The C-terminal Region of TIM17 Links the Outer and Inner Mitochondrial Membranes in Arabidopsis and Is Essential for Protein Import*

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The translocase of the inner membrane 17 (AtTIM17-2) protein from Arabidopsis has been shown to link the outer and inner mitochondrial membranes. This was demonstrated by several approaches: (i) In vitro organelle import assays indicated the imported AtTIM17-2 protein remained protease accessible in the outer membrane when inserted into the inner membrane. (ii) N-terminal and C-terminal tagging indicated that it was the C-terminal region that was located in the outer membrane. (iii) Antibodies raised to the C-terminal 100 amino acids recognize a 31-kDa protein from purified mitochondria, but cross-reactivity was abolished when mitochondria were protease-treated to remove outer membrane-exposed proteins. Antibodies to AtTIM17-2 inhibited import of proteins via the general import pathway into outer membrane-ruptured mitochondria, but did not inhibit import protein via the carrier import pathway. Together these results indicate that the C-terminal region of AtTIM17-2 is exposed on the outer surface of the outer membrane, and the C-terminal region is essential for protein import into mitochondria.

Mitochondria were derived from a single endosymbiotic event that occurred approximately one billion years ago (1, 2). The loss of genes from the mitochondrial progenitor and transfer of genes to the host nucleus require that mitochondria rely on the import of nuclear-encoded cytosolic-synthesized proteins (3). This necessitated the development of specific targeting signals to direct proteins to the mitochondrion and an import apparatus to recognize and translocate the mitochondrial subset of proteins from all cytosolic-synthesized proteins (4–6). The completion of several genome sequencing projects allows a comparative genomic approach to be taken to identify and characterize the import apparatus of various organisms using the extensively studied yeast system as a template (7–10).

The import of proteins into mitochondria is achieved by a number of oligomeric protein complexes on the outer and inner mitochondrial membranes. A single translocase of the outer membrane (TOM) 1 recognizes all proteins destined for mitochondria, mediates their passage through the outer membrane, and subsequently passes them to other proteins complexes (11, 12). β-Barrier proteins destined for the outer membrane are passed to the sorting and assembly machinery of the outer membrane (SAM) (13–16). Signal anchored proteins in the outer membrane are imported via a distinct pathway (17). The general import pathways describes the import of proteins targeted to the translocase of the inner membrane 17:23 (TIM17:23) complex that is responsible for the import of proteins that contain N-terminal, generally cleavable, targeting sequences termed presequences. Proteins can be sorted to the inner membrane by this pathway via conservative or stop-transfer mechanisms (7, 11). The carrier import pathway describes the insertion of inner membrane proteins that contain internal targeting signals and that contain multiple transmembrane regions. These proteins are passed from TOM to small intermembrane space proteins, which act as chaperones to mediate transfer to the TIM22 complex on the inner membrane (18). TIM22 and TIM23 are voltage-activated channels on the inner membrane that are activated by internal and N-terminal targeting signals respectively (10, 19).

Comparison of the protein import apparatus of yeast with that in mammals and plants indicated that the basic machinery is generally well conserved between diverse eukaryotic lineages (8, 9, 20, 21). However differences do exist in specific components. Notably, orthologs for TOM70, TIM12, -18, -54, and TOB38 have not been identified in the Arabidopsis genome (22). Additionally, even when components appear shared at a functional level between organisms, they can be unrelated at a sequence level e.g. TOM20 from plants is not orthologous to yeast TOM20 as it displays low significant sequence similarity and significant structural differences (23). Interestingly, plant TOM22 lacks the cis-receptor domain that in yeast is a binding point for precursor proteins that have been recognized by TOM20. This modification may have been necessary to ensure the specificity of targeting to mitochondria and plastids in plant cells (24, 25). After initial recognition of precursor proteins by TOM20 in yeast they are proposed to be passed onto a series of binding sites located in the TOM and TIM17:23 complexes. This model was initially called the acid chain hypothesis but subsequently modified to the binding chain hypothesis to account for the important role of hydrophobic as well as ionic...
interactions (26–29). Characterization of TIM17 and TIM23 from Arabidopsis indicated that although they are clearly orthologous to yeast import components they appear to be quite distinct. Structurally Arabidopsis TIM23 lacks a complete pre-protein and amino acids transporter (PRAT) domain and does not contain the heptad leucine repeat domain (20, 26, 30). Furthermore, TIM17 contains a significant extension at the C-terminal end not present in fungi and mammals, but genome sequence information indicates this extension is present in other plant TIM17. The Arabidopsis orthologs can only complement yeast mutants when these differences are changed to resemble the yeast structure (30). This suggests that these components play different or additional roles in plants.

Here we characterize the topology of TIM17 from plants in particular reference to the large C-terminal extension that is absent in yeast and mammalian TIM17. We show that this extension is located in the outer membrane and is essential for import of proteins via the general import pathway in plants.

MATERIALS AND METHODS

Isolation of Mitochondria and in Vitro Import of Radiolabeled Precursor Proteins—Precursor proteins for the alternative oxidase (AOX) (31), the F$_o$/F$_c$ subunit of ATP synthase (F$_o$/F$_c$) (32), adenine nucleotide translocator (ANT), phosphate translocator (Pic) (33), and translocase of the inner membrane 17 (ATIM17-2) (30), were produced in a coupled TnT transcription-translation system according to the manufacturer’s instructions (Promega).

Percoll-purified Arabidopsis mitochondria were isolated according to Millar et al. (34). Outer membrane-ruptured mitochondria (Mit*OM) were prepared for two purposes: (i) to carry out in vitro imports to test for insertion into or across the inner membrane, and (ii) after the import assay to test for the intraganelle location of imported protein. Rupture of the outer membrane allowed access of added protease to intermembrane space components or inner membrane proteins exposed to the inner membrane space. Mit*OM were prepared by resuspending 100 $\mu$g of mitochondrial protein in 10 $\mu$L of SEH buffer (250 mM sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.4) and then adding 155 $\mu$L of 200 mM Hepes, pH 7.4 and incubating on ice for 20 min. To restore osmolarity, 25 $\mu$L of 2 M sucrose and 10 $\mu$L of 3 M KCl were added and mixed, re-pelleted, and washed in import buffer. This procedure removes cytochrome $c$ but not outer membrane components as determined by blotting with outer membrane markers (35). These outer membrane-ruptured mitochondria were either used in in vitro import assays or were subjected to protease treatment.

Import assays into mitochondria were carried out with 200 $\mu$g of mitochondrial protein in each import assay. Import assays were carried out at 26 °C for 20 min unless otherwise stated. Import assays were carried out in a modified hypophosphate tube buffer containing 150 mM succinate, 50 mM KCl, 10 mM MOPS, pH 7.2, 5 mM KH$_2$PO$_4$, 1% (w/v) bovine serum albumin, 1 mM MgCl$_2$, 1 mM methionine, 0.2 mM ADP, 0.75 mM ATP, 5 mM succinate, and 5 mM diethiothreitol. Assays were stopped by placing on ice for 3 min, dividing samples into two 100-$\mu$L aliquots, and adding proteinase K (PK) to one aliquot to a final concentration of 40 $\mu$g/mL. PK digestion was stopped by the addition of phenylmethanesulfonyl fluoride to 2 mM after 15 min. Mitochondria were diluted into 1 mL of ice-cold import buffer and pelleted by centrifugation for 3 min in a microcentrifuge. Mitochondrial pellets were resuspended in SDS-PAGE gel sample buffer, subject to SDS-PAGE, gels were dried, and products were visualized by exposing to a BAS TR2040 plate for 24 h and reading in a BAS 2500 Bio imaging analyzer (Fuji, Tokyo). A similar procedure was utilized for import into outer membrane-ruptured mitochondria except that the mitochondrial outer membrane was ruptured as outlined above before adding to the assay mixture.

Likewise these assays were similarly treated with PK unless otherwise stated. Import assays in the presence of difference amounts of malonate, a competitive inhibitor to complex II, were carried out by first adding the desired amount of malonate to mitochondria and then the import buffer and equilibrated for 3 min on ice prior to addition of precursor protein and commencement of import assay (32). For chase experiments import assays were kept on ice with added precursor, washed in ice-cold import buffer without proteinase, and resuspended in 200 $\mu$L of import buffer without precursor. These mitochondria were incubated at 26 °C for 20 min as for a standard import assay.

Tagging of ATIM17-2 and TIM23 with c-Myc and His$_{6}$ and in Vitro Import—The Rapid Translation System RTS-500 (Roche Applied Science) was used for in vitro production of epitope-tagged ATIM17-2. The coding region was cloned into PIVEX2.3-MCS (Roche Applied Science) ensuring that the C-terminal His$_{6}$ tag was in the correct reading frame. Addition of the N-terminal c-Myc tag (MEQKLISEEDL) was carried out using PCR with the N-terminal primer containing the coding sequence for the c-Myc tag. The resulting product was cloned into PIVEX2.3-MCS and confirmed by DNA sequencing. The proteins were synthesized in the RTS-500 system according to the manufacturer’s instructions for 24 h at 30 °C; the reaction vessel was mixed with magnetic agitation at 120 rpm. The synthesized protein was purified via two rounds of nickel affinity chromatography according to the manufacturer’s instructions (Qiagen). The final elution buffer was 8 $\mu$L urea, 0.1 M NaH$_2$PO$_4$, 10 mM Tris-HCl, pH 4.3. The protein was stored at −80 °C until use. Import assays using this precursor protein were carried out by thawing the purified protein followed by sonication for 10 s for three times on a microtip setting of 4 (Branson sonifier B-12). This protein was immediately added to the in vitro import assay; 4 $\mu$L was added to a 400-$\mu$L assay to dilute the urea to below 100 mM so as not to inhibit the import assay. The import assays were treated as outlined above except that after SDS-PAGE, the products were detected by Western blot analysis using antibodies to c-Myc and His$_{6}$ (Roche Applied Science).

Production of Antibodies and Western Blot Analysis—Immunodetection of various proteins was carried out by transferring proteins separated on PAGE to support nitrocellulose membranes (Amersham Biosciences). Proteins were probed with a variety of antibodies and visualized and quantitated using chemiluminescence (Roche Applied Science) using a LAS 1000 (Fuji, Tokyo). Antibodies to Arabidopsis TOM20 (36), Uncoupling protein (UCP) (37), cytochrome $c$ (BD Pharmingen), and ATIM17-2 were used to probe membranes. Inoculating rabbits with the C-terminal 100 amino acids of TIM17 as antigen resulted in rabbit antibodies (38). The C-terminal extension of ATIM17-2 (amino acids 143–243) was cloned into pDest15 (Invitrogen) protein expression vector using the Gateway Cloning Technology (Invitrogen). A PreScisson protease cleavage site (LEVLFQGP) was introduced inbetween the N-terminal GST tag and TIM17. Recombinant GST-LEVFLQGP-TIM17 expression was induced in the Escherichia coli strain BL21(DE3)pLyso5 via isopropyl-1-thio-β-D-galactopyranoside (Promega). A PreScisson-protected product was isolated and resuspended in SDS-PAGE gel sample buffer, subjected to SDS-PAGE, gels were dried, and products were visualized by exposure to an autoradiogram. A similar procedure was utilized for import into mitochondria except that the GST tag by digestion with PreScisson protease (Amersham Biosciences), as the recombinant protein remained bound to glutathione-Sepharose. The 15-kDa protein was dialyzed in phosphate-buffered saline and sequenced by mass spectrometry to confirm identity prior to inoculation of rabbits.

RESULTS

In Vitro Imported TIM17 Remains Sensitive to Externally Added Protease—TIM17 is encoded by three genes in Arabidopsis, designated ATIM17-1 (At1g20350), ATIM17-2 (At2g37410), and ATIM17-3 (At5g11690) (9, 30). Although all predicted proteins display 50% sequence similarity with yeast TIM17 (158 amino acids in length) the predicted proteins differ in length; ATIM17-1 is 218 amino acids, ATIM17-2 is 243 amino acids, and ATIM17-3 is 133 amino acids. Sequence alignments show ATIM17-1 and ATIM17-2 both contain long C-terminal extensions not present in ATIM17-3 or the yeast TIM17. The ATIM17-2 transcript was expressed at 10-fold higher levels than the other two genes in all Arabidopsis tissues examined and its product, ATIM17-2, was the only TIM17 paralog identified in isolated Arabidopsis mitochondria by tandem mass spectrometry (39). Therefore we investigated the intraorganelle location and function of ATIM17-2 with particular reference to its C-terminal extension that appears to be plant specific. In vitro mitochondrial uptake of radiolabeled ATIM17-2 resulted in a protease-protected product with an apparent molecular mass of 31 kDa. An additional band of 28 kDa was generated on protease treatment of mitochondria compared with the precursor alone (Fig. 1A, lanes 1–3). The generation of this 28-kDa product required an inner membrane potential as increasing malonate concentration to 50 mM, to dissipate the mitochondrial membrane potential (Δψ), resulted in no protease generated product (Fig. 1, lane 9). Notably, the protease-protected product of 31 kDa was still evident in the absence of Δψ (Fig. 1, lanes 8 and 9). We also tested the import of a protein
imported via the carrier import pathway, ANT. The N-terminal extension of the plant ANT was cleaved generating a mature form of 31 kDa from a 39-kDa precursor (33, 35). At 50 mM malonate, no processing of ANT to the mature form was detected suggesting dependence. AOX, a protein imported via the general import pathway, was imported and processed as previously documented except at 50 mM malonate, where no import occurred because of the lack of /H9004/H9274 (Fig. 1A) (31). Thus we concluded that all precursors tested were imported in a membrane potential-dependent manner. The protease-protected forms of AtTIM17-2 and ANT at 50 mM malonate likely represent stage III import intermediates (see below). Translocation of proteins via the carrier pathway to a protease insensitive location in the absence of a membrane potential has been shown in mitochondria (40, 41) and a reconstituted vesicle system (42). From these observations it appeared that a portion of AtTIM17-2 remained protease accessible when imported into the inner mitochondrial membrane.

To further characterize the location of imported AtTIM17-2 we increased the amount of added protease and also ruptured the outer membrane after the import assay but prior to protease treatment (Fig. 1B). Increasing of protease to four times the amount normally used to remove unimported precursor proteins did not further convert the 31-kDa AtTIM17-2 precursors
form to the 28-kDa form. Confirmation that the imported At-TIM17-2 was located in the inner membrane was demonstrated by the fact that upon rupture of the outer membrane a 16-kDa inner membrane protease protected form was evident as previously reported (30). AOX was used as a control because it is located on the inner surface of the inner membrane, and mature imported protein was protected from increasing protease concentration in both intact and outer membrane-ruptured mitochondria (Fig. 1B). In addition to the 28 kDa protein generated by externally added protease, a product with an apparent molecular mass of 21 kDa was generated during incubation of At/TIM17-2 with mitochondria (Fig. 1B, lane 2, marked with an asterisk). This product was partially sensitive to protease treatment and is probably attributed to translation initiation at internal methionines. Previously we have demonstrated that the four radiolabeled bands with apparent molecular masses of 31, 27.5, 24.5, and 23 kDa, which are present in the translation mixture of At/TIM17-2, represent translations commencing at methionine residues 1, 25, 57, and 80, respectively. Additionally the 18-kDa product represents translation commencing at methionine residue 143 (30). The translation products of 27.5, 24.5, and 23 kDa still contain transmembrane regions 3 and 4, which contain the mitochondrial targeting activity of At/TIM17-2 (30). Thus the 21-kDa product generated upon incubation with mitochondria likely represents these imported products that have been subjected to endogenous proteolysis. Like the full-length translation product of 31 kDa, the 21-kDa product is only partially sensitive to externally added protease in intact mitochondria. The 18-kDa translation product beginning at methionine 143 has no mitochondrial targeting activity (30).

In combination, these data suggested the undigested 31-kDa form of At/TIM17-2 could represent one of two forms, either it is an imported product located in the inner membrane with a fraction also in the outer membrane, or the protease-protected 31-kDa form is a stage III import intermediate of the carrier import pathway not yet inserted into the inner membrane, as suggested by the fact it is imported into a protease-insensitive location in the absence of a Δψ (Fig. 1) (40–42). To confirm the latter we bound At/TIM17-2 to mitochondria and then chased it into the inner membrane (Fig. 1C). Incubation of At/TIM17-2 with mitochondria at 4 °C resulted in only a very small amount of 31-kDa At/TIM17-2 been protected from protease digestion, which suggested external binding to mitochondria (Fig. 1C, lane 3). Similarly, no import of AOX was observed under these conditions because no protease-resistant products were detected (Fig. 1C, lane 3). These mitochondria were washed in import buffer without precursor protein and incubated at 26 °C for 20 min. It was observed that ~90% of the bound 31-kDa At/TIM17-2 protein was converted to the 28-kDa form. Thus we concluded that the 28-kDa form of At/TIM17-2 represents the truly imported form, and the protease-protected 31-kDa band is a stage III-like import intermediate that is imported across the outer membrane to a protease-insensitive location but not inserted into the inner membrane.

The integrity of mitochondria, the rupture of the outer membrane and efficacy of the protease treatments performed in these experiments was monitored using antibodies raised to TOM20 (outer membrane marker), cytochrome c (intermembrane space marker), and the uncoupling protein (UCP; inner membrane marker). UCP was resistant to protease and outer membrane rupture, cytochrome c was only digested by protease upon outer membrane rupture, whereas TOM20 was digested by protease in intact and outer membrane-ruptured mitochondria (Fig. 1D).

The C-terminal Region of AtTIM17-2 Is Protease-sensitive in the Outer Membrane—To determine which region of AtTIM17-2 was protease-sensitive in mitochondria, the N terminus was tagged with c-Myc and the C-terminal with His6. These proteins were expressed in an in vitro translation system, purified using nickel affinity chromatography, denatured in urea, and used in import assays with purified mitochondria. The imported and protease-protected proteins were detected by Western blotting (Fig. 2A). Protease treatment resulted in the removal of the C-terminal His6 tag from AtTIM17-2 because no signal was detected when anti-His6 antibodies were used to detect imported products (Fig. 2A). In contrast, antibodies to c-Myc detected a protease product of 31 kDa compared with the undigested form of 34 kDa. The small differences in apparent molecular mass between the tagged proteins (Fig. 2) and radiolabeled proteins (Fig. 1) are caused by the presence of the tags. Deletion of the 100 amino acids at the C-terminal region of At/TIM17-2 resulted in both the C-terminal and N-terminal tags being protease-protected after import. We conclude that the C-terminal His6 tag can be efficiently imported across the outer membrane and that the 100 amino acids of the C-terminal region of At/TIM17-2 were responsible for the protease sensitivity in the outer membrane.

To confirm the location of the C-terminal region of At/TIM17-2 in the outer membrane we also raised antibodies to the C-terminal 100 amino acids. Purification and protease treatment of mitochondria were carried out. Antibodies to the outer membrane protein import receptor, TOM20, detected a single band with an apparent molecular mass of 23 kDa, which is sensitive to the lowest amount of protease added (Fig. 2B). Antibodies raised to the 100 C-terminal amino acids of At/TIM17-2 detected a protein with an apparent molecular mass of 31 kDa. This product was also sensitive to protease digestion but was somewhat more protected than TOM20 (Fig. 2B). Typically, 75% of the signal was removed by digestion with 40 μg/ml of proteinase K and increasing PK to 80 μg/ml with the remaining 25% digested in 90% digestion. However, complete digestion was not achieved with higher amounts of added protease. This indicates that a small proportion (~10%) of At/TIM17-2 is not exposed in the outer membrane. The intactness of the outer membrane was confirmed because cytochrome c was protected even with higher protease concentrations (Fig. 2B). Rupture of the outer membrane resulted in complete digestion of TOM20, At/TIM17-2, and cytochrome c. Note that the antibody raised against At/TIM17-2 will only recognize the C-terminal region and not the inner membrane-located region. Attempts to raise antibodies to the predicted inner membrane regions of At/TIM17-2 have proven unsuccessful to date.

Antibodies to the C Terminus of AtTIM17-2 Specifically Inhibit the General Import Pathway—The antibodies raised against the C-terminal 100 amino acids of At/TIM17-2 did not inhibit protein import into intact mitochondria, perhaps because of difficulties in the antibodies accessing the outer membrane component of At/TIM17-2 (data not shown). Therefore we tested whether the antibody could inhibit import into outer membrane-ruptured mitochondria. Import was conducted with two proteins by the general import pathway (AOX, FAd) and one carrier protein via the carrier import pathway (Pic) into outer membrane-ruptured mitochondria (Fig. 2C, lane 3) (33, 35). Preincubation of outer membrane-ruptured mitochondria with preimmune serum had no effect on the import of these proteins (Fig. 2C, lanes 4 and 5). However antibodies to At/TIM17-2 completely abolished the import of AOX and FAd (Fig. 2C, lanes 6 and 7). In contrast no inhibition of import of Pic was observed (Fig. 2C, lanes 6 and 7). We used this precursor because it also contains an N-terminal cleavable extension that is removed upon import, but nonetheless
is imported via the carrier pathway (33). Thus the C-terminal 100 amino acids of TIM17-2 are essential for protein import via the general import pathway but are not necessary for import via the carrier pathway.

Import via the General Import Pathway Requires an Outer Membrane Protease-sensitive Component—The binding chain hypothesis proposes that there are a series of binding sites on components of the import apparatus that achieve the unidirectional transport of precursor proteins across both mitochondrial membranes (29). It has been demonstrated in yeast that TIM17:23 can recognize and import proteins via the general import pathway when the outer membrane and the TOM complex are disrupted or absent (43, 44). Rupture of the outer membrane of plant mitochondria followed by import assays indicated that import via the general import pathway (AOX) and carrier import pathway (ANT) was still possible (Fig. 3A). This import was reduced in abundance compared with intact mitochondria (Fig. 3, A and C). For the carrier import pathway we have previously shown that import can be stimulated to levels observed with intact mitochondria by re-addition of intermembrane space fractions (35). However it is possible that import is still taking place via the TOM complex in the attached but disrupted outer membrane. To ensure the TOM complex was bypassed, we pretreated mitochondria with protease that resulted in complete abolition of import via the general and carrier import pathways (Fig. 3B, lanes 4 and 5, 11 and 12). When the outer membrane was ruptured, import of ANT was restored (Fig. 3B, lanes 6 and 7), thus bypassing the

![Image](https://example.com/image1.png)

**Fig. 2. Immunodetection of AtTIM17-2 in mitochondria and immunoinhibition of protein import.** A. AtTIM17-2 was tagged at the N terminus with c-Myc and at the C terminus with His6. Also tagged in a similar manner was AtTIM17-2 that had the coding region for the 100 C-terminal amino acid-deleted AtTIM17-243–243. The proteins were expressed in an in vitro expression lysate, purified via nickel affinity chromatography, and imported into mitochondria. The imported products were detected with antibodies to c-Myc and His6. Lane 1, precursor protein detected with c-Myc antibody. Lane 2, precursor protein incubated with mitochondria under conditions that support import and precursor detected with c-Myc antibodies. Lane 3, as lane 2 except that mitochondria were treated with PK to a final concentration of 40 μg/ml. The TIM17 precursor is reduced in apparent molecular mass from 34 to 31 kDa. Lanes 4–6 as for lanes 1–3 except that antibodies to His6 were used to detect precursor proteins. In the case of AtTIM17-2, no product was detected on protease treatment (lane 6). B, antibodies raised against the 100 amino acids of AtTIM17-2 were used to probe mitochondria and outer membrane-ruptured mitochondria (Mit*OM) treated with increasing amounts of protease as indicated. C, effect of AtTIM17-2 antibodies on import of AOX, FAd, and Pic precursor proteins into outer membrane-ruptured mitochondria. Lane 1, precursor protein alone. Lane 2, precursor protein incubated with mitochondria under conditions that support import. Lane 3, as lane 2 except outer membrane-ruptured mitochondria were used in the in vitro import assay and treated with PK. Lanes 4 and 5, as lane 3 except that outer membrane-ruptured mitochondria were incubated with 100 and 200 μg of preimmune serum (PI) prior to addition of the uptake assay. Lanes 6 and 7, as lanes 4 and 5 except 100 and 200 μg of antibodies to AtTIM17-2 was added to outer membrane-ruptured mitochondria prior to addition of the uptake assay. AtTIM17-2 Ab, AtTIM17-2 antibodies.
TOM complex could be achieved with a protein imported via the carrier import pathway. In contrast no import of AOX could be detected with protease-treated outer membrane-ruptured mitochondria. Thus a protease-sensitive outer membrane-exposed component was essential for import via the general import pathway.

**DISCUSSION**

The complete sequencing of the Arabidopsis genome allows a comparative genomic approach to be taken to identifying the components of the mitochondrial import apparatus using the model yeast system as a template (7, 10, 45). Using sequence similarity as a primary tool it has been possible to quickly identify many of these components in other organisms, albeit organism-specific components will not be identified using this approach (8, 9, 22). In Arabidopsis and other organisms it has emerged (in contrast to yeast) that many of the import components are encoded by small gene families (8, 9). Evidence is emerging that these genes are expressed in a tissue, developmental, or inducible manner and thus the functional charac-

**Fig. 3. Import of precursor protein into mitochondria and outer membrane-ruptured mitochondria.** A, import of ANT and AOX into mitochondria and outer membrane-ruptured mitochondria. Lane 1, precursor protein. Lane 2, precursor protein incubated with mitochondria, an additional 31-kDa mature form is evident. Lane 3, as lane 2 with PK added to 40 μg/ml. Lanes 4 and 5, as lanes 2 and 3 except that mitochondria that had the outer membrane-ruptured were used in import assays. Import was reduced (see C). Lanes 6–10, as lanes 1–5 except that the AOX precursor protein was used. B, import of ANT and AOX into mitochondria, mitochondria that were treated with PK to 40 μg/ml, and outer membrane-ruptured mitochondria where the mitochondria were treated with PK to 40 μg/ml prior to rupture of the outer membrane. Lane 1, precursor protein. Lane 2, precursor protein incubated with mitochondria. Lane 3 as lane 2 with PK added to 40 μg/ml after the import assay. Lanes 4 and 5, as lanes 2 and 3 except that mitochondria were treated with PK to 40 μg/ml prior to import assay, no imported products are detected. Lane 6, as lane 4, except that the outer membrane of protease-treated mitochondria was ruptured prior to the import assay, a mature form of 31 kDa is visible. Lane 7, as lane 6, with Mit*OM treated with PK to 40 μg/ml after the import assay. Lanes 8–14, as lanes 1–7, except that AOX was used in the import assay. No import was detected into PK-pretreated mitochondria or outer membrane-ruptured mitochondria that had been treated with PK to 40 μg/ml. C, quantitation of import into mitochondria, outer membrane-ruptured mitochondria (Mit*OM), and PK-treated mitochondria that had outer membrane-ruptured (PK-Mit*OM).
A physical link between the outer and inner membrane via AtTIM17 suggests a novel role in the import of proteins. Import of proteins into plant mitochondria that had the outer membrane-ruptured (but not removed) can take place via the general and carrier import pathways (Fig. 3A) (33, 35). However, prior protease digestion of mitochondria with 40 μg/ml PK followed by outer membrane rupture abolished the import of proteins via the general import pathway, but not via the carrier import pathway (Fig. 3, B and C). Thus an outer membrane protease-sensitive component was essential for import via the general import pathway in Arabidopsis. Antibodies to the C-terminal 100 amino acids of AtTIM17-2 inhibited import into outer membrane-ruptured mitochondria of proteins that are imported via the general import pathway (Fig. 4B). Together these lines of evidence suggest that a portion of the C-terminal 100 amino acids of AtTIM17-2 plays a novel and direct role in protein import, either binding precursor proteins or interacting with components that bind precursor and undertake an essential role in transferring them to TIM17:23.

The reason why plants have this important function associated with AtTIM17-2 is not clear. We have previously demonstrated that AtTIM17-2 can complement a yeast mutant but only when the C-terminal 100 amino acids were removed (30). Thus in yeast, this extension appears to interfere with the inner membrane function of TIM17. Interestingly, the binding partner for TIM17, known as TIM23, has an N-terminal region located in the outer membrane in yeast (44), but TIM23 does not contain this extension in the Arabidopsis sequence (30). The C-terminal extension in TIM17 in plants may conceivably play an early role in the import process; after the precursors are bound to TOM20 they could be passed to TIM17:23.

To determine if AtTIM17-2 plays a novel and direct role in protein import, either binding precursor proteins or interacting with components that bind precursor and undertake an essential role in transferring them to TIM17:23.

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