lister et al., 2003).

Expression of Arabidopsis *AtTIM17-1*, *AtTIM17-2*, *AtTIM17-3*, *AtTIM23-1*, *AtTIM23-2*, and *AtTIM23-3*

We analyzed the expression of all of the genes for *AtTIM17* and *AtTIM23* and compared them with three *AtTIM22*, which represents the other TIM (Pfanner and Geissler, 2001). The expression of three nuclear-encoded ribosomal genes were analyzed, with AtRPS15a located in the cytosol, AtRPS1 located in the plastid, and AtRPS13 located in the mitochondrion (Adams et al., 2002; Fig. 2). All *TIM17*, *TIM22*, and *TIM23* genes were expressed. However changes in expression were evident between tissues and with development (Supplementary Figs. 2 and 3). Notable differences were that *AtTIM17*-1was expressed at very low levels in roots compared with all other TIM genes analyzed. In fact, it was similar to *AtRPS1*, which encoded a chloroplast-located ribosomal protein (Supplementary Fig. 2). *AtTIM17-2* and *AtTIM23-1* and *AtTIM23-2* were the highest expressed forms for these genes and peaked at the earliest and latest stages of cotyledon development. The peak at the later stages of cotyledon development was not observed for *AtTIM22-2*, the highest expressed form for this translocase (Supplementary Fig. 3).

Complementation of Yeast Deletion Strains for ScTIM17 and ScTIM23 with Arabidopsis Homologs

Yeast TIM17 and TIM23 are essential proteins for viability, so we tested the ability of the Arabidopsis homologs to complement these genes using a gene replacement strategy (Winzeler et al., 1999; Rehling et al., 2001). This strategy also had the advantage of being less harsh in that the ability of the Arabidopsis homologs to complement under normal growth conditions and was not dependent on the ability of the Arabidopsis homologs to support sporulation. The constructs tested and the ability to complement are shown in Figure 2. *AtTIM17-3* and *AtTIM17-2* were not able to complement a defect in the corresponding yeast gene. When the region encoding the 85-amino acid extension of *AtTIM17-2* was deleted, it supported growth of the deleted *ScTIM17* strain. *AtTIM23-2* was unable to complement a yeast deletion in *ScTIM23*. Examination of the predicted *At*TIM23 proteins indi-

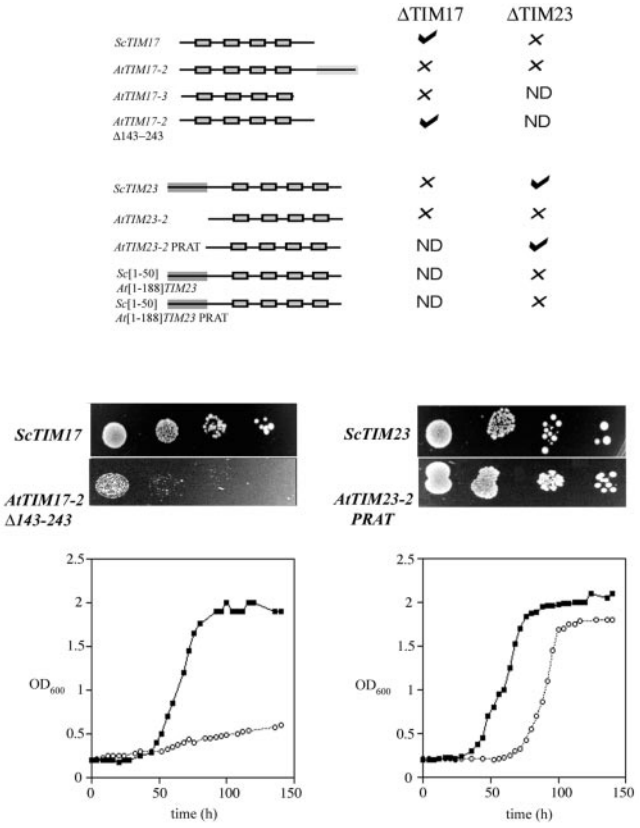

Figure 2. Ability of Arabidopsis *TIM17* and *TIM23* homologs to complement yeast deletion strains. The ability of *AtTIM17* and *AtTIM23* and various chimerics and mutants to complement yeast deletion strains was tested by their ability to support growth. A, The constructs tested and ability to complement are indicated. *ScTIM*17 and *ScTIM*23 on plasmids were used as positive controls. *AtTIM17-2* and *AtTIM17-3* failed to complement, but a deletion of *AtTIM17-2* with 100 amino acids at the C terminus removed supported growth. *AtTIM23-2* failed to complement growth, but insertion of a PRAT domain as is evident in *AtTIM23-3* supported growth. The presence of the first 50 amino acids of *ScTIM23* did not support growth with *AtTIM23-2* or *AtTIM23-2*(PRAT). B, Growth of the complemented strains at 22°C. The top panels represent the growth of yeast supported by the corresponding gene on a plasmid, 10-fold serial dilutions of the inoculum are shown after growth for 7 d. C, Growth rates for the complemented strains compared with that supported by the yeast genes on a plasmid over a period of 7 d.

cated that although *At*TIM23-3 has a preprotein and amino acid transporters (PRAT) domain, *At*TIM23-1 and *At*TIM23-2 lack this domain (Rassow et al., 1999). This domain, $G/A_{X2}F/Y_{X10}R_{X3}D_{X6}[G/A/S]GX_3G$, is present in all predicted TIM23 proteins from yeast and mammalian systems (Rassow et al., 1999). In *At*TIM23-1 and *At*TIM23-2, the Arg is replaced by a Thr. When this Thr codon at position 132 was changed to an Arg codon in *AtTIM23-2*, complementation of a yeast deletion in *TIM23* was observed. Therefore complementation was dependent on the presence of the PRAT domain.

We made a chimeric construct consisting of the region encoding first 50 amino acids of *ScTIM*23 fused to *AtTIM23-2* (*y[1-50]At[1-188]TIM23*), because it had been previously reported that this portion of *Sc*TIM23 connects the inner and outer membranes and is necessary for efficient protein import and cell growth (Donzeau et al., 2000). This construct failed to complement the deleted *ScTIM*23 strain, even when it was modified to contain a PRAT domain. Therefore although an Arabidopsis *TIM23* gene with a PRAT domain can complement a deletion of *ScTIM23*, it cannot do this if the region encoding first 50 amino acids of *ScTIM23* are placed in front. This suggests that while functionally similar, the plant and yeast proteins are structurally different.

The Δ*ScTIM*17 and Δ*ScTIM*23 deletion strains complemented with *AtTIM17-2*Δ143-243 and *AtTIM23-2*(PRAT) displayed better growth at 22°C compared with 30°C. In both cases, this was slower than growth supported by the yeast gene on a plasmid, most dramatically for *TIM17*.

Import of *At*TIM17 and *At*TIM23 into Mitochondria

We investigated the import of *At*TIM17 and *At*TIM23 into isolated soybean (*Glycine max*) cotyledon mitochondria. Additionally, we used yeast mitochondria and *Sc*TIM17 and *Sc*TIM23 to compare the import and topology of the plant TIMs to this well-characterized system. We then characterized the topology and import characteristics of *At*TIM17-2 and *At*TIM23-2 in detail because the genes encoding these proteins were highly expressed. To verify the location and intactness of mitochondria used in the in vitro import assays, we carried out western-blot analysis against proteins present in different mitochondrial locations (Fig. 3). The outer membrane protein TOM20 was susceptible to protease digestion in both mitochondria and mitoplasts. The inter membrane space protein cytochrome c was only accessible to protease digestion in mitoplasts, whereas the inner membrane uncoupling protein and matrix-located HSP60 were resistant to protease digestion. These results verify the intactness of mitochondria, the rupture of the outer membrane by osmotic shock, and that the concentration of protease used did not digest known integral membrane proteins.

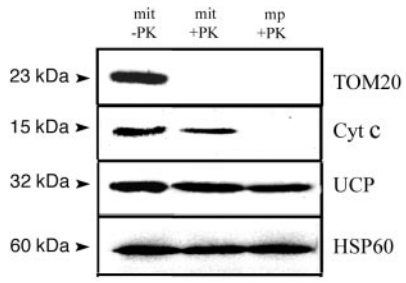

Figure 3. Western-blot analysis of mitochondria, mitochondria treated with PK, and mitoplasts treated with PK. The apparent molecular mass of the protein detected upon probing with various antibodies is indicated in kilodaltons. Cyt c, The inter membrane space protein cytochrome *c*; UCP, the inner membrane uncoupler protein; HSP60, matrix heat shock protein 60; TOM20, TOM of the outer membrane 20.

Because *Sc*TIM23 links the inner and outer membrane, we carried out import studies on *At*Tim23-2 to investigate if it was also accessible to externally added protease with intact mitochondria. *At*TIM23-2 contains a single Met at residue 1, which provides a convenient point to investigate the location of the N terminus of this protein. We either placed two additional Met residues in front of the start codon to produce *At*TIM23-2 (2M, −2, −1) or changed two Ile residues at positions 116 and 117 to Met residues to produce *At*TIM23-2 (2M, 116, 117), and a combination of both, *At*TIM23-2 (4M, −2, −1, 116, 117). *At*TIM23-2 (4M, −2, −1, 116, 117) was imported into isolated mitochondria and produced a membrane-protected fragment of 16 kD when the outer membrane was ruptured by osmotic swelling to allow access of added protease to the inter membrane space (Fig. 4, i). Import studies with *At*TIM23-2 (2M, −2, −1) yielded the same results except that no protected fragment was evident when the outer membrane was ruptured (Fig. 4, ii). This was due to the position of the radiolabeled Met residues at the N terminus of the protein, making them accessible to added protease when the outer membrane was ruptured. However an inner membrane-protected fragment was detected with *At*TIM23-2 (2M, 116, 117), because the added Met labels in this case were located in the predicted transmembrane region 2 and thus would be protected from digestion by added protease (Supplementary Fig. 1; Fig. 4, iii). This indicated that under normal import conditions with intact mitochondria, the N terminus of *At*TIM23-2 was not accessible to externally added protease, in contrast to what has been observed with *Sc*TIM23 (Donzeau et al., 2000; Fig. 4, v). A chimeric protein between the first 50 amino acids of *Sc*TIM23 and *At*TIM23-2 (y[1–50]*At*[1–188]TIM23) was imported into both soybean and yeast mitochondria and produced an inner membrane-protected fragment upon rupture of the outer membrane. It also generated a small breakdown fragment with protease-treated mitochondria, indicating that the first 50 amino acids of *Sc*TIM23

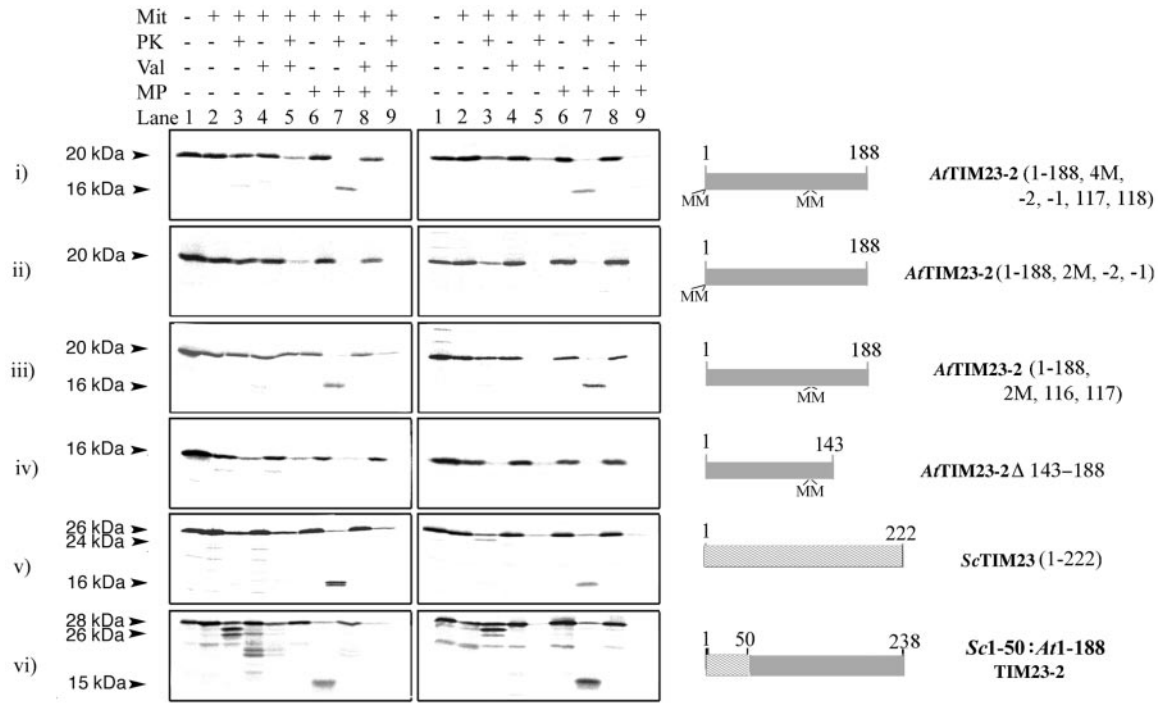

Figure 4. Import of *At*TIM23-2 into soybean and yeast mitochondria. *At*TIM23-2 precursor protein synthesized in a rabbit reticulocyte translation system was used with in vitro import assays with purified soybean or yeast mitochondria. The left set of panels represent import assays into soybean mitochondria, and right panels represent import assays into yeast mitochondria. The apparent molecular mass of the precursor and products generated upon import and/or protease treatment are indicated in kilodaltons. The constructs used to produce the precursor protein are indicated on the right of the figure, inserted Met residues are indicated by M, and positions are indicated relative to the start Met residue of *At*TIM23-2. The additions or treatment to each import assay is indicated on the top. MP, Mitochondria ruptured after import assay by osmotic shock; Val, valinomycin added before commencement of import assay; PK, addition of Proteinase K after import assay or mitoplast preparation.

also appear to have some sequence at the N-terminal exposed on the outer membrane upon import into plants (Fig. 4, vi). However, this smaller breakdown product was not observed in soybean mitochondria when *Sc*TIM23 alone was used, only when the four membrane-spanning segments of TIM23 were from *At*TIM23-2 (Fig. 4, soybean v and vi). The import signal for insertion into the inner membrane of *At*TIM23-2 is located at the C terminus of the protein as deletion of residues 143 to 188 abolishes import (Fig. 4, iv). In summary, *At*TIM23-2 contains an internal targeting sequence between residues 143 and 188 similar to *Sc*TIM23. However, in contrast to the situation in yeast, the N terminus of the plant protein does not appear to be accessible to protease in intact mitochondria.

Import studies were carried out with *At*TIM17-2 to investigate its topology and to elucidate the signals that define mitochondrial localization. In contrast to *At*TIM23-2, *At*TIM17-2 contains numerous Met residues and a Met-rich region beginning at residue 143. This causes the production of several products upon in vitro translation. Using site-directed mutagenesis to change Met residues 1, 25, 57, and 90 to Ile residues, it was concluded that the first four bands with apparent molecular masses of 31, 28, 26, and 25 kD arose from precocious translation initiation at each of these sites, respectively (Fig. 5A). Additionally, the major band at 18 kD likely corresponds to translation initiated at the triple Met residues (position 143–146) based on a similar size comparison when this fragment was expressed alone (Fig. 5A).

Translation of *At*TIM17-2 produced a precursor protein with an apparent molecular mass of 31 kD, which upon import into mitochondria and protease treatment produced a breakdown fragment of 28 kD in a membrane potential-dependent manner (Fig. 5B, i). Rupture of the outer membrane before protease treatment yielded an inner membrane-protected fragment with an apparent molecular mass of 16 kD. With yeast mitochondria import of *At*TIM17-2 was reduced and thus only a faint breakdown fragment was apparent with intact mitochondria, but an inner membrane-protected fragment was produced upon protease treatment of mitoplasts (Fig. 5B, i). The C-terminal extension of *At*TIM17-2 apparently does not contain mitochondrial targeting information because it was not imported into either soybean or yeast mitochondria when expressed (Fig. 5B, ii). The first 143 amino acids contained the mitochondrial

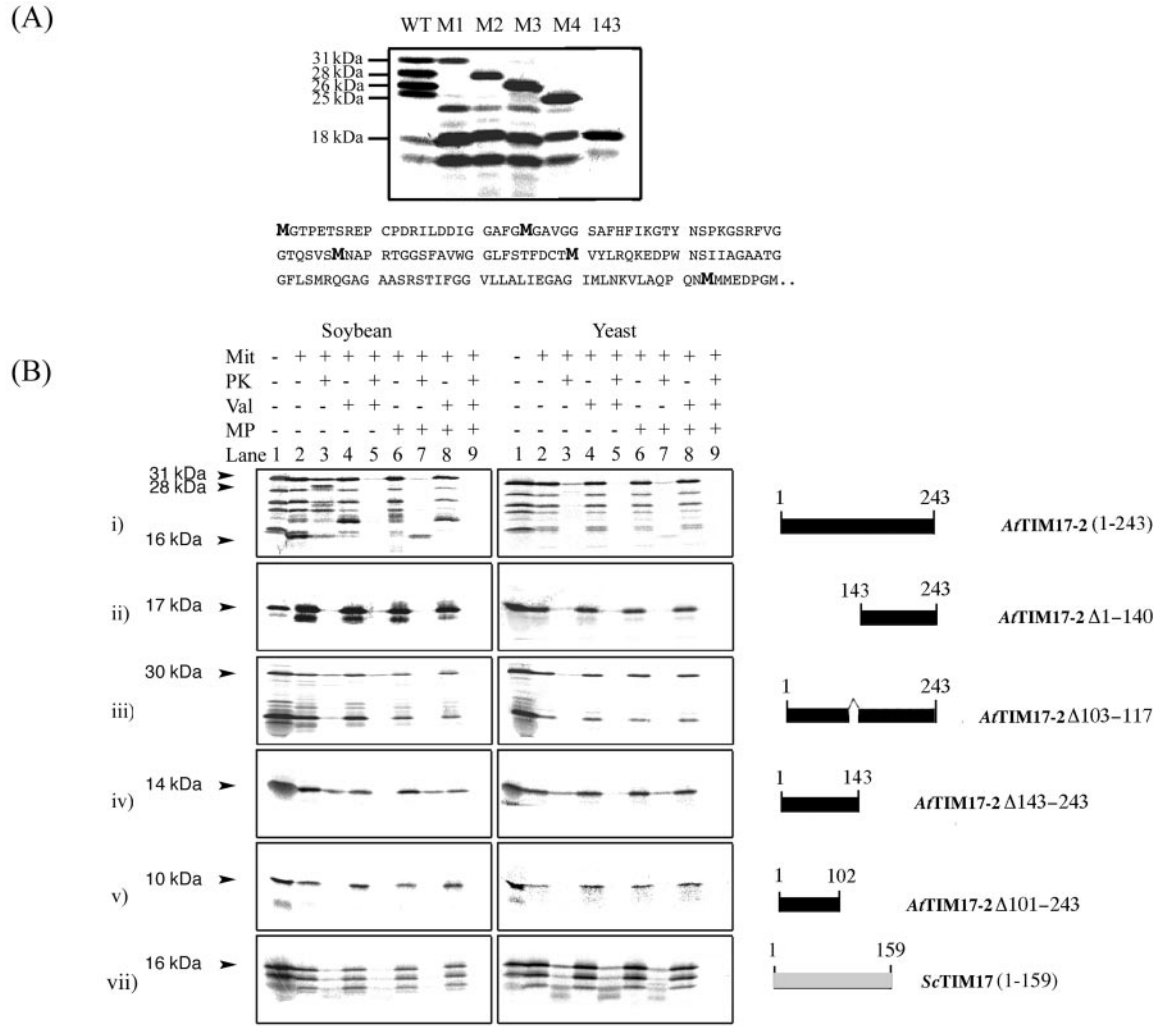

Figure 5. Characterization of the translation products of *At*TIM17-2 (A) and import of *At*TIM17-2 into soybean and yeast mitochondria (B). A, To elucidate the nature of the several products observed with translation of *At*TIM17-2, Met residues at various positions were changed to Ile residues. Each Met in the sequence was simply designated by which Met it represented. M1, Met residues at position 1; M2, Met residues at position 25; M3, Met residues at position 57; and M4, Met residue at position 90. Lane WT, Wild-type *At*TIM17-2 with the apparent molecular mass of the main translation products indicated. Lane M1, Met residues M2, M3, and M4 have been converted to Ile. Lane M2, Met residues M1, M3, and M4 have been converted to Ile residues. Lane 3, Met residues M1, M2, and M4 have been converted to Ile. Lane M4, Met residues M1, M2, and M3 have been converted to Ile. Lane 143, A gene fragment where the coding sequence for the first 142 amino acids have been deleted so that translation may commence at Met residue 143. B, As in Fig 4 except that *At*TIM17-2 was used in import assays.

targeting signal for insertion into the inner membrane between residues 103 and 117 (Fig. 5B, iii–v). *AtTIM17-2Δ143-243*, which complemented a yeast deletion in *ScTIM17*, upon translation was imported into both soybean and yeast mitochondria with equal efficiency and protected in mitoplasts (Fig. 5B, iv). This indicates that the first 143 amino acids contained the targeting signal for insertion into the inner membrane between residues 103 and 117 (Fig. 5B, iii–v). There was no change in apparent molecular mass of this product in mitoplasts treated with protease because it was indistinguishable in size from the inner membrane-protected product seen with *At*TIM17-2.

In summary, under the import conditions used, *At*TIM17-2 is imported into mitochondria under the direction of an internal targeting signal in a membrane potential-dependent manner. A portion of the protein appears to be accessible to externally added protease. A similar pattern of import for TIM17 and TIM23 was observed with purified Arabidopsis mitochondria, however, we routinely used purified soybean cotyledon mitochondria due to the availability of antibodies to verify mitochondrial purity and intactness and to probe intramitochondrial location. All three TIM17 and TIM23 proteins were tested for uptake into mitochondria. All proteins except *At*TIM17-3 were

imported into mitochondria, but none were imported into chloroplasts (data not shown).

DISCUSSION

The complete genome sequence from Arabidopsis allows the characterization of components using homology-based approaches from well-characterized organisms such as yeast. Using this approach, it was apparent that although the central components of both TIM complexes were present, major differences existed in the predicted proteins for *At*TIM17 and *At*TIM23. *At*TIM17 displays high homology with *Sc*-TIM17, especially in the region encompassing the four inner membrane transmembrane regions (Supplementary Fig. 1A). One gene for *At*TIM17 present on chromosome 2 (*At*TIM17-2) contains an 85-amino acid extension at the C terminus, it is 243 amino acids long compared with the 158 amino acids of *ScTIM*17 (Fig. 1A). In contrast, *At*TIM17-3 is only 133 amino acids long. The C-terminal extension of *At*TIM17-2 does not display homology with any other proteins in GenBank but was characterized by a tripeptide repeat of GMQ/P from amino acids 149 to 178. The remainder of the protein of 65 amino acids was enriched with 16 hydroxylated amino acids and 14 charged residues. The only noticeable hit on searching GenBank with this region was that the tripeptide repeat displayed high homology with the M region of the signal recognition particle protein of 54 kD (SRP54). This protein, and in particular this region, is involved in binding mRNA in the signal recognition particle translation arrest cycle and in protein-protein interactions between the chloroplast homolog of SRP54 and chloroplast signal recognition particle protein of 43 kD (Jonas-Straube et al., 2001; Keenan et al., 2001). A similar extension is present on TIM17 homologs from several other plant species, including *Medicago* spp. (GenBank accession no. Aw559460) and soybean (GenBank accession no. Aw309106). TIM17 from these species display 67% and 60% identity with *At*TIM17-2, although the C-terminal extension is less conserved displaying 49% and 47% identity. Structural predictions of the C-terminal extension indicate an α-helix, β-sheet, and α-helix structure, with maximum hydrophobicity in the β-sheet-forming region (Chou and Fasman, 1979; Deleage and Roux, 1987; Cserzo et al., 1997).

In contrast to *At*TIM17-2, *At*TIM23 predicted proteins are 34 and 35 amino acids shorter than their yeast counterpart. The *At*TIM23 proteins do not appear to contain the N-terminal region corresponding to the domain shown to be present in the outer membrane in yeast (Fig. 1B; Supplementary Fig. 1; Donzeau et al., 2000).

To characterize the function of these various isoforms in plants, we investigated the expression, function, and topology of *At*TIM17 and *At*TIM23 of the plant inner mitochondrial membrane. Expression analysis indicated that all homologs of *AtTIM17* and *AtTIM23* were transcribed, but all were not expressed in the same pattern or amount. Notably, gene expression for the *TIM17*:*23* translocase did not show the same developmental profile to that of TIM22. This may indicate differential regulation of the general and carrier import pathways.

Functional analysis of the Arabidopsis homologs was carried out by testing their ability to complement yeast deletion mutants. The abundantly expressed genes of *AtTIM17* and *AtTIM23* could not complement deletions of these genes in yeast. However, a truncated version of *AtTIM17-2* and *AtTIM23-2* encoding a PRAT domain could complement yeast. The latter did not require the yeast N-terminal region for complementation. Insertion of the coding region for the first 50 amino acids of *ScTIM23* in front of the *AtTIM23-2* gene prevented complementation.

TIM17 and TIM23 are imported via the carrier import pathway in yeast. This pathway uses the TIM22 translocase on the inner membrane and differs from the general import pathway that uses TIM17:23. Import via the carrier pathway is defined via internal import signals as we have demonstrated here for Arabidopsis TIM17 and TIM23. The carrier import pathway can be conveniently divided into a number of stages, from synthesis in the cytosol (stage I), interaction with receptor on the mitochondrial surface (stage II), partial membrane potential-independent translocation across the outer membrane (stage IIIa), and membrane potential interaction with the small TIMs of the inter membrane space (stage IIIb). Membrane potential-dependent insertion into the inner membrane (stage IV) and assembly (stage V) together result in a functional protein complex (Rehling et al., 2001). However, for different carrier proteins, variations on this theme occur at stage IIIa where membrane potential-independent translocation to a soluble intermediate in the inter-membrane space can be observed. This is seen with the dicarboxylate carrier in yeast (Zara et al., 2001). Consequently, in import assays of *At*TIM17-2 and *At*TIM23-2 into soybean mitochondria, we deemed import had occurred only when products were protease protected after rupture of the outer membrane and when import was membrane potential dependent. This indicated insertion into the inner membrane. We observed that *At*TIM23-2 and *At*TIM17-2 did display some protease insensitivity even in the absence of a membrane potential with soybean but not yeast mitochondria (Figs. 4 and 5). Notably, when *At*TIM23 was only radiolabeled at the N terminus, this signal was much reduced. Thus, these products protected from protease in the absence of a membrane potential may represent import intermediates. The differences observed in this study are between the sources of mitochondria. The differences in the plant TOM complex, together with the apparent absence of TIM54, TIM18, and TIM12 from plants, may contribute to this difference between organisms

(Jansch et al., 1998; Werhahn et al., 2001; Lister et al., 2002).

In vitro import assays suggested that the C terminus of *At*TIM17-2 was accessible to protease digestion in intact mitochondria, whereas the remainder of the protein was present in the inner membrane. Kinetic and chase experiments with radiolabeled precursor protein did not result in all of the precursor being converted to this cleaved product (data not shown). Notably this degradation of *At*TIM17-2 was only observed in the presence of a membrane potential, indicating that insertion into the inner membrane was a prerequisite for the generation of this product. Removal of 85 amino acids from the C-terminal of *At*TIM17-2 resulted in no fragment being generated. Thus, this region appears to be inhibitory to the function of *At*TIM17-2 in yeast and appears to have an inhibitory effect on import of *At*TIM17-2 in yeast compared with soybean mitochondria. Because Arabidopsis TIM23 isoforms do not appear to be inserted into the outer membrane as yeast TIM23, it is possible that *At*TIM17-2 exerts this function in Arabidopsis and links the inner and outer membrane, which has been reported to be necessary for efficient import in yeast (Donzeau et al., 2000). To date, several attempts to raise antibodies to *At*TIM17-2 using either antibodies or overexpressed protein to verify the topology of the protein in vivo have been unsuccessful.

The TIM17:23 translocase of plant mitochondria appears to display significant differences to that in yeast and mammalian systems. Structurally, TIM17 appears to be different with a C-terminal extension that is accessible to external protease when the four predicted transmembrane regions are in the inner membrane. In yeast, the outer membrane-exposed domain is on *Sc*TIM23. A chimeric construct that placed the yeast N-terminal 50 amino acids in front of *At*TIM23-2 indicated that the plant TIM23 did not appear to function when the N terminus of the protein was located in the outer membrane. A C-terminal-deleted *At*TIM17-2, on the other hand could complement a yeast deletion mutant. Overall, this may indicate that anchoring in the outer membrane may occur via different proteins of the translocase in yeast and plants. The role of the C-terminal extension in plants is unclear, but the homologous Met-rich region in SRP54 binds to RNA. Plant mitochondria import tRNA (Dietrich et al., 1996b), in a process that may require the components of the protein import apparatus (Dietrich et al., 1996a,b; Kolesnikova et al., 2000). The C-terminal extension of TIM17, which appears to be present in several plants, may serve the biological function of importing RNA. Because the type and extent of tRNA import differs dramatically between plant species, the C-terminal extension on plant TIM17 may not be highly conserved (Kumar et al., 1996).

MATERIALS AND METHODS

Identification of Arabidopsis Homologs of the Yeast (*Saccharomyces cerevisiae*) Mitochondrial Protein Import Apparatus

All bioinformatic programs were used with the default settings unless specified otherwise. BioNavigator (http://www.entigen.com) was used to facilitate the analysis, including the use of programs by the Genetics Computer Group (University of Wisconsin, Madison). Sequence information for the yeast import components was obtained from the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/). The yeast gene and protein sequences were used to search GenBank (http://www.ncbi.nlm.nih.gov/) and The Institute for Genomic Research (http://www.tigr.org/) Arabidopsis sequence databases for homologs by BLASTN, BLASTP, and TBLASTN alignment (Altschul et al., 1997). Protein sequences were deduced from nucleic acid sequences using Translate (Genetics Computer Group, University of Wisconsin). Protein alignments were generated using PileUp (Genetics Computer Group, University of Wisconsin). Percentage identity and similarity between yeast and Arabidopsis proteins was calculated using Gap (Genetics Computer Group, University of Wisconsin). Prediction of transmembrane regions was performed using the DAS transmembrane prediction server (Cserzo et al., 1997; http://www.sbc.su.se/approximately miklos/DAS/).

Gene Expression of TIM Components

Cloning of TIM Components

Total RNA was isolated from Arabidopsis tissue using the RNeasy Plant mini protocol (Qiagen, Clifton Hill, Australia). Total RNA was reverse transcribed with the appropriate reverse primer (refer to PCR primers below) using Expand Reverse Transcriptase as per the manufacturer's recommendations (Roche Diagnostics, Sydney). Five microliters of the resulting cDNA was used in a PCR reaction with the appropriate forward and reverse primers (30 pmol each) using the Expand High Fidelity PCR system (Roche Diagnostics) according to the manufacturer's instructions. The amplification profile consisted of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, and a final extension cycle of 72°C for 5 min and then 22°C for 6 min. Fragments amplified by PCR were purified by either the QIAquick Gel Extraction Kit or QIAquick PCR purification kit (Qiagen) and cloned into the pCR2.1 vector (Invitrogen, Sydney) according to the manufacturers' instructions.

The following PCR primers were used to clone the genes from plants and yeast: *AT TIM17-1 fwd*, CCCTAAAAAGTTACTTGTAG; *AT TIM17-1 rev*, TCAGACCTCAATAATTCCATC; *AT TIM17-2 fwd*, ATGGGAACACCAGAGACATC; *AT TIM17-2 rev*, TTACTTGAACTCAAATGATGGCACCG; *AT TIM17-3 fwd*, ATGGACACTAAGAAGAAATC; *AT TIM17-3 rev*, TTACTTGCTCCCGAACGGAGGG; *AT TIM22-1 fwd*, ATGGCTGATTCGAGTGCTGC; *AT TIM22-1 rev*, CTACTCAGGGATGCTAGTTG; *AT TIM22-2 fwd*, ATGGCGGCGAACGATTCTTC; *AT TIM22-2 rev*, TTAGAACTTCCTTGGTTTAGCTAAGG; *AT TIM22-3 fwd*, ATGGCGGCCGAGAATTCTTC; *AT TIM22-3 rev*, TCAACGAGCATGAGGAAATTTGAGC; *AT TIM23-1 fwd*, CGTCTCCCGTCTTCTTCTAATG; *AT TIM23-1 rev*, GGGCTCTAAAGATCACTCCGG; *AT TIM23-2 fwd*, ATGGCGGCTAATAACAGATC; *AT TIM23-2 rev*, TCAAATGGGCACATACCGC; *AT TIM23-3 fwd*, ATGGCGGATCCGATGAACCATAG; *AT TIM23-3 rev*, TTAGATTGGAACGAATCGC; *Yeast TIM17 fwd*, AATAGAGTACACGGGAGCG; *Yeast TIM17 rev*, CTAAGCTTGCAGAGGTTGAGAG; *Yeast TIM23 fwd*, ATGTCGTGGCTTTTTGGAGATAAG; and *Yeast TIM23 rev*, TCATTTTTCAAGTAGTCTTTTC.

Isolation of Total RNA and cDNA Synthesis

Total RNA was isolated three separate times from Arabidopsis tissue using the RNeasy Plant mini protocol (Qiagen). Each batch of total RNA was treated with DNaseI (Roche Diagnostics) to remove contaminating DNA and reverse transcribed using Expand Reverse Transcriptase as per the manufacturer's recommendations (Roche Diagnostics). Primers used in the reverse transcription were random primers (Roche Diagnostics) for the analysis of transcripts in different tissues and poly-A primer (Roche Diagnostics) for the cotyledon transcript analysis. This yielded three separate lots of cDNA. The QIAquick

PCR purification kit (Qiagen) was used to purify the cDNA before real-time PCR analysis. Three ribosomal protein genes, one for a cytosolic ribosomal protein *RPS15A*, one for a nuclear encoded plastid ribosomal protein *RPS1*, and one for a nuclear encoded mitochondrial ribosomal protein *RPS13*, were used for comparison against the gene expression profiles of the TIM components (Adams et al., 2002).

Real-Time PCR Primers

The primers used in the real-time PCR reactions for analysis of gene expression are listed below: *AT TIM17-1 fwd*, CGTTCAAGCTTTGAGAATG; *AT TIM17-1 rev*, GCTCGTTATGCGCAGTAC; *AT TIM17-2 fwd*, GTGAGCATGAACGCACCTCG; *AT TIM17-2 rev*, CAGGCATTCCTTGCATTCCAGG; *AT TIM17-3 fwd*, CTAAGGAACATGGCCTATACC; *AT TIM17-3 rev*, CAGAACTCCACCTGTAGC; *AT TIM22-1 fwd*, GATAGGCATCTCTTGTATGG; *AT TIM22-1 rev*, CTCTTGGTGTATGGGAAACG; *AT TIM22-2 fwd*, CACTGACGGTGACGAAGC; *AT TIM22-2 rev*, CAGACTGCCCTGCATCTG; *AT TIM22-3 fwd*, ACAAACCAGAAGTCCCCAAC; *AT TIM22-3 rev*, TCAACGAGCATGAGGAAATTTG; *AT TIM23-1 fwd*, CGTAGCTCCGATCATGAATCC; *AT TIM23-1 rev*, GGCTAGATAACCCGTCCCTG; *AT TIM23-2 fwd*, ATCCGATCATGGGTCAGACG; *AT TIM23-2 rev*, TGACCAGAAGAGTTCAAGATCC; *AT TIM23-3 fwd*, ATGGCGGATCCGATGAACC; *AT TIM23-3 rev*, CCGCAATTGTACCCTTGAAG; *AT RPS1 fwd*, TATCGCAACTGTTCTTCAGCC; *AT RPS1 rev*, TCAGAACTCAGCGTCAGTCC; *AT RPS15A fwd*, CACTGGAGGCAAGCAGAAGC; *AT RPS15A rev*, AAGTGTCTTAGTACGTACAAGC; *AT RPS13 fwd*, ATCAGCCAGTCTCTGCTTCG; and *AT RPS13 rev*, TCAGTTCATCACCATGCTGG.

Real-Time PCR Analysis of Transcript Levels

Transcript levels were assayed using the LightCycler and FastStart DNA Master SYBR Green I kit (Roche Diagnostics). Reactions were carried out in a total volume of 10 μL with a final concentration of 0.008% (w/v) bovine serum albumin under conditions optimized to minimize primer-dimer formation and to maximize amplification efficiency. The LightCycler protocol consisted of four programs: denaturation, 95°C for 10 min; amplification, 40 cycles at 95°C for 15 s, touchdown annealing from 85°C to 45°C decreasing 2°C per cycle for 5 s, 72°C for 15 s with single data acquisition; melting curve analysis, 95°C for 0 s, 70°C for 60 s, 95°C for 0 s with a transition rate of 0.1°C s^{-1} and continuous data acquisition; cooling, 40°C for 30 s.

DNA was amplified from the cloned genes by PCR using the cloning primers and purified with the QIAquick PCR purification kit (Qiagen) for use as standard template DNA in real-time PCR reactions. The concentration of standard template DNA was quantitated using the PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. A standard curve was generated by real-time PCR analysis of 10-fold serial dilutions of the standard template DNA. Transcript levels were assessed using 10^{-1} dilutions of the cDNA prepared from Arabidopsis tissue of different ages. Absence of primer-dimer and/or nonspecific product accumulation was checked by melting curve analysis and confirmed by agarose gel electrophoresis. Template abundance was quantified using the second derivative maximum method of the LightCycler v3.5 software to determine the cycle at which each PCR reaction reached exponential amplification (Roche Diagnostics). From the three independent cDNA preparations, each transcript was analyzed twice giving a minimum of six replicates for each data point. The SE was calculated for every data point. Absolute transcript levels were calculated from the standard curve of known DNA concentration. The data for each gene was normalized by setting the data point with the most abundant transcript to 100 and adjusting the other data points of that gene relative to it.

Complementation of Yeast Deletion Mutants Using Arabidopsis TIM17 and TIM23 Homologs

Diploid deletion strains for *ScTIM17* (YJL143w) and *ScTIM23* (YNR017w) were obtained from EUROSCARF (http://www.uni-frankfurt.de/fb15/mikro/euroscarf; Winzeler et al., 1999).

BY4743; Mat a/α′3b his3Δ1/his3Δ1′3b leu2Δ0/leu2Δ0; lys2Δ0/LYS2Δ′3b MET15/met15Δ0′3b ura3Δ0/ura3Δ0′3b YJL143w::kanMX4/YJL143w.

BY4743; Mat a/α′3b his3Δ1/his3Δ1′3b leu2Δ0/leu2Δ0; lys2Δ0/LYS2Δ′3b MET15/met15Δ0′3b ura3Δ0/ura3Δ0′3b YNR017w::kanMX4/YNR017w.

The KANMX4 insertion in the appropriate gene was confirmed by PCR. Confirmation of the strains was carried out using the primer sets: *Sc-TIM17*-fwd, GGGTAACCCGGCATTCTTGCG; *Sc-TIM17*-rev, CATGGTTGAGAGAAATGGTTGG; Sc-TIM23-fwd, GGTTATTGCATTCGCCCTCC; *Sc-TIM23*-rev, CCGGGTTTCTACTTTCATTGG; and *kanMX4*-rev, CAATCATAGATTGTCGCACC.

The ability of Arabidopsis homologs to complement the deletion in the yeast genes was tested by a gene replacement approach. The yeast genes for *TIM17* and *TIM23* were cloned into Yep352 plasmid under the control of the alcohol dehydrogenease promoter (Hill et al., 1986). The Arabidopsis homologs and various deletions and chimerics to be tested for their ability to complement were cloned into p425 Gal1 (Mumberg et al., 1994). The yeast genes were transformed into the appropriate deletion strains (*ScTIM17* into YJL143w and *ScTIM23* into YNR017w), and transformants were selected on Western Australia-Glc minus uracil plates. These diploid cells were induced to sporulate, and tetrad dissection was carried out for each strain (Sherman and Hicks, 1991). Tetrads were grown on WA minus uracil and Met or WA minus uracil and Lys to ensure the accuracy of the tetrad dissection. Tetrads that showed a 2:2 segregation for above were grown on WA minus uracil with Geneticin G418 (400 μg mL^{-1}), and resistant colonies were used to transform with p425 Gal1 containing the Arabidopsis gene construct. Transformants were selected on WA minus uracil and Leu plates and replica plates onto WA-Gal minus Leu plus uracil (50 mg L^{-1}), plus 5-fluoro-orotic acid (1 g L^{-1}). The ability to grow on plates containing 5-fluoro-orotic acid was monitored at 22°C and 30°C, and colonies were replica plated at least three subsequent times over a period of 1 month so that the ability to grow was accurately determined. At least 10 individual transformants were monitored for each gene construct.

Colonies that displayed growth were cultured in liquid WA-Gal minus Leu medium and were used to determine the growth rate from an A_{600} of 0.2 over a period of a week. This growth rate was compared with the parental haploid that was Geneticin resistant so the only difference between the two strains was the plasmid that carried either the *ScTIM* gene (Yep 352) or the plasmid that carried the Arabidopsis gene (p425 Gal1) that supported growth.

Synthesis of Precursor Proteins

cDNA for *AtTIM17-2*, *AtTIM23-2*, *ScTIM17*, and *ScTIM23* in pCR 2.1 (Invitrogen) where subcloned into pGEM-3Zf(+) (Promega, Melbourne). The various constructs and deletions of *TIM17* and *TIM23* were produced using site-directed mutagenesis (Stratagene, La Jolla, CA) and standard molecular biology techniques. Precursor proteins where translated using the TNT-coupled transcription-translation in reticulocyte lysate in the presence of [^{35}S]Met under either the SP6 or T7 promoter (Promega).

Isolation of Mitochondria

Mitochondria and mitoplasts from 7-d-old soybean (*Glycine max*) cotyledons and yeast were isolated as outlined previously (Moro et al., 1999; Donzeau et al., 2000; Lister et al., 2002).

In Vitro Import Assays

Import assays were performed as previously described for soybean (Whelan et al., 1996). Import assays into yeast mitochondria varied to contain 2 mM ATP, 2 mM GTP, and 2 mM NADH. Fifty milligrams of proteinase K was added to import assays where indicated to determine whether the precursor protein was imported into mitochondria, Protease treatments of mitochondria and mitoplasts were carried out as previously described (Murcha et al., 1999; Lister et al., 2002). Proteins were separated by 12% or 16% (w/v) SDS-PAGE, and gels were stained, dried, exposed to a BAS TR2040S plate for 24 h, and detected using a BAS 2500 (Fuji, Tokyo).

Preparation of Mitoplasts after Import

Mitoplasts were prepared after import via osmotic swelling. The mitochondria were pelleted after import and resuspended in 10 μL of SEH buffer (250 mM Suc, 1 mM EDTA, 10 mM HEPES, pH 7.4). One hundred and fifty-five microliters of 20 mM HEPES, pH 7.4, was added and incubated on

ice for 20 min, and 25 μL of 2 M Suc and 10 μL of 3 M KCl was added and mixed. The sample was aliquoted into two where to one proteinase K was added and incubated on ice for 30 min. Phenylmethylsulfonyl fluoride was added to stop the reaction, and mitoplasts were pelleted.

Immunodetection

Mitochondrial proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Sydney) using a semidry blotting apparatus (Millipore, Sydney) and were incubated with primary antibodies against matrix HSP60 (StressGen, Canada), intermembrane space cytochrome *c* (BD Biosciences, San Diego), inner membrane uncoupling protein (Considine et al., 2001) and TOM20 (Dr H.-P. Braun, University of Hannover, Germany). Chemiluminescence was used for detection using a LAS 1000 (Fuji).

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