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Functional Definition of Outer Membrane Proteins Involved in Preprotein Import into Mitochondria[W]

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The role of plant mitochondrial outer membrane proteins in the process of preprotein import was investigated, as some of the principal components characterized in yeast have been shown to be absent or evolutionarily distinct in plants. Three outer membrane proteins of *Arabidopsis thaliana* mitochondria were studied: TOM20 (translocase of the outer mitochondrial membrane), METAXIN, and mtOM64 (outer mitochondrial membrane protein of 64 kD). A single functional *Arabidopsis TOM20* gene is sufficient to produce a normal multisubunit translocase of the outer membrane complex. Simultaneous inactivation of two of the three *TOM20* genes changed the rate of import for some precursor proteins, revealing limited isoform subfunctionalization. Inactivation of all three *TOM20* genes resulted in severely reduced rates of import for some but not all precursor proteins. The outer membrane protein METAXIN was characterized to play a role in the import of mitochondrial precursor proteins and likely plays a role in the assembly of β-barrel proteins into the outer membrane. An outer mitochondrial membrane protein of 64 kD (mtOM64) with high sequence similarity to a chloroplast import receptor was shown to interact with a variety of precursor proteins. All three proteins have domains exposed to the cytosol and interacted with a variety of precursor proteins, as determined by pull-down and yeast two-hybrid interaction assays. Furthermore, inactivation of one resulted in protein abundance changes in the others, suggesting functional redundancy. Thus, it is proposed that all three components directly interact with precursor proteins to participate in early stages of mitochondrial protein import.

INTRODUCTION

The mitochondrial protein import machinery has been most comprehensively characterized in yeast (*Saccharomyces cerevisiae*), *Neurospora crassa*, and to a lesser extent in mammalian systems. Hetero-oligomeric translocation complexes in the outer and inner membranes mediate the recognition, import, and suborganellar sorting of mitochondrial precursor proteins (Neupert, 1997; Pfanner and Geissler, 2001; Hoogenraad et al., 2002; Truscott et al., 2003; Wiedemann et al., 2004). The translocase of the outer mitochondrial membrane (TOM) complex facilitates the recognition of precursor proteins and their translocation through the outer membrane (Taylor and Pfanner, 2004). In yeast, the outer membrane receptors TOM20, TOM22, and TOM70 associate with the general import pore that consists of the pore-forming TOM40, and TOM7, TOM6, and TOM5. TOM20 and TOM70 are N-terminal anchored primary receptor proteins that recognize precursor proteins with N-terminal and internal targeting information, respectively (Wiedemann et al., 2004). Precursor proteins subsequently interact with the TOM22 receptor, which delivers them to the general import pore (Taylor and Pfanner, 2004). Operating in concert with the TOM complex, the sorting and assembly machinery (SAM) complex (also called topogenesis of mitochondria outer membrane β-barrel proteins [TOB]) in the outer mitochondrial membrane inserts proteins into the outer membrane (Pfanner et al., 2004; Taylor and Pfanner, 2004; Habib et al., 2005; Paschen et al., 2005). Although initially thought to be a protein import receptor of the TOM complex (Gratzer et al., 1995), SAM37 (also called MAS37/TOM37) was subsequently reported to be located in a distinct outer membrane complex, SAM, and was demonstrated to function in the sorting and assembly of β-barrel proteins (Wiedemann et al., 2003). The SAM complex, consisting of SAM50 (TOB55), SAM35 (TOB38), SAM37, and MDM10 (TOM13), in yeast and *N. crassa*, is required for the correct assembly of complex β-barrel proteins into the outer membrane after import through the TOM complex, but the precise molecular functions of SAM35 and SAM37 have not yet been elucidated (Wiedemann et al., 2003; Paschen et al., 2005; Neupert and Herrmann 2007). Proteins destined for the inner membrane and matrix interact with two discrete TIM (translocase of the inner mitochondrial membrane) complexes (Wiedemann et al., 2004).

Experimental isolation of mitochondrial proteins coupled with genome sequence analysis in animals and plants has revealed that much of the import apparatus is conserved throughout diverse eukaryotic lineages (Herrmann, 2003; Dyall et al., 2004; Lister et al., 2005; Dolezal et al., 2006). However, considerable differences exist in the TOM complex subunit composition between

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species; only the core translocase module of TOM40, TOM22, and TOM7 is conserved (Dolezal et al., 2006), and the plant TOM22 has lost the N-terminal receptor domain present in yeast and animals (Macasev et al., 2004). Isolation of the plant TOM complex also identified small proteins analogous to yeast TOM5 and TOM6 and a 23-kD protein (plant TOM20) analogous to yeast/animal TOM20, but none displayed significant protein sequence similarity to the yeast or mammalian counterparts (Heins and Schmitz, 1996; Jansch et al., 1998; Werhahn et al., 2003). Subsequent elucidation of the solution structure of the plant TOM20 showed it to have a similar tertiary structure but reverse domain arrangement to yeast/animal TOM20 (Perry et al., 2006). Given that there is no significant sequence similarity between plant TOM20 and that of yeast or mammals, it is proposed that convergent evolution has lead to a receptor with the same function but reversed orientation (Lister and Whelan, 2006; Perry et al., 2006). As plant TOM20 is not orthologous to yeast or mammalian TOM20, it is of interest to determine if it plays the same role in the TOM complex.

No clear plant homolog of the yeast and animal TOM70 receptor can be identified in the genomes of *Arabidopsis thaliana* or rice (*Oryza sativa*; Chan et al., 2006), in agreement with the lack of any biochemical evidence for this component in the purified plant TOM complex (Jansch et al., 1998). Interestingly, anchored in the outer membrane of *Arabidopsis* mitochondria is mtOM64, a paralog of the chloroplast outer envelope protein import receptor At TOC64-III (translocase of the outer chloroplast envelope) (Chew et al., 2004; Qbadou et al., 2006). Notably, inactivation of both plastid-localized *TOC64* orthologs in *Physcomitrella patens* and inactivation of TOC64 in *Arabidopsis* had no effect on protein import into plastids (Hofmann and Theg, 2005; Aronsson et al., 2007). To date, no functional role has been demonstrated for mtOM64 in *Arabidopsis*. Plants also display differences in the SAM complex in comparison to yeast; in plants, only SAM50 can be clearly identified by sequence similarity (Lister et al., 2005). *Arabidopsis* METAXIN was identified by sequence similarity to the human METAXIN 1 protein; the latter displays limited sequence similarity to yeast SAM37 and has been implicated in mitochondrial protein import (Armstrong et al., 1997; Abdul et al., 2000). METAXIN1 and METAXIN2 in mammals have been reported to be involved in the import of β-barrel proteins but in a different complex compared with SAM50 (Kozjak-Pavlovic et al., 2007). METAXIN protein has been shown to be present in *Arabidopsis* mitochondria (Lister et al., 2004), but no role in mitochondrial protein import or sorting has been demonstrated.

Another notable difference between yeast and higher eukaryotes, such as plants and mammals, is that the yeast subunits of the TOM, TIM, and SAM complexes are encoded by single nuclear genes, while in higher organisms, components of TOM, TIM, and SAM are often encoded by small multigene families. It is not clear what the function of these multigene families are, whether they represent functionally distinct isoforms or provide greater ability for regulation of these components at a transcriptional level (i.e., subfunctionalization verses neofunctionalization). In *Drosophila melanogaster*, it has been documented that TOM20 and TOM40 are each encoded by two differentially expressed genes (Hwa et al., 2004). Detailed expression analysis of almost all genes encoding mitochondrial protein import components in *Arabidopsis* indicates differential expression patterns of genes within each family (Lister et al., 2004). Furthermore, functional analysis of the *Arabidopsis* TIM17 gene family suggests some differences in function based on the ability to complement a *tim17* mutant in yeast (Murcha et al., 2003).

Although yeast is an excellent model to provide the basic mechanism of how proteins are imported into mitochondria, it cannot give insights into the functions of nonhomologous components in other organisms, such as plants, in which the putative receptor components are not orthologous to yeast receptor subunits. Furthermore, the yeast model system cannot be used to explore any functional diversification of these multiple genes encoding import components. Here, we investigate the function of three outer mitochondrial membrane proteins in *Arabidopsis* with respect to protein import into mitochondria. We demonstrate that the three highly expressed TOM20 isoforms in *Arabidopsis* are predominantly functionally equivalent but display limited specialization and together are important, but not essential, for the import of a wide range of mitochondrial proteins. Furthermore, we demonstrate that mtOM64 is a mitochondrial protein that can interact with a variety of precursor proteins, increases in abundance when two or more TOM20 isoforms are inactivated, and plays a role in the import for at least some mitochondrial proteins. Finally, we identify that METAXIN interacts with a wide variety of precursor proteins and is involved in their import, and results suggest that it plays more than one role in the import and assembly of proteins into mitochondria.

RESULTS

The TOM20 Gene Family Encodes Functionally Redundant Proteins Involved in Mitochondrial Protein Import

TOM20 is encoded by four paralogous genes in the *Arabidopsis* genome, *TOM20-1* to *TOM20-4* (Werhahn et al., 2001). *TOM20-1* and *TOM20-3* are tandemly duplicated genes, the predicted proteins of which display 60% amino acid identity. *TOM20-2*, *TOM20-3*, and *TOM20-4* are highly expressed in diverse plant organs (Lister et al., 2004), whereas by contrast, *TOM20-1* transcript is rarely detectable (see Supplemental Figure 1A online) (Lister et al., 2004; Murcha et al., 2007), and TOM20-1 is the only TOM20 protein that has not been directly identified in isolated plant mitochondria (Werhahn et al., 2001; Heazlewood et al., 2004; Lister et al., 2004). Antibodies raised against TOM20-3 cross-react with in vitro–translated TOM20-1 (see Supplemental Figure 1B online), but TOM20-1 could not be detected with this antibody in mitochondria isolated from whole seedlings of any of the *Arabidopsis* genotypes used in this study. Mitochondria were isolated from wild-type *Arabidopsis* (ecotype Columbia-0) and two independent T-DNA insertion lines of each highly expressed *TOM20* gene (Sessions et al., 2002; Alonso et al., 2003; Rosso et al., 2003), and the absence of transcript derived from the specific TOM20 isoforms was verified by RT-PCR (data not shown) and immunodetection of total mitochondrial protein samples with specific antibodies raised to each of these TOM20 isoforms (Figure 1A). No severe phenotypic abnormalities were observed in any of the single insertion mutants; however, *tom20-2* had a slightly delayed flowering time, 4 to 7 d later than wild-type plants (Figure 2A).

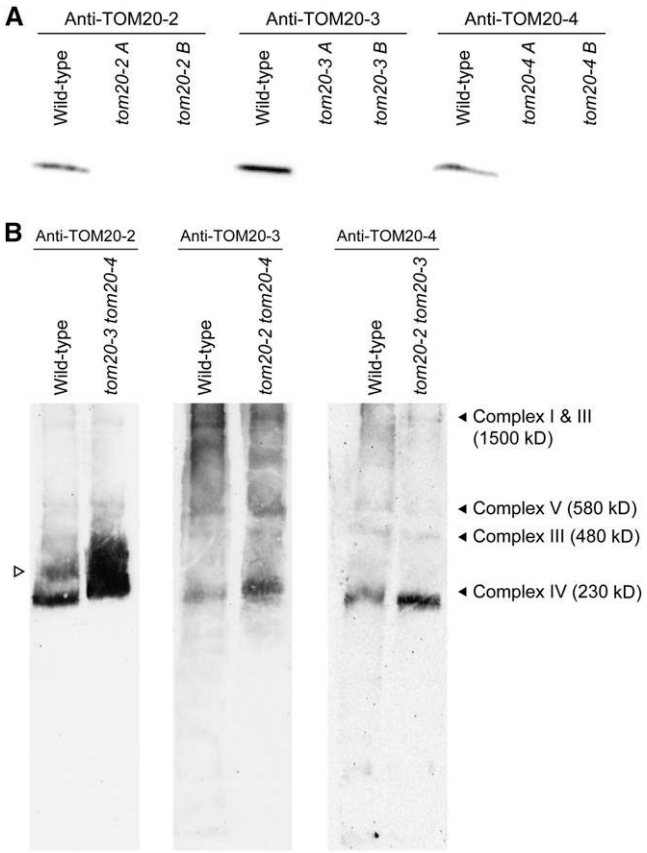

Figure 1. The TOM Complex Can Form with Only One TOM20 Isoform.

(A) Immunodetection of Tom20 isoforms in mitochondrial protein samples isolated from wild-type and *tom20* T-DNA insertional mutant plants and separated by SDS-PAGE.

(B) Immunodetection of Tom20 proteins in mitochondrial protein samples isolated from wild-type and *tom20* double knockout plants after digitonin solubilization and separation of protein complexes by first dimension BN-PAGE. The open arrowhead indicates the higher molecular weight complex containing TOM20-2. The position and approximate molecular mass of the inner membrane respiratory complexes I, III, IV, and V are indicated, as detected by the residual Coomassie blue staining and reported in a previous study of potato mitochondrial protein complexes (Jansch et al., 1998).

In vitro import of radiolabeled plant mitochondrial precursor proteins into mitochondria isolated from wild-type and *tom20* plants was performed. While the selection of precursor proteins represented a wide range of mitochondrial protein import pathways (general import pathway, alternative oxidase [AOX] and the 10-kD protein of the small mitochondrial ribosomal subunit [RPS10]; carrier import pathway, mitochondrial phosphate carrier [PiC]; dual-targeted proteins, glutathione reductase [GR]; plant specific protein, F_Ad-subunit of mitochondrial ATP synthase) (Murcha et al., 1999; Heazlewood et al., 2003), no significant differences in the rate of protein import were observed between wild-type and *tom20* mitochondria for any precursor protein (see Supplemental Figure 2 online). Therefore, none of the Tom20 proteins alone appeared to have an essential function in mitochondrial protein import.

Different TOM20 Isoforms Display Some Precursor Recognition Specificity

The single *TOM20* isoform mutant plants were crossed to generate double *TOM20* knockout plants deficient in each combination of the three highly expressed *TOM20* isoforms. Notably, the resulting double knockout plants were viable and did not display severe phenotypic abnormalities, although again slight delays in flowering time were noticeable (Figure 2A). Mitochondria were isolated from wild-type and double *tom20* knockout plants and the protein complexes solubilized by digitonin treatment and separated by one-dimensional blue native PAGE (BN-PAGE), followed by immunodetection of the remaining TOM20 protein (Figure 1B). TOM20-3 in both wild-type mitochondria and the *tom20-2 tom20-4* mitochondria was detected in a protein complex of a similar apparent molecular mass, indicating that the loss of two TOM20 isoforms did not disrupt the integrity or structure of the TOM complex. This was also observed for TOM20-4 in wild-type and *tom20-2 tom20-3* mitochondria. Residual Coomassie blue staining of the mitochondrial inner membrane respiratory complexes I, III, IV, and V enabled estimation of the TOM complex molecular mass at ~230 kD, based on a comparison with a previous study of potato (*Solanum tuberosum*) mitochondrial membrane protein complexes (Jansch et al., 1998). Interestingly, a second complex containing TOM20-2 was always detected in wild-type mitochondria (Figure 1B, open arrowhead), indicating that some TOM20-2 is located in a higher molecular weight complex. Furthermore, in mitochondria isolated from *tom20-3 tom20-4* plants, a larger proportion of TOM20-2 was present at a higher molecular weight, abolishing the two discrete complexes and forming a continuous distribution of larger complexes. Thus, when mitochondria only contain TOM20-2 it appears to lead to an ectopic size distribution of TOM complexes.

The rate of protein import into mitochondria with only one functional TOM20 protein was measured in vitro for a range of plant mitochondrial precursor proteins (Figure 2B). The import of TOM40 and PiC was unaffected; however, for the other precursor proteins, significant differences in the rate of protein import in various mutants were observed ($P < 0.05$). AOX import into *tom20-2 tom20-3* and *tom20-2 tom20-4* mitochondria was reduced to 71 and 64%, respectively, compared with AOX import into wild-type mitochondria. F_Ad was imported into *tom20-2 tom20-4* mitochondria at 85% of the wild-type rate of import. Finally, the import of the dual-targeted protein GR into *tom20-2 tom20-3* mitochondria was 31% higher after 10 min and 71% higher after 20 min, at which the rate was still linear. Therefore, generation of mutant plants with only one functional TOM20 protein revealed that a functional TOM complex can be formed with a single TOM20 and that different TOM20 isoforms display some precursor recognition specificity that is evident when import of a variety of precursor proteins is assessed.

Tom20 Is a Nonessential Protein

Plants were generated that lacked all the three highly expressed TOM20 isoforms, *tom20-2 tom20-3 tom20-4* (*tom20* triple knockout).

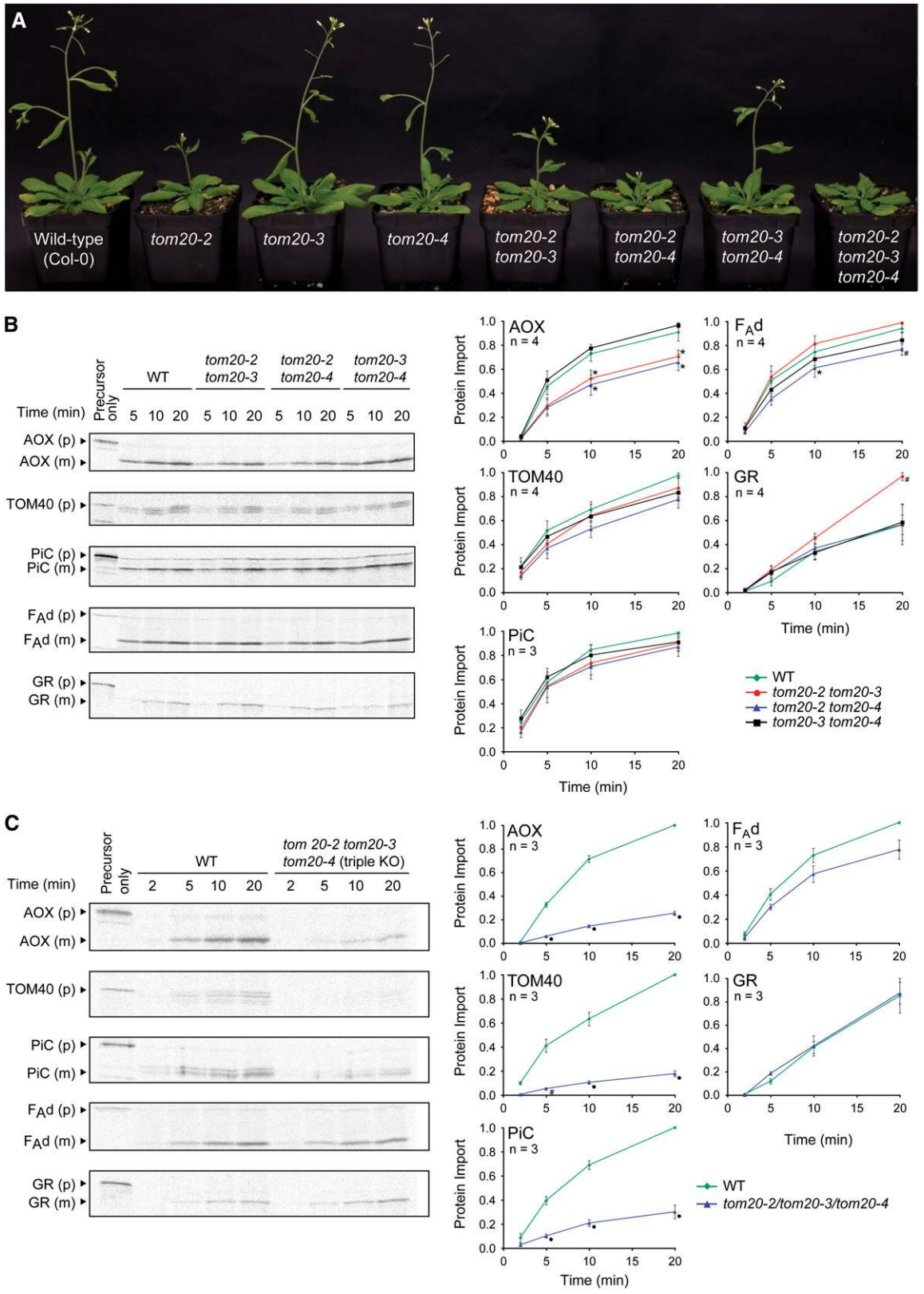

Figure 2. *Arabidopsis* TOM20 Is Encoded by a Multiple Gene Family of Predominantly Functionally Equivalent Isoforms That Plays a Role in the Import of Preproteins into Mitochondria.

These plants were viable but displayed a slightly slower growth rate (Figure 2A). In vitro import of radiolabeled precursor proteins into mitochondria isolated from *tom20* triple knockout plants indicated that TOM20 is involved in the import of a diverse range of mitochondrial precursor proteins (Figure 2C). Import of AOX, TOM40, and PiC was reduced to only 20 to 30% of the amount of import into wild-type mitochondria ($P < 0.01$), indicating that TOM20 is involved in the general, carrier, and outer membrane β-barrel protein import pathways. However, the import of GR was unaffected, and import of F_Ad did not decrease significantly ($P > 0.05$), indicating that TOM20 has little or no involvement in the import of these precursor proteins or that its absence can be compensated for completely by another import component.

mtOM64 Is Involved in Mitochondrial Protein Import

mtOM64 is a protein anchored in the mitochondrial outer membrane that has 67% protein sequence identity to At TOC64-III, a chloroplast outer envelope protein import receptor (Chew et al., 2004; Qbadou et al., 2006). Sequence analysis indicated that mtOM64 has three C-terminal tetratricopeptide repeat (TPR) motifs, resembling the C-terminal TPR domains of At TOC64-III and TOM70 that function to recognize chloroplast and mitochondrial proteins bound to HSP90 (Young et al., 2003; Qbadou et al., 2006). To investigate the role of mtOM64, two *Arabidopsis* lines with independent T-DNA insertions in the coding region of *mtOM64* were obtained and a specific antibody was raised to verify the absence of mtOM64 protein in the knockout plants (Figure 3A). BN-PAGE analysis revealed that mtOM64 did not migrate with a complex, but rather it was consistently detected at the bottom of the gel, indicating that it did not form part of a larger complex under the conditions tested (data not shown). In vitro protein import reactions were conducted with a wide range of mitochondrial precursor proteins into mitochondria isolated from the two *mtom64* plant lines (Figure 3B). In comparison with wild-type mitochondria, no difference in the rate of protein import was observed for AOX, PiC, GR, and TOM40. However, the import of F_Ad was consistently 30 to 40% lower in plants lacking mtOM64 ($P < 0.05$), indicating that it is involved in the import of this plant-specific protein. This is in agreement with a previous study that suggested that F_Ad does not solely rely on TOM20 for import (Dessi et al., 1996; Murcha et al., 1999).

To investigate the interaction of mtOM64 with mitochondrial precursor proteins, import competition experiments were performed with mtOM64 competitor protein that was synthesized in an in vitro wheat germ lysate transcription/translation system. First, radiolabeled precursor proteins were preincubated with expressed competitor proteins in a wheat germ lysate mix (wheat germ lysate resuspended in wheat germ reconstitution buffer), either mtOM64 or an equivalent volume, and a quantity of β-glucuronidase (GUS). Following preincubation, the precursor/competitor protein mix was added to wild-type mitochondria under conditions that support import, and the amount of precursor protein imported after 10 min was quantitated (Figure 3C). Preincubation with the wheat germ reconstitution buffer alone decreased protein import of AOX and F_Ad by 69 and 53%, respectively (see Supplemental Figure 3 online). Preincubation with wheat germ lysate mix inhibited AOX import by 65%, indicating that the wheat germ reconstitution buffer, and not the wheat germ lysate, was responsible for the decrease in AOX import. By contrast, preincubation with the wheat germ lysate mix did not inhibit F_Ad import, indicating that a factor present in the wheat germ lysate stimulates F_Ad import, thus abrogating the inhibitory effect of the wheat germ reconstitution buffer. To control for the effects of wheat germ lysate, wheat germ reconstitution buffer, and protein overexpression, an identical volume of wheat germ lysate mix in which GUS was overexpressed was used as a control competitor protein; thus, the specific effect of mtOM64 on the import of a variety of proteins could be assessed. Preincubation of AOX or TIM23 with the mtOM64 competitor protein did not result in a significant change in the amount of precursor protein import relative to the GUS control. However, preincubation of F_Ad with mtOM64 resulted in a significant reduction ($P < 5.0 \times 10^{-4}$) of F_Ad import into mitochondria of 32% compared with GUS (Figure 3C), in close agreement with the decrease in the amount of import into *mtom64* mitochondria (Figure 3B). Therefore, exogenous mtOM64 protein was able to specifically compete with the F_Ad precursor protein but not other precursors tested. Preincubation of F_Ad with the chloroplast import receptor At TOC64-III did not affect F_Ad import, in contrast with the 32% reduction after preincubation with mtOM64 (Figure 3D), again indicating a specific effect of mtOM64. The ability of mtOM64 to compete with the F_Ad precursor could result either from a direct interaction with this precursor or interaction with a common component on the outer membrane that enables import into mitochondria.

METAXIN Is an Outer Mitochondrial Membrane Protein Essential for Normal Cellular Development, Starch Metabolism, and Plant Growth

Although the *tom20* triple and *mtom64* knockout plants appeared to interact with a range of precursor proteins, the lack of a

Figure 2. (continued).

(A) Five-week-old *Arabidopsis* plants with different genotypes indicated. Plants lacking Tom20-2 display a delayed-growth phenotype.

(B) [^{35}S]-labeled precursor proteins AOX, F_Ad, TOM40, GR, and PiC were incubated with mitochondria isolated from wild-type and mutant plants deficient in two of the TOM20 isoforms under conditions that support import. Aliquots were removed at 2, 5, 10, and 20 min and treated with PK. PK-protected mature radiolabeled protein was quantitated at each time point and normalized to the highest time point for replicate experiments ($n \geq 3 \pm$ SE). Where indicated, mutant mitochondria had a significantly lower amount of protein import than wild-type mitochondria, with a P value < 0.05 (*) and 0.01 (#) using Student's *t* test. m, mature protein; p, precursor protein.

(C) [^{35}S]-labeled precursor proteins AOX, F_Ad, TOM40, GR,x and PiC were incubated with mitochondria isolated from wild-type and *tom20-2 tom20-3 tom20-4* plants (triple KO). The import time course and analysis were performed as above. Where indicated, mutant mitochondria had a significantly lower amount of protein import than wild-type mitochondria, with a P value < 0.01 (#) and 0.005 (circles) using Student's *t* test.

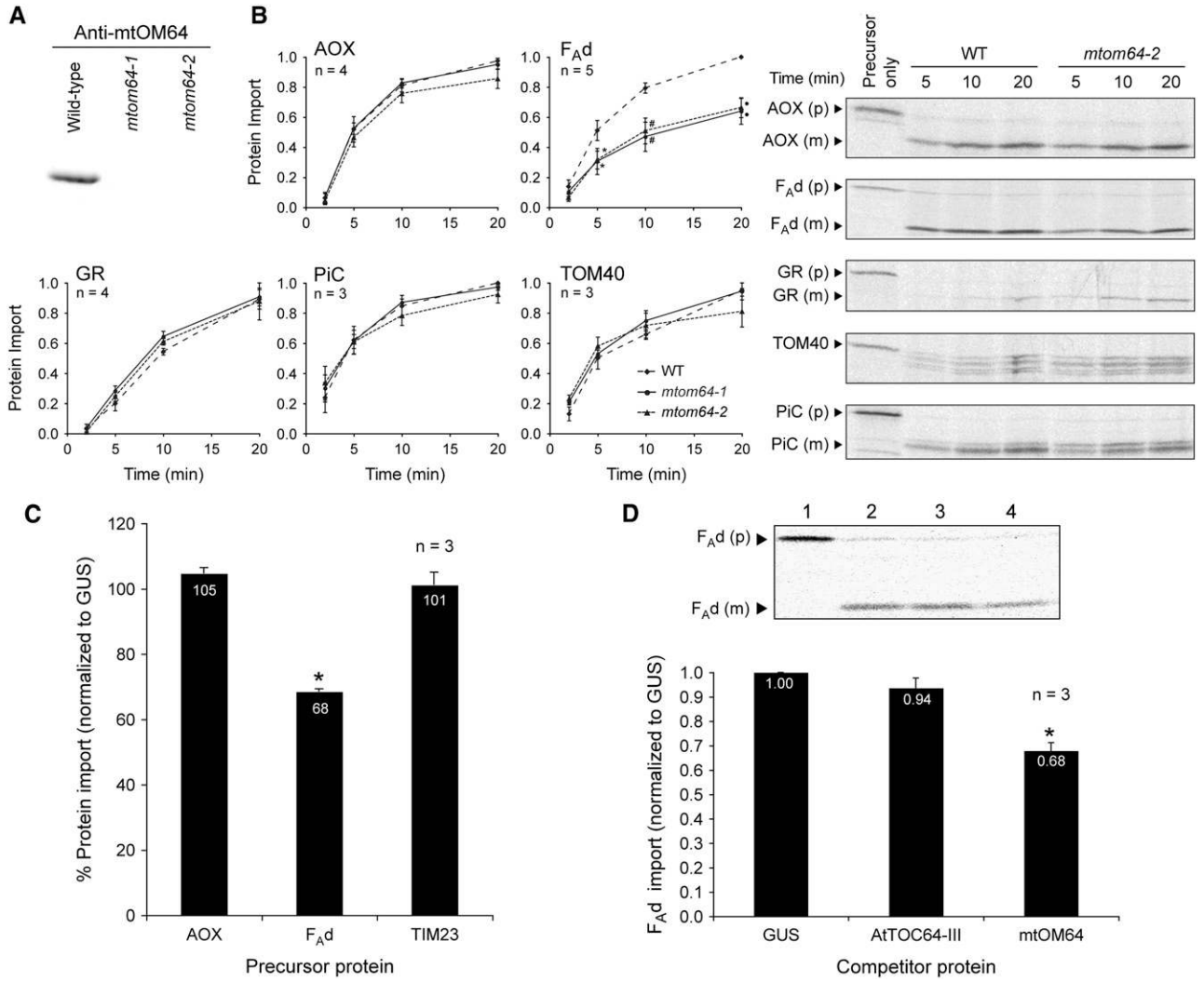

Figure 3. mtOM64 Is a Mitochondrial Preprotein Import Component That Is Involved in the Import of F_Ad into Mitochondria.

(A) Immunodetection of mtOM64 in mitochondrial protein samples isolated from wild-type and *mtom64* T-DNA insertional mutant plants and separated by SDS-PAGE.

(B) [^{35}S]-labeled precursor proteins AOX, F_Ad, Tom40, GR, and PiC were incubated with mitochondria isolated from wild-type and *mtom64* plants under conditions that support protein import. Aliquots were removed at 2, 5, 10, and 20 min and treated with PK. PK-protected mature radiolabeled protein was quantitated at each time point and normalized to the highest time point for replicate experiments ($n \geq 3 \pm$ SE). Where indicated, mutant mitochondria had a significantly lower amount of protein import than wild-type mitochondria, with a P value < 0.05 (*), 0.02 (#), and 0.01 (circles) using Student's *t* test.

(C) [^{35}S]-labeled precursor proteins AOX, F_Ad, and TIM23 were preincubated with overexpressed GUS or mtOM64 competitor protein prior to incubation with mitochondria from wild-type plants. PK-protected mature radiolabeled protein was quantitated and normalized against the amount of protein imported after preincubation with GUS ($n = 3 \pm$ SE). Where indicated, the mtOM64 competitor protein decreased protein import significantly compared with GUS, with a P value < 5.0×10^{-4} (*) using Student's *t* test.

(D) [^{35}S]-labeled F_Ad precursor protein was preincubated with overexpressed GUS, At TOC64-III, or mtOM64 competitor proteins prior to incubation with mitochondria from wild-type plants. PK-protected mature radiolabeled F_Ad was quantitated and normalized against the amount of protein imported after preincubation with GUS. Lane 1, AOX precursor only; lane 2, AOX precursor incubated with GUS; lane 3, AOX precursor incubated with At TOC64-III; lane 4, AOX precursor incubated with mtOM64. Where indicated, the competitor protein decreased protein import significantly compared with GUS, with a P value < 0.01 (*).

striking phenotype, as observed previously when plastid import receptors were inactivated (Soll and Schleiff, 2004; Bedard and Jarvis, 2005; Kessler and Schnell, 2006), suggested that additional components may also be present. Experiments were conducted to identify additional mitochondrial outer membrane proteins involved in protein import. As human METAXIN1 has previously been implicated in mitochondrial protein import (Armstrong et al., 1997), the role of *Arabidopsis* METAXIN was investigated. *Arabidopsis* lines with independent T-DNA insertions in the *METAXIN* coding sequence were obtained and the

absence of *METAXIN* transcript confirmed (see Supplemental Figure 4 online). *metaxin* plants displayed severe phenotypic abnormalities following leaf emergence, including diminished growth, abnormal leaf morphology and ectopic floral development and sterility (Figures 4A and 4B). Iodine staining of plants at the end of both the light and dark photoperiods revealed that *metaxin* plants accumulated higher levels of starch (Figure 4C). Light microscopy of leaf cross sections indicated that the mesophyll cells of the *metaxin* plants contained more chloroplasts compared with the wild type (Figures 4D and 4E). Transmission electron microscopy of leaf mesophyll cells revealed large starch deposits within the chloroplasts of *metaxin* cells, which was not observed in wild-type cells (Figures 4F and 4G). Genetic transformation of the *METAXIN* cDNA sequence under the 35S promoter of *Cauliflower mosaic virus* into *metaxin* plants rescued the mutant phenotype (data not shown). Sequence analysis indicated that METAXIN has two highly hydrophobic segments near the C terminus, possibly acting as transmembrane regions that anchor the protein in a membrane (Figure 4H). *Arabidopsis* suspension cell culture was biolistically transformed with a chimeric construct that fused green fluorescent protein (GFP) to the N terminus of METAXIN. The full-length METAXIN protein was able to direct N-terminal GFP to mitochondria, as indicated by the colocalization of GFP with the mitochondrial-targeted AOX-RFP (red fluorescent protein) control construct (Figure 4H). Interestingly, the GFP signal formed hollow circular structures, suggesting that the GFP was targeted only to the outer mitochondrial membrane. These structures closely resemble those observed by Setoguchi et al. (2006) upon immunofluorescence microscopy–based detection of the mammalian mitochondrial outer membrane proteins TOM22 and Bak.

To independently confirm the outer membrane localization of METAXIN, intact wild-type mitochondria were isolated and incubated in increasing concentrations of proteinase K (PK). Immunodetection of TOM20-2 and TOM20-4 using specific polyclonal antibodies raised to their cytosolic domains revealed that 1.7 μg/mL PK resulted in the complete digestion of the cytosolic portion of these proteins (Figure 4I). Immunodetection of METAXIN by a specific polyclonal antibody raised to the predicted METAXIN cytosolic domain indicated that the cytosolic portion of METAXIN was completely degraded in the presence of 53.3 μg/mL of PK (Figure 4I). At up to 106.7 μg/mL PK, there was no noticeable degradation of VDAC (voltage-dependent anion channel) or AOX, which are located in the outer membrane and on the matrix side of the inner mitochondrial membrane, respectively, indicating that the PK did not compromise the integrity of either of the mitochondrial membranes at the concentrations used. Therefore, METAXIN is accessible to externally added PK, indicating that the predicted cytosolic domain to which the antibody was raised is exposed to the cytosol. Membrane protein complexes from both wild-type and *metaxin* mitochondria were solubilized by digitonin and separated by one-dimensional BN-PAGE, followed by immunodetection of METAXIN, TOM20, and the COXII subunit (cytochrome c oxidase) of mitochondrial respiratory Complex IV (Figure 4J). COXII colocalized with Complex IV, as determined by Coomassie blue staining of the respiratory complexes (data not shown), whereas METAXIN was detected in a high molecular weight complex that is distinct from the smaller TOM complex (Figure 1B). Furthermore, this high molecular weight complex could not be detected in *metaxin* mitochondria, indicating that it is an authentic mitochondrial protein complex containing METAXIN. Therefore, while METAXIN is exposed to the cytosol on the outer mitochondrial membrane, it is located in a distinct complex that has not previously been characterized in plant mitochondria.

Mitochondria from Metaxin Plants Have Reduced Rates of Protein Import

A range of radiolabeled mitochondrial proteins were incubated with mitochondria isolated from wild-type and *metaxin* plants (Figure 5). As the yeast SAM37 forms part of the SAM complex, the import of two β-barrel proteins, TOM40 and VDAC, was also studied. The rate of protein import into *metaxin* mitochondria was dramatically reduced for all precursors tested, especially TOM40 and VDAC. This indicates that METAXIN is involved in the import of these proteins into mitochondria, either because it is directly required for import by all of these mitochondrial proteins or because it is required for correct TOM40 import and assembly and thus needed for generation of a functional general import pore, as is observed for SAM37 in yeast (Paschen et al., 2003; Wiedemann et al., 2003). Although the decrease in protein import of all the precursor proteins may be due to insufficient import or assembly of TOM40, two-dimenstional PAGE analysis indicated that TOM40 was present in the same amount in *metaxin* plants as in the wild type (see Supplemental Figure 6 online). This indicates that the inactivation of METAXIN does not completely block the import of TOM40.

As it cannot be ruled out that the decrease in the rate of protein import into *metaxin* mitochondria was a downstream consequence of the disruption of TOM complex structure/organization or a process in mitochondria not directly related to protein import, an alternative approach was required to determine if it played any direct role in the import of a variety of precursor proteins destined to various intramitochondrial locations. METAXIN was expressed in a wheat germ lysate and preincubated with radiolabeled mitochondrial precursor proteins before incubation with mitochondria to determine if exogenous METAXIN could compete with mitochondria for interaction with the precursor proteins. The addition of METAXIN resulted in a large decrease in the import of diverse precursor proteins into mitochondria, relative to the amount of protein import when precursors were incubated with GUS (Figure 6A). METAXIN preincubation inhibited the import of the general pathway proteins AOX and RPS10, the carrier pathway protein PiC, and the dual-targeted GR by 50 to 70%, while the β-barrel proteins TOM40 and VDAC were imported 30 to 40% less. Notably, very little reduction (~10%) was observed for F_Ad, and TIM23 import was not significantly reduced ($P > 0.1$), indicating that the addition of METAXIN to the in vitro protein import reaction was not causing general disruption of the protein import machinery or general import pore but was likely specifically interacting with certain precursor proteins.

Comparative sequence alignment of plant METAXIN, animal METAXIN1, and fungal SAM37 protein sequences from nine diverse species revealed that the sequence of SAM37 from the

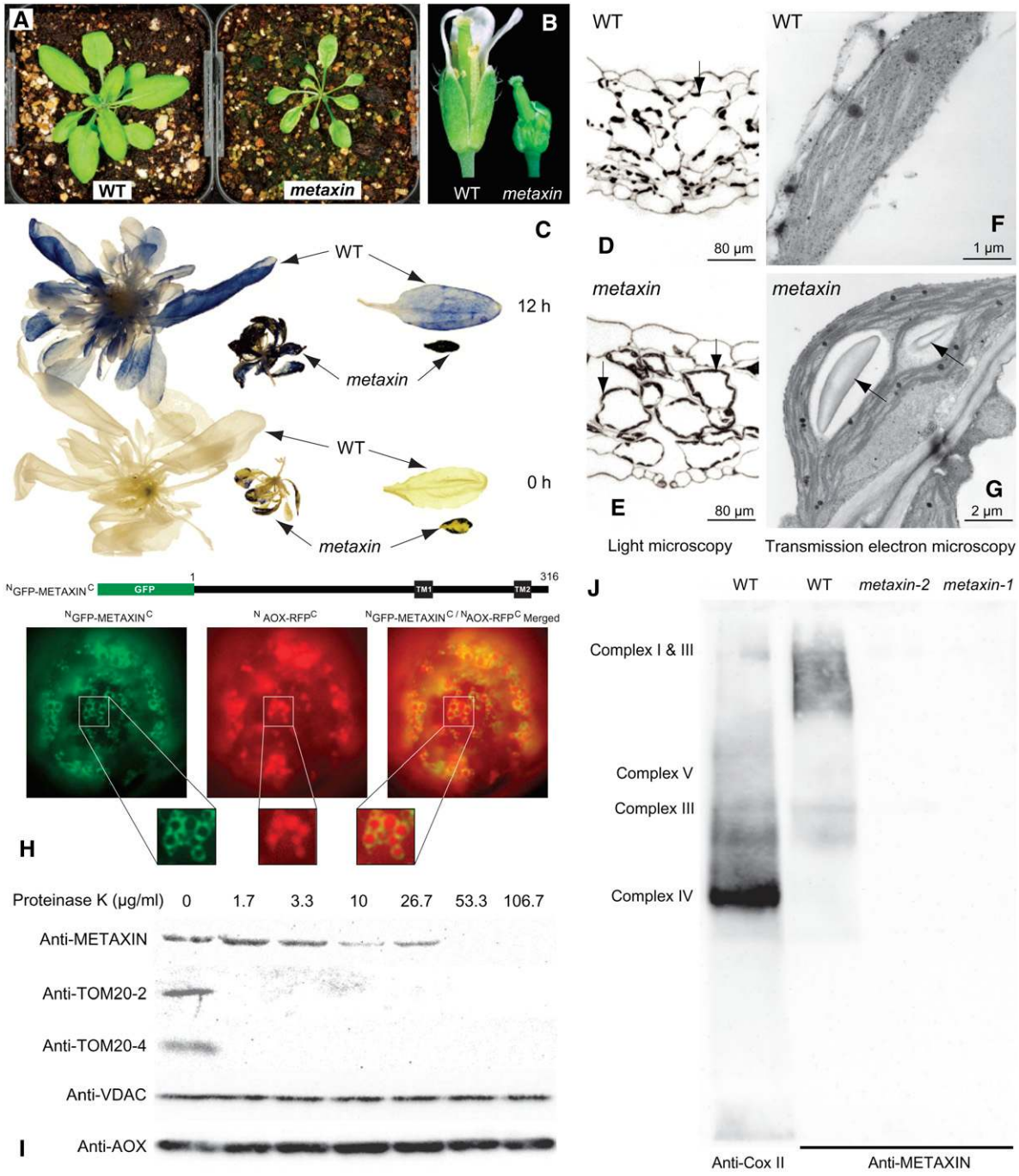

Figure 4. METAXIN Is an Outer Mitochondrial Membrane Protein Required for Normal Plant Metabolism, Growth, and Development.

(A) METAXIN-deficient *Arabidopsis* (*metaxin*) displays retardation of growth.
(B) *metaxin* is sterile and displays floral abnormalities, including incomplete anther and style maturation.
(C) Iodine staining of starch at 0 and 12 h after initiation of the light photoperiod.
(D) Light microscopy of wild-type leaf cross section (arrow indicates chloroplast).
(E) Light microscopy of *metaxin* leaf cross section (arrows indicate chloroplasts).
(F) Transmission electron microscopy of wild-type leaf mesophyll cell.
(G) Transmission electron microscopy of *metaxin* leaf mesophyll cell (arrows indicate starch granules within chloroplast).
(H) Subcellular localization of METAXIN was tested by fluorescence microscopy visualization of *Arabidopsis* suspension cells biolistically cotransformed with plasmids encoding ^{N}AOX-RFP^{C} and ^{N}GFP-METAXIN^{C}. TM, transmembrane domain.

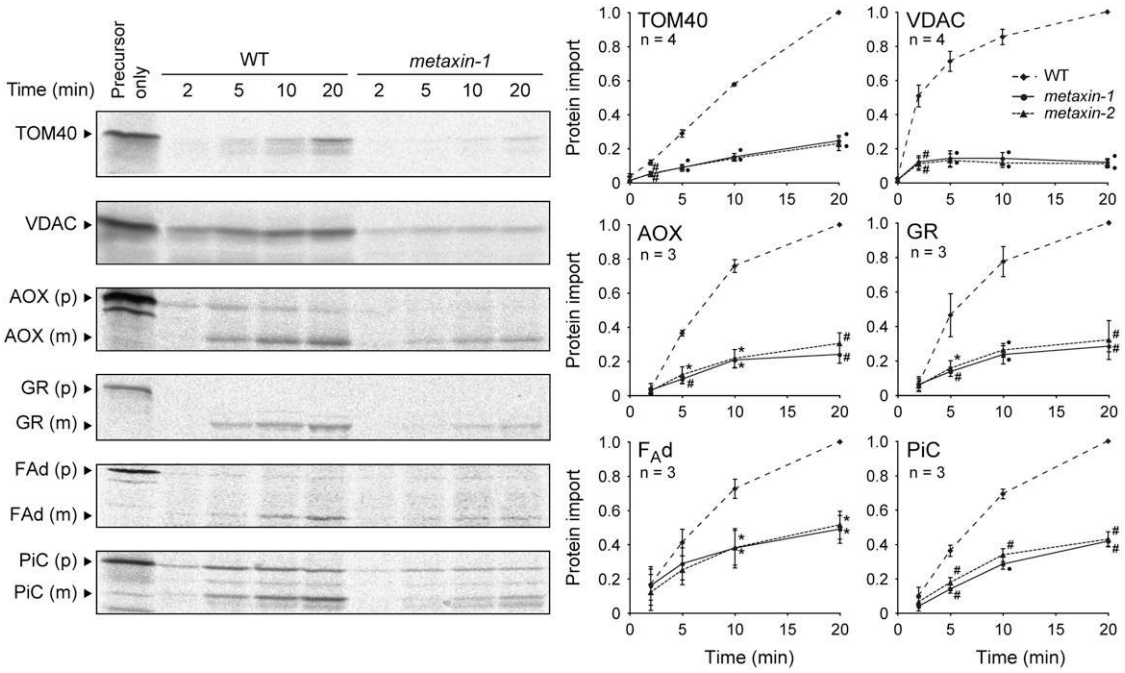

Figure 5. METAXIN-Deficient Mutant Plants Have Reduced Rates of Mitochondrial Protein Import.

[^{35}S]-labeled precursor proteins TOM40, VDAC, AOX, F_Ad, PiC, and GR were incubated with mitochondria isolated from wild-type and METAXIN-deficient mutants under conditions that support protein import. Aliquots were removed at 2, 5, 10, and 20 min and treated with PK. PK-protected mature radiolabeled protein was quantitated at each time point and normalized to the highest time point for replicate experiments ($n \geq 3 \pm$ SE). Where indicated, mutant mitochondria had a significantly lower amount of protein import than wild-type mitochondria, with a P value < 0.05 (*), 0.01 (#), and 0.001 (circles) using Student's *t* test.

fungi *Schizosaccharomyces pombe* and *Aspergillus clavulata* were more similar to the animal METAXIN1 proteins than to yeast SAM37 (Figure 6B; see Supplemental Figure 5 online). Two putative conserved domains were identified at similar positions in each protein, in plant METAXIN the N-terminal GST-N-METAXIN, and C-terminal GST-C-METAXIN domains, and in animals/fungi the N-terminal GST-N-METAXIN1-like and C-terminal GST-C-METAXIN1-3 domains (Figure 6B) (Marchler-Bauer and Bryant, 2004). Alignment of the METAXIN/SAM37 protein sequences revealed regions of significant sequence similarity in these domains. Furthermore, a region of 40 to 52 amino acids from the C-terminal end of the GST-N-METAXIN domain displayed significant similarity between all species, suggesting it may be important for protein function (see Supplemental Figure 5 online). Progressive truncations of METAXIN from the C terminus were made to disrupt the three putative conserved domains, and the proteins were translated in a wheat germ lysate (Figure 6C). These METAXIN deletions were used in competition import assays with wild-type mitochondria to ascertain which regions of the METAXIN protein were required for it to compete with mitochondrial-located METAXIN for the import of radiolabeled AOX (Figure 6C). The first 112 amino acids of METAXIN inhibited AOX import; however, further deletion to only the first 72 or 37 residues of METAXIN abolished the competitive inhibition of AOX import, suggesting that the conserved region (or competitor domain) identified between amino acids 72 and 112 may be important for interaction of the METAXIN protein with AOX (Figure 6C). Preincubation with METAXIN that lacked only the competitor domain resulted in a significant decrease in import compared with GUS, but the amount of import inhibition was less than when full-length METAXIN was used as a competitor protein, further supporting a role for this region in the import of preproteins across the outer membrane. Finally, increasing the concentration of the METAXIN1-112 competitor protein resulted in increased levels of AOX import inhibition (Figure 6D).

Figure 4. (continued).

(I) Mitochondria were purified from wild-type plants and incubated with increasing concentrations of PK. The mitochondrial proteins were separated by SDS-PAGE and probed with specific antibodies as indicated.

(J) Immunodetection of CoxII and METAXIN in mitochondrial protein samples isolated from wild-type and *metaxin* plants after digitonin solubilization and separation of protein complexes by first dimension BN-PAGE. The position and approximate molecular mass of the inner membrane respiratory complexes I, III, IV, and V are indicated.

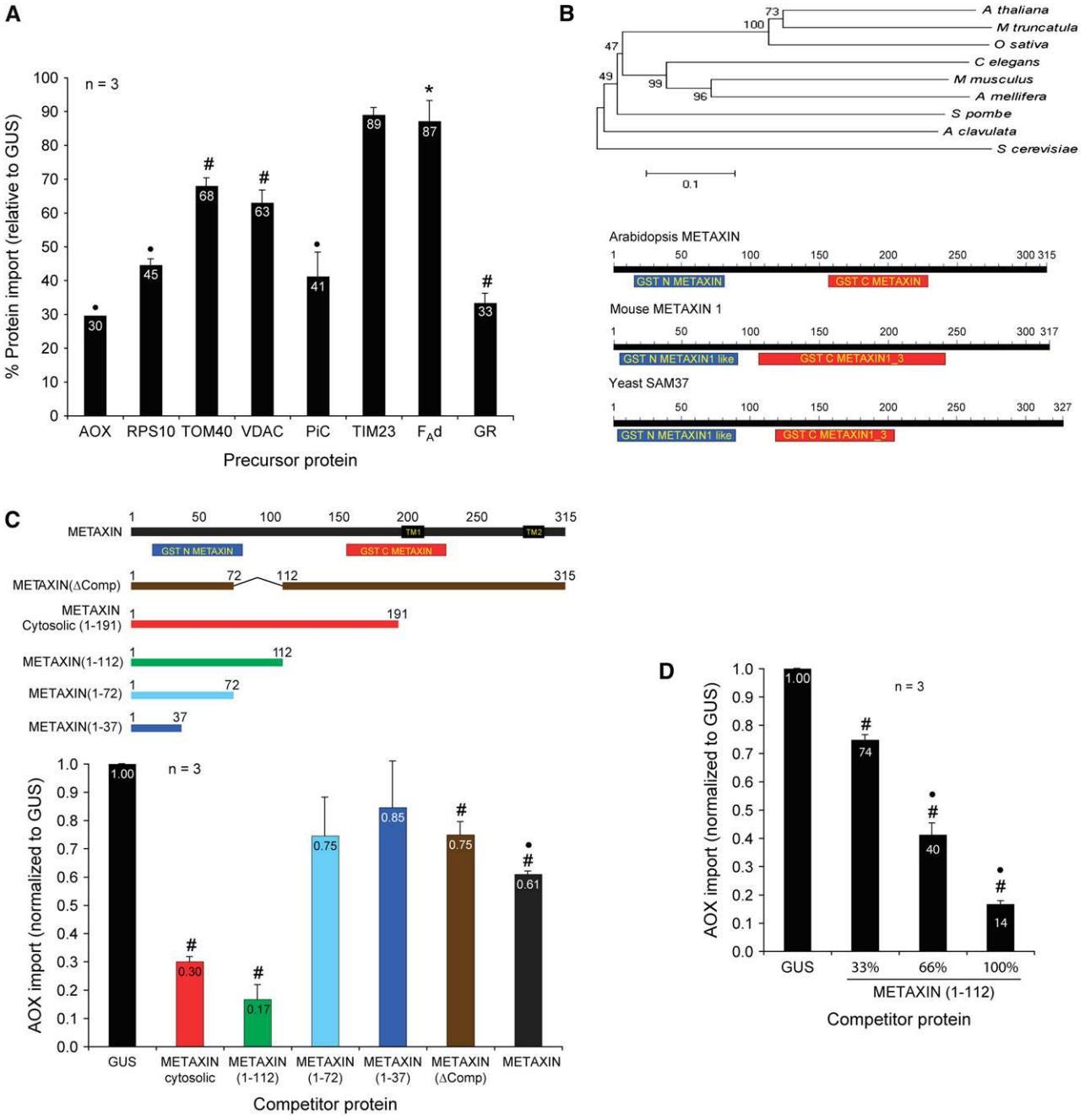

Figure 6. The Cytosolic Portion of METAXIN Can Interact with Mitochondrial Precursor Proteins.

(A) [^{35}S]-labeled precursor proteins AOX, RPS10, TOM40, VDAC, PiC, TIM23, F_Ad, and GR were preincubated with overexpressed GUS or metaxin competitor protein prior to incubation with mitochondria from wild-type plants under conditions that support protein import. PK-protected mature radiolabeled protein was quantitated and normalized against the amount of protein imported after preincubation with GUS ($n = 3 \pm$ SE). Where indicated, the METAXIN competitor protein decreased protein import significantly compared with GUS, with a P value < 0.05 (*), 0.02 (#), 0.001 (circles) using Student's *t* test.

(B) Phylogenetic tree of protein sequences of plant METAXIN, animal METAXIN1, and fungal SAM37/METAXIN from *Arabidopsis*, *M. truncatula*, *O. sativa*, *C. elegans*, *A. mellifera*, *M. musculus*, *A. clavulata*, *S. pombe*, and *S. cerevisiae*. METAXIN/SAM37 proteins from plants, animals, and fungi display overlapping GST-N-METAXIN/GST-N-METAXIN1-like and GST-C-METAXIN/GST-C-METAXIN1-3 conserved domains.

(C) [^{35}S]-labeled AOX precursor protein was preincubated with overexpressed GUS or truncated/deleted METAXIN proteins prior to incubation with mitochondria from wild-type plants under conditions that support protein import. METAXIN (ΔComp) refers to a recombinant protein comprised of the full-length METAXIN protein with the putative competitor domain between amino acids 72 and 112 removed. PK-protected mature radiolabeled

TOM20, mtOM64, and METAXIN Interact with a Variety of Precursor Proteins

To determine if these proteins could interact directly with precursor proteins, pull-down and yeast two-hybrid interaction assays were performed. For the pull-down assays, full-length TOM20-4, METAXIN, and mtOM64 proteins were synthesized in a wheat germ lysate and added to a variety of radiolabeled precursor proteins. The interactions of each of these proteins with various precursors was tested by the ability to pull down radiolabeled precursor protein with antibodies raised against TOM20-4, METAXIN, and mtOM64, respectively (Figure 7A). All three proteins interacted with various precursor proteins, AOX, PiC, F_Ad, TIM23, and TOM40. However, the precursor protein GR could not be pulled down in this assay with any of the antibodies tested (Figure 7A). Although it is not possible to compare how efficiently each antibody pulls down each precursor due to variable antibody affinities, it was apparent that although the precursor protein F_Ad could be pulled down by all three antibodies, it was weak in comparison to the other precursor proteins. For both AOX and F_Ad, removal of the mitochondrial targeting signal resulted in no interaction (Figure 7A). In contrast with the positive interaction detected with the various precursor proteins and TOM20, METAXIN, and mtOM64, using the same lysate programmed to synthesize GUS, or using the lysate alone (Figure 7A) resulted in no detectable interaction, indicating that the antibodies were not directly binding to any of the precursor proteins (Figure 7A). The binding of the Protein A Sepharose to the antibody was confirmed with protein gel blotting (Figure 7B). To further determine if these proteins interacted with mitochondrial precursor proteins, a yeast two-hybrid interaction screen was performed. The cDNAs encoding the three TOM20 proteins, METAXIN, mtOM64, and At TOC64-III, were each cloned to produce a recombinant fusion protein with the GAL4 binding domain (bait), while cDNAs encoding the precursor proteins were cloned to produce a fusion protein with the GAL4 activation domain (prey). Interactions were determined by the ability to grow without the addition of His to the media and the ability to grow in the absence of adenine to give a red/orange color. All mitochondrial precursor proteins supported growth in the absence of His when TOM20, METAXIN, or mtOM64 was used as bait, and colony numbers were typically 5- to 10-fold higher than that obtained when At TOC64-III was used as bait (data no shown). To visualize the difference, colonies were grown in the absence of adenine (Figure 7C). Whereas a red/orange color is clearly evident for all mitochondrial precursor proteins with the mitochondrial proteins as bait, At TOC64-III colonies are clearly white. Notably, nonrecombinant bait and/or prey constructs did not produce any interaction (see Supplemental Figure 7 online). Removing the mitochondrial targeting signal from AOX or F_Ad resulted in no interaction. One exception was when GR was used as the prey fusion protein; a weak orange color was evident with At TOC64-III as bait. *Arabidopsis* GR is a dual-targeted protein (Chew et al., 2003a), although the plastid receptor for this protein has not yet been determined. It contains the consensus phosphorylation motif in the targeting signal that has been reported to increase targeting ability to chloroplasts (Chew et al., 2003a, 2003b; Martin et al., 2006). Thus, it is possible that it can interact with At TOC64-III, although the color was weak compared with the color development with mitochondrial precursor proteins and mitochondrial outer membrane components. In summary, the two assays show the ability of the three mitochondrial proteins, TOM20, METAXIN, and mtOM64, to interact with a variety of mitochondrial precursor proteins.

A Flexible Regulatory Response to Mitochondrial Protein Import Dysfunction

Adaptive responses of the plant cell to ablation of the different protein import components were monitored using antibodies raised to each TOM20 isoform, mtOM64, METAXIN, and several other mitochondrial proteins (Figure 8A). Overall, these results indicated a complicated series of responses. The absence of any one of the TOM20 isoforms did not result in an increase in the abundance of either of the remaining two TOM20 proteins (Figure 8A). However, in plants lacking two TOM20 isoforms, the amount of the remaining TOM20 protein increased at least twofold, supporting the observation from in vitro protein import experiments (Figure 2B) that the TOM20 isoforms are largely functionally redundant. Furthermore, the amount of METAXIN was increased in each TOM20 double mutant. The level of the inner mitochondrial membrane translocase component TIM17 increased in *tom20-2 tom20-4*, *tom20-3 tom20-4*, and the *tom20* triple knockout, indicating that the retrograde regulatory mechanism that increases TOM20 abundance may also control other mitochondrial protein import components. Interestingly, mtOM64 abundance increased in all *tom20* plants except *tom20-2* (Figure 8A). Together, the alteration in abundance of each component is consistent with the proposal of overlapping roles for these proteins. Notably, no signal was evident in the *tom20* triple knockout when probed with the TOM20-3 antibody. As this antibody clearly interacts with TOM20-1 (see Supplemental Figure 1B online), and as the abundance of TOM20-2, TOM20-3, and TOM20-4 increased in the respective TOM20 double knockout lines, it appears that TOM20-1 abundance is not being increased

Figure 6. (continued).

AOX was quantitated and normalized against the amount of protein imported after preincubation with GUS. Where indicated, the METAXIN competitor protein decreased protein import significantly compared with GUS, with a P value < 0.01 (#) using Student's *t* test. The circle indicates that the full-length METAXIN competitor protein inhibited AOX import significantly more than METAXIN (ΔComp), with a P value < 0.02 using Student's *t* test.

(D) [^{35}S]-labeled AOX precursor protein was preincubated with overexpressed GUS or METAXIN1-112 (the N-terminal 112 amino acids of METAXIN) prior to incubation with mitochondria from wild-type plants under conditions that support protein import. PK-protected mature radiolabeled AOX was quantitated and normalized against the amount of protein imported after preincubation with GUS. Where indicated, the METAXIN1-112 competitor protein decreased protein import significantly compared with GUS, with a P value < 0.01 (#) using Student's *t* test. Circles indicate that addition of METAXIN1-112 competitor protein inhibited AOX import significantly more than the next lower concentration of competitor, with a P value < 0.01 using Student's *t* test.

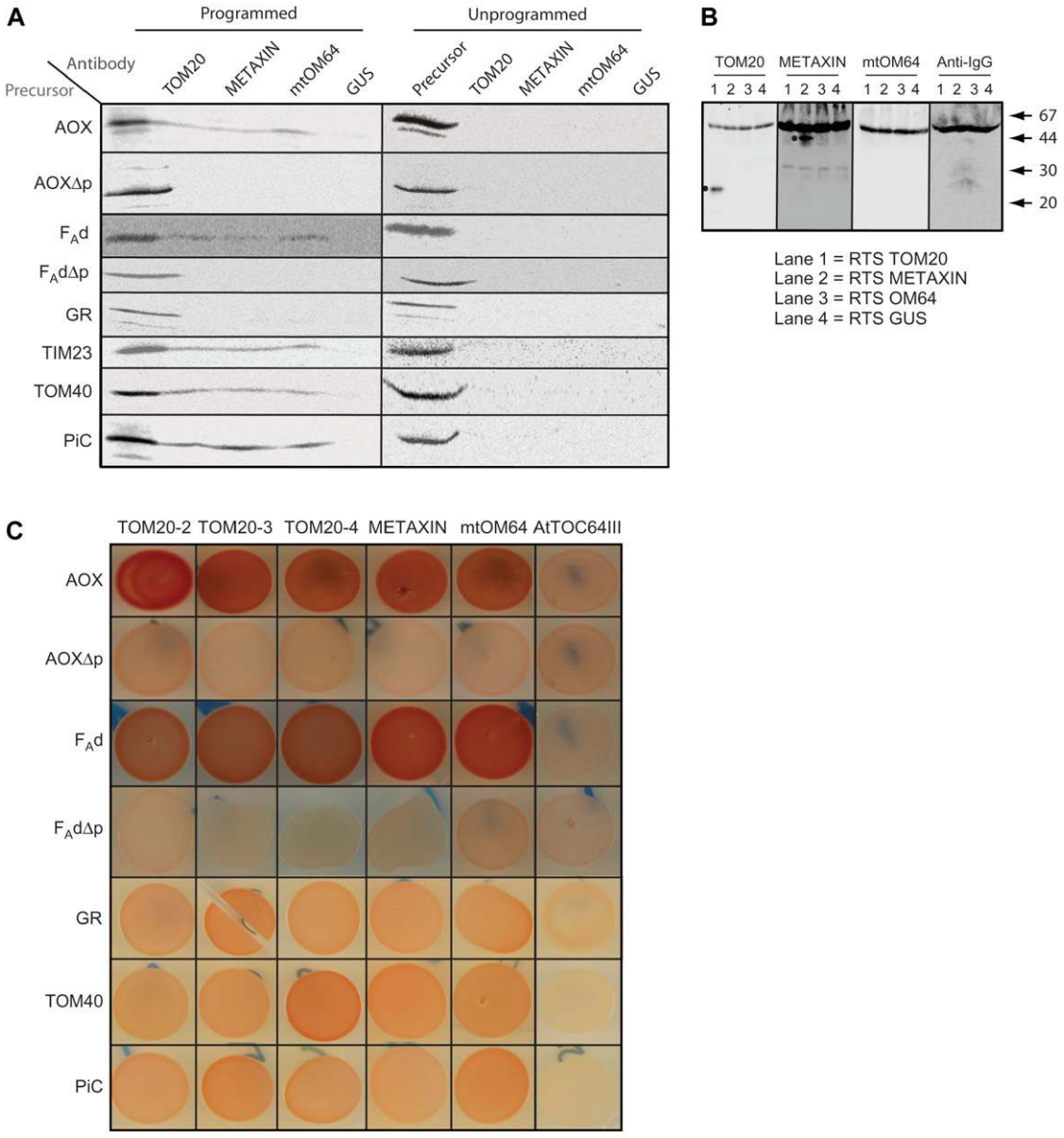

Figure 7. Interaction of Precursor Proteins with TOM20, mtOM64, or METAXIN.

(A) The ability of TOM20, mtOM64, or METAXIN to pull down precursor protein in solution was tested for various precursor proteins. The lanes indicate the antibody used to pull down the precursor proteins from a solution containing the corresponding protein synthesized in the RTS lysate. The left-hand panel represents the reaction where the RTS lysate was programmed to synthesize the target protein. The right-hand panel represents the negative control, where RTS lysate was not programmed to synthesize any protein, and thus the respective pull-down reactions contained no TOM20, METAXIN, mtOM64, or GUS, respectively. AOXΔp and F_AdΔp represent altered precursor proteins where the mitochondrial targeting presequence is not present.
(B) Protein gel blot analysis of the pull-down reactions to verify that the target protein was being specifically pulled down. Pull-down reactions were performed and 10% of this reaction was analyzed by protein gel blotting. For each protein synthesized in the RTS reaction, the pull-down reaction was performed and separated by SDS-PAGE, blotted, and probed with antibodies to TOM20, METAXIN, mtOM64, and anti-rabbit IgG. Lanes 1 to 4 in each panel represent lysate programmed to synthesize TOM20, METAXIN, mtOM64, and GUS, respectively. A positive product was detected with the TOM20 and METAXIN antibody (indicated by cirlces). As the mtOM64 protein has a similar molecular mass to that of the heavy IgG chains, a specific product for mtOM64 could not be resolved; the rabbit IgG molecules are present as these were used to pull down the target proteins.
(C) Yeast two-hybrid interaction assays. The mitochondrial outer membrane proteins TOM20-2, TOM20-3, TOM20-4, mtOM64, and METAXIN and the chloroplast outer envelope protein At TOC64-III were cloned into the bait vector and tested for interaction with the five precursor proteins cloned into the prey vector. A red/orange color indicates an interaction. AOXΔp and F_AdΔp represent these precursor proteins where the mitochondrial targeting presequence had been removed.

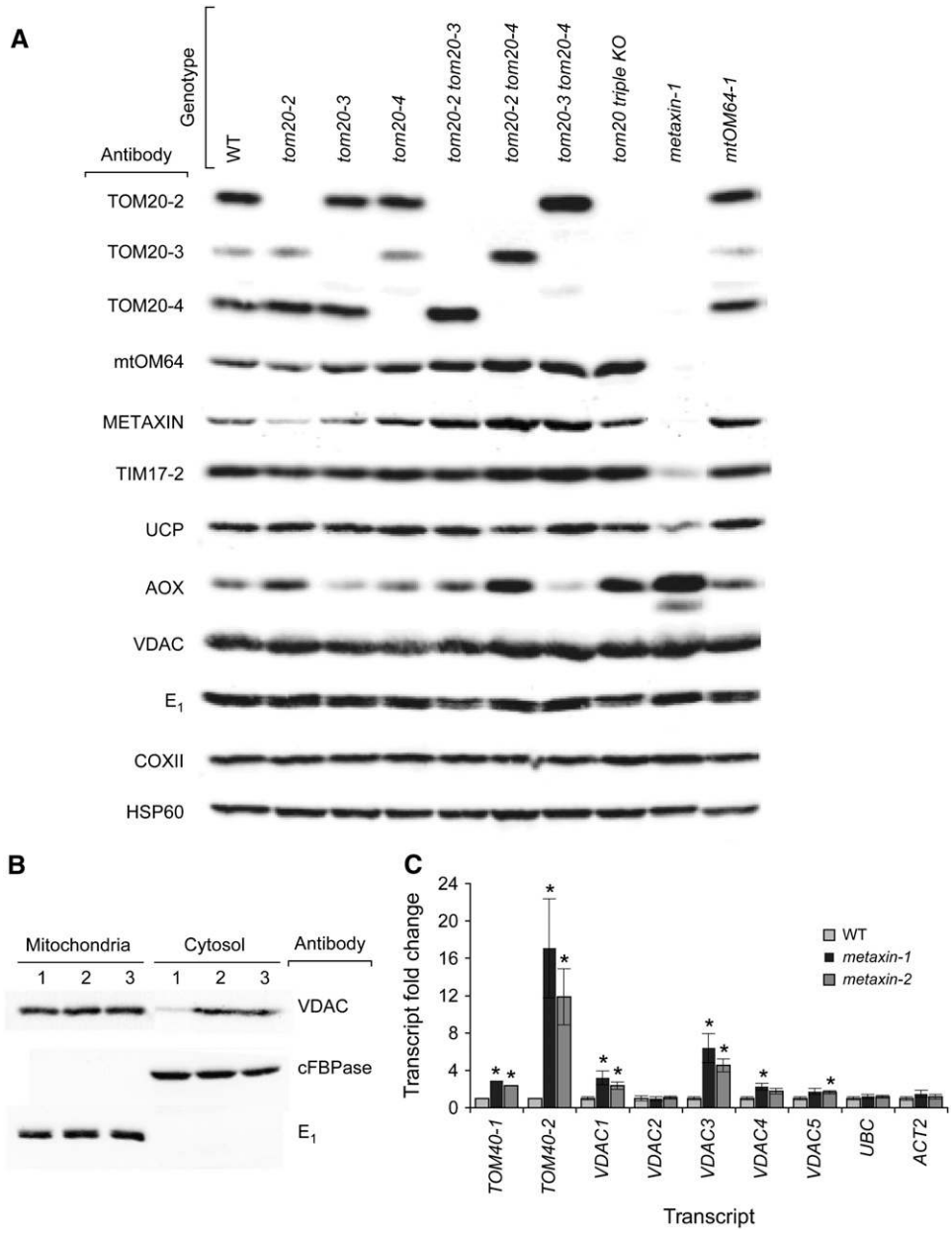

Figure 8. Protein Levels in Mutant Plants Indicate Retrograde-Regulated Compensation by TOM20 Isoforms, Disruption of the TOM Complex in *metaxin*, VDAC Accumulation in the Cytosol of *metaxin* Cells, and Upregulation of mtOM64 and METAXIN When Multiple TOM20 Isoforms Are Depleted.

(A) Mitochondria were purified from wild-type and mutant plants, and the mitochondrial proteins separated by SDS-PAGE and probed with specific antibodies to import components and other mitochondrial proteins.

(B) Detection of marker proteins in mitochondrial and cytosolic protein fractions from wild-type and METAXIN-deficient plants. Lane 1, the wild type; lane 2, *metaxin-1*; lane 3, *metaxin-2*.

(C) Abundance of TOM40, *VDAC*, *UBC* (ubiquitin conjugating enzyme), and *ACT2* (Actin2) transcripts in *metaxin-1* and *metaxin-2* rosette leaves relative to wild-type levels. Where indicated, the transcript abundance in *metaxin* was significantly higher than in the wild type, with a P value < 0.05 (*) using Student's *t* test. cFBPase, cytosolic fructose-1,6-bisphosphatase; $E_1\alpha$, E1 α-subunit of pyruvate dehydrogenase.

perceptibly to compensate for the loss of the three highly expressed isoforms. As *TOM20-1* transcript abundance is extremely low (see Supplemental Figure 1C online) and detection of the TOM20-1 protein has not been reported, it appears that this isoform does not play an important role in mitochondrial protein import.

AOX protein abundance, a marker for retrograde signaling in plant mitochondria (Rhoads et al., 2006), increased in all mutants lacking TOM20-2, most noticeably in *tom20-2*/*tom20-4* and the *tom20* triple knockout. The in vitro protein import experiments revealed that TOM20-2 plays a specialized role in AOX

recognition and import (Figure 2B). AOX is a marker of stress-induced mitochondrial retrograde pathways in plants, and its upregulation in *tom20-2* indicates that this isoform alone appears to trigger this response (Rhoads et al., 2006). Similarly, the $E_1\alpha$ subunit of pyruvate dehydrogenase ($E_1\alpha$) was less abundant in mitochondria isolated from *tom20-2/tom20-3* and the *tom20* triple knockout, indicating that TOM20-2 and TOM20-3 may both have a higher affinity for this mitochondrial protein. The levels of the inner membrane uncoupling protein (UCP), VDAC, cytochrome c oxidase subunit II (COXII), and heat shock protein 60 (HSP60) were unchanged in the *tom20* mutant plants, including the *tom20* triple knockout, demonstrating that despite lacking all TOM20 receptors, the mitochondria are able to import a wide range of proteins to a final abundance very similar to wild-type levels. This again demonstrates that TOM20 is not an essential protein import component and that the plant cell lacking TOM20 is able to generate a mitochondrial proteome quite similar to wild-type mitochondria. This may be achieved by the operation of the other protein import components mtOM64 and METAXIN. Furthermore, it suggests that the upregulation of TOM20, mtOM64, and TIM17 in the *tom20* double and triple knockout plants is not the consequence of an extensive alteration of a wide range of mitochondrial proteins, but rather the outcome of a precise retrograde regulatory pathway that specifically targets the mitochondrial protein import apparatus.

The *mtom64* mitochondria did not display significant changes in the abundance of the proteins tested, except that the abundance of METAXIN was consistently higher (Figure 8A). The *metaxin* mitochondria showed dramatic decreases in the abundance of several mitochondrial proteins, including an almost total absence of all TOM20 isoforms and mtOM64 and a large reduction in TIM17 and UCP abundance (Figure 8A). Interestingly, AOX protein levels increased significantly in the *metaxin* mitochondria, as did the alternative respiratory pathway activity catalyzed by AOX (Ho et al., 2007), suggesting that this protein is required at a greater abundance in the mitochondria in response to aberrant mitochondrial behavior. This is in agreement with the suggestion that elevated AOX abundance in mitochondria lacking TOM20-2 is an adaptation to increased stress. By contrast, the level of VDAC, $E_1\alpha$, COXII, and HSP60 were unaltered compared with wild-type mitochondria, suggesting that despite a partially dysfunctional protein import apparatus in *metaxin*, the plant cell can achieve normal levels of many mitochondrial proteins. To determine if the abundance of VDAC throughout the plant cell was unaltered by ablation of METAXIN, cytosolic proteins were isolated from wild-type and *metaxin* leaves and the amount of VDAC measured by immunodetection (Figure 8B). *metaxin* plants had a much higher abundance of VDAC in the cytosol, in contrast with the very low levels of VDAC in the wild-type cytosolic fraction. Equivalent abundance of the cytosolic fructose-1,6-bisphosphatase between wild-type and *metaxin* cytosolic fractions demonstrated that not all cytosolic proteins in the mutant had increased abundance. Cytosolic accumulation of mitochondrial $E_1\alpha$ was not observed, indicating that the cytosolic accumulation does not occur for all mitochondrial proteins and that METAXIN may be particularly important for the recognition and import of VDAC. Together with the increased *VDAC* and *TOM40* transcript abundance in *metaxin* plants (Figure 8C), this suggests that the *metaxin* cells upregulate the expression of this critical protein to attain normal levels in mitochondria, potentially by saturating the residual import capability of the *metaxin* mitochondria. To determine if TOM40 was present in the *metaxin* and *tom20* triple knockout lines, two-dimensional isoelectric focusing (IEF)/SDS-PAGE analysis was performed with purified mitochondrial proteins, and the amount of TOM40 was determined. Overall, there appeared to be no significant changes in TOM40 abundance in any of the mutant plant lines (see Supplemental Figure 6 online). Likewise, for the F_Ad subunit, it was evident that it was present at normal levels in the mt*om64* plants (see Supplemental Figure 6 online). Finally, it was evident that AOX was more abundant in *tom20-2*, *tom20-2 tom20-4*, *tom20-2 tom20-3 tom20-4*, and *metaxin* lines (Figure 8A), even though import was reduced from ~30 to 80%, respectively. Thus, the steady state levels of proteins present in mitochondria isolated from 4-week-old mutant plants was not affected by the absence of the respective import components, even though a reduced rate of protein import for these proteins was measured in vitro.

DISCUSSION

Investigation of the function of TOM20, mtOM64, and METAXIN using a variety of approaches indicated that they all play a role in the import of proteins into mitochondria. Insertional inactivation is a powerful tool to determine function but cannot distinguish between primary (direct) and secondary (indirect) effects. Here, we observed that (1) altering any one of the three components resulted in changes in abundance of at least one of the other two that could possibly compensate for the function (Figure 8); and (2) in the *metaxin* mutant, a reduced amount of other components was observed (Figure 8). To overcome these limitations, a variety of alternative approaches was undertaken. The potential for all three components to directly interact with precursor proteins was demonstrated (Figure 7), which combined with the import and competition assays, strongly suggests a model of overlapping ability to interact with precursor proteins on the outer surface of mitochondria. Thus, although the insertional inactivation of mtOM64 only affected the import of the F_Ad precursor (Figure 3), the interaction assays indicate that it can bind and affect the import of a variety of precursor proteins (Figure 7). However, as METAXIN abundance increased in the *mtom64* mutants (Figure 8) and TOM20 is present, an in vitro assay where excess precursor protein is added the absence of a nonlimiting component may result in normal rates of import being observed even if that component can interact with precursor proteins when present. It was notable that in the *mtom64* mutants, only the import of the F_Ad precursor was affected (Figure 3), and the competition assays indicated that METAXIN competitor protein could only compete weakly for the import of this precursor protein (Figure 6). Thus, the upregulated abundance of METAXIN and the fact that the import of the F_Ad precursor is still high in the presence of the triple *tom20* knockout (Figure 2C) are consistent with a role for mtOM64 in the import of this precursor protein. However, TOM20 and METAXIN can also play a role, as they can interact directly with this precursor protein (Figure 7). mtOM64 may play a role in the import of other precursor proteins, but due to the apparent functional redundancy of the import machinery,

this is not detected in knockout mutants, and other approaches are required to elucidate its interaction with these precursor proteins, such as direct interaction assays. In the case of GR, only the yeast two-hybrid assay indicated an interaction. The in vitro import assays in the triple *tom20* and *mtom64* mutants revealed no effect on import. Addition of full-length METAXIN could compete for import, but this may be due to competition for a common import component. Alternatively, as GR is located in the intermembrane space and the matrix (Chew et al., 2003a, 2003b), it may interact with METAXIN via the import route to the intermembrane space or directly with GR via the intermembrane space exposed domains of METAXIN. A recent study of METAXIN from human cell lines using coimmunoprecipitation revealed that it interacted with an inner membrane protein present in a larger complex with several other proteins (Xie et al., 2007), while another study in humans using BN-PAGE concluded that it was not in a complex with SAM50 (Kozjak-Pavlovic et al., 2007). Thus, the role of METAXIN may differ considerably between species and/or it may play a variety of roles in various organisms.

The Tom20 Multiple Gene Family

Duplication of the *TOM20* gene family has apparently resulted in limited functional specialization, as evidenced by the formation of a second and larger complex containing TOM20-2 and differences in the rate of import of some precursor proteins. As the rice genome encodes only a single TOM20 isoform, this gene family duplication and subfunctionalization has most likely occurred subsequent to the divergence of monocotyledonous and dicotyledonous plants. Studies on duplicated genes suggest that subfunctionalization is an important transition state to neofunctionalization and acts to increase the preservation of duplicated genes (Rastogi and Liberles, 2005). TOM20 was identified in plants from direct protein analysis of the isolated TOM complex that contained TOM40 (Jansch et al., 1998). Subsequent analysis of TOM20 indicated that it is not orthologous to yeast or mammalian TOM20 (Perry et al., 2006), thus necessitating reevaluation of its role in mitochondrial protein import. Based on the fact that the majority of the protein is exposed on the cytosolic side of the outer membrane (Figure 4), that the nuclear magnetic resonance structure of TOM20-3 indicates a very similar presequence binding fold to mammalian TOM20 (Likic et al., 2005; Perry et al., 2006), that inactivation of all three TOM20 isoforms leads to a substantial decrease in the rate of protein import (Figure 2), that TOM20 directly interacts with mitochondrial precursor proteins (Figure 7), and that overexpressed *Arabidopsis* TOM20-3 can compete for protein import into yeast mitochondria (Perry et al., 2006), we conclude that TOM20 likely functions as a mitochondrial protein import receptor.

Although we could not detect TOM20-1 protein and transcript was not detected in a variety of materials we have previously examined (see Supplemental Figure 1 online) (Lister et al., 2004; Murcha et al., 2007), it is possible that its expression at a protein level is below the limits of detection or limited to specific cell types. An examination of 2509 arrays in Genevestigator indicates that it is called present in 347 (14%) (Zimmermann et al., 2004), although this does not incorporate false discovery rate correction that should be used (Nettleton, 2006). Analysis of these arrays indicated that TOM20-1 transcript abundance was higher in roots compared with other organs. Thus, we performed quantitative RT-PCR and could detect some expression (see Supplemental Figure 1C online); however, the corresponding protein could not be detected in mitochondria isolated from the same triple *tom20* mutant plants (water culture) used to carry out the in vitro import assays (Figure 2) or from a variety of other mitochondrial preparations (cell culture, water culture or plant or root material; data not shown). Thus, given the reduction we observe in import with the *tom20* triple knockout for several precursor proteins, combined with the fact that we cannot detect the protein, it is unlikely that TOM20-1 is highly expressed and could compensate to function as the predominant import receptor.

Is mtOM64 a Receptor?

The absence of a plant homolog of TOM70 has frequently been noted as one of the fundamental differences in the plant mitochondrial import apparatus (Lister et al., 2005; Chan et al., 2006). In this study, mtOM64 could not be identified in the TOM complex by immunodetection (data not shown), suggesting that any attachment is peripheral and that it is dynamically associated with the TOM complex, as observed for the interaction of At TOC64-III with the TOC complex (Schleiff et al., 2003). Insertional inactivation, in vitro competition experiments with overexpressed mtOM64, antibody inhibition assays, and direct interaction assays all suggest a role for mtOM64 in the import of proteins into mitochondria. For the competition experiments, mtOM64 (or METAXIN) was synthesized in a wheat germ transcription/translation system where waste products are continuously removed to achieve high levels of protein expression. It has been reported that a different wheat germ lysate system than the one used in this study can have an inhibitory effect on protein import into mitochondria and plastids (Schleiff et al., 2002; Dessi et al., 2003). However, the following should be noted: (1) the F_Ad precursor is as efficiently imported into mitochondria from a wheat germ lysate compared with a rabbit reticulocyte lysate (Dessi et al., 1996; Tanudji et al., 2001), and (2) a study analyzing the inhibitory effect of wheat germ on import indicated that it is due to the folding status of the mature part of the protein (Dessi et al., 2003). Other precursor proteins can also be imported into mitochondria from a wheat germ lysate (Biswas and Getz, 2004). Thus, it appears that some formulations of the wheat germ translation lysate contain factors that inhibit import, and, notably, it has been reported that other factors, such as mitochondrial import stimulating factor, can relieve this inhibition (Hachiya et al., 1993). The type of wheat germ translation system used here is optimized to produce enzymatically active protein. Overall, although addition of this lysate mix alone reduces the rate of protein import, it can be readily used, as it is a plant-based lysate, and the inhibition observed in these studies was dependent on the mRNA used to program the lysate and thus was not a general effect of the lysate itself on import as was the topic of investigation in previous studies. Furthermore, as evidenced by the immunodetection (Figure 8A) and two-dimensional IEF/SDS-PAGE (see Supplemental Figure 6 online) analyses, the steady state level of mitochondrial proteins is not directly related to the in vitro rate of import.

Several independent approaches used here all indicate a role for mtOM64 in the import of at least some mitochondrial proteins; however, its exact role cannot yet be concluded. Although it can clearly interact directly with a variety of precursor proteins, the binding chain hypothesis for mitochondrial import proposes that many components contain such binding properties. For instance, it has been recently shown that the pore-forming SAM50 (TOB55) from yeast contains such a domain (Rehling et al., 2001; Habib et al., 2007). The localization of mtOM64 on the cytosolic face of the outer membrane is evidenced by digestion with externally added protease (Chew et al., 2004) and supports an interaction with precursor proteins on the outer face of mitochondria. At TOC64-III, TOM70, and mtOM64 have three C-terminal TPR motifs that are predicted to form a superhelical structure (Scheufler et al., 2000). Recently, the C-terminal TPR motifs of *Arabidopsis* At TOC64-III were demonstrated to mediate its recognition of chloroplast precursor proteins via interaction with the chaperone HSP90 (Qbadou et al., 2006). In this study, the competitive inhibition of F_Ad import by mtOM64 was not abolished by addition of geldanamycin, a chemical that binds to HSP90 and inhibits its chaperone activity (data not shown) (Young et al., 2003). Thus, it can be concluded that mtOM64 does specifically interact with mitochondrial precursor proteins and therefore may function as a receptor in the early stages of precursor recognition and import. mtOM64 is a rate-limiting component for the import of the plant-specific protein F_Ad (Figure 3), although this may be an indirect affect due to the inactivation of mtOM64 altering the level of as yet unknown component involved or rate-limiting for the import of this precursor protein.

A Multifunctional METAXIN?

As the yeast SAM complex contains two of the three outer membrane proteins known to be essential for cell viability, SAM50 and SAM35 (Milenkovic et al., 2004), it was surprising to find that only SAM50 is highly conserved in diverse eukaryotic organisms. Animals possess METAXIN1 and METAXIN2, which display just 36 and 28% sequence similarity to yeast SAM37 and SAM35, respectively (*Mus musculus* sequences). METAXIN2 was demonstrated to interact with METAXIN1 and to be peripherally associated with the mitochondrial outer membrane (Armstrong et al., 1999), akin to yeast SAM35 (Waizenegger et al., 2004). Taken together, the sequence similarity and protein–protein interactions suggest that the animal METAXIN proteins are orthologs of SAM35 and SAM37. By contrast, *Arabidopsis* possesses only one METAXIN protein, which displays 21% sequence similarity to mouse METAXIN1 and only 11% similarity to yeast SAM35. Thus, it appears that the plant METAXIN is a highly diverged form of yeast SAM37. The location of Metaxin on the outer mitochondrial membrane and sensitivity to externally added protease (Figure 4), the ability of various regions of the METAXIN protein to inhibit import of some precursor proteins (Figure 6), and the ability to interact with precursors directly (Figure 7) together strongly suggest that METAXIN can interact with precursor proteins on the outside of mitochondria. However, given that METAXIN is not located in the TOM complex with TOM20 (Figure 4), it would be premature to conclude that it plays a primary role as a preprotein receptor.

The accumulation of VDAC in the cytosol of *metaxin* plants (Figure 8) and the negligible rate of VDAC and TOM40 import into *metaxin* mitochondria in vitro (Figure 5) suggest that METAXIN also performs a role in β-barrel protein import. The competition and interaction experiments indicated that plant METAXIN can bind both VDAC and TOM40 (Figures 6 and 7), and the abundance of transcripts encoding these proteins increased significantly in *metaxin* plants (Figure 8). Therefore, plant METAXIN likely functions in a plant SAM complex, which remains to be characterized. The significant sequence similarity of plant and animal METAXIN proteins and the conservation of the plant GST-N-METAXIN and GST-C-METAXIN motifs and the functional Competitor domain in animal METAXIN and yeast SAM37 suggests that animal METAXIN and yeast SAM37 may have some functional similarities to the plant METAXIN. Mutant yeast cells lacking SAM37 displayed reduced import of several mitochondrial proteins, and anti-SAM37 antibodies were able to inhibit protein import, leading to the initial characterization of SAM37 as an import receptor that cooperated with TOM70 to recognize a range of mitochondrial proteins (Gratzer et al., 1995). Subsequently it was reported that SAM37 was not involved in the initial binding to mitochondria of the inner membrane metabolite carrier protein AAC, and its depletion did not affect the general or carrier import pathways (Ryan et al., 1999). However, if TOM20 and TOM70 were still present, their receptor capabilities may have compensated for the absence of SAM37. Indeed, inactivation of SAM37 was synthetically lethal with the deletion of either TOM20 or TOM70 (Gratzer et al., 1995). Thus, the role of the cytosolic domain of METAXIN remains unclear in other organisms; however, its ability to interact with a variety of precursor proteins suggests it may play another role in addition to the assembly of β-barrel proteins and thus interact with proteins on both the outside and inside of the outer membrane. Notably, At TOC64-III in chloroplasts has been proposed to play a role in precursor binding on the cytosolic and inter envelope space (Qbadou et al., 2007).

A Flexible Import Apparatus with Overlapping Specificity

Overall, TOM20, mtOM64, and METAXIN possess cytosolic domains that are located on the outer face of the mitochondria and are required for normal rates or protein import. Overexpressed protein competes for import, and they can interact with a variety of precursor proteins, all indicating that it is likely that TOM20, mtOM64, and METAXIN directly interact with mitochondrial precursor proteins to function in the initial stages of the import process. Given the NMR structure of TOM20 combined with the results presented here, it is likely that it is a receptor for at least some precursor proteins, while mtOM64 and METAXIN may play similar roles.

These findings provide a unique insight into the complexity of mitochondrial preprotein import machinery in a multicellular eukaryote. Analysis of genome sequences from a variety of organisms suggests that plants do not have orthologs to TOM20 and TOM70 and that the receptor domain of TOM22 is lacking, compared with yeast and mammalian systems (Macasev et al., 2004). Here, we have defined three plant outer mitochondrial membrane proteins that may fulfill the roles of preprotein receptors to produce a

flexible and redundant set of receptor subunits in plants. SAM37/35 and METAXIN appear to play a role in β-barrel assembly in all lineages but may have acquired lineage-specific functions as a receptor subunit, especially evident in plants. Finally, the use of very similar protein import components in both plant mitochondria and plastids suggests coevolution of the import machineries of both organelles, which may have been a significant impetus in the development of this unique import apparatus.

METHODS

Plant Growth

All plants were grown at 22°C under long-day conditions (16 h of 100 μE m^{-2} s^{-1} light, 8 h dark), except for *metaxin* plants that were iodine stained for starch content analysis, which were grown with a 12-h-light/12-h-dark photoperiod. For T-DNA insertion line genotyping, *Arabidopsis thaliana* seeds were grown on soil after stratification for 2 d. For mitochondrial isolation, *Arabidopsis* seeds were sterilized in 70% (v/v) ethanol and 5% (v/v) bleach/0.1% (v/v) Tween 20 and grown for 14 d on an orbital shaker at 80 rpm in 80 mL of sterile liquid growth media (0.5× Murashige and Skoog media, 0.5× Gamborgs B5 vitamins, 2% [w/v] sucrose, 50 μg/mL cefotaxime, and 2 mM MES KOH, pH 5.7). As *metaxin* homozygotes are infertile, seeds from plants heterozygous for the *metaxin-1* or *metaxin-2* null allele were sterilized as above and grown for 14 d on agar growth media (1× Gamborgs B5 salts, 3% [w/v] sucrose, 50 μg/mL cefotaxime, 2 mM MES KOH, pH 5.7, and 0.75% [w/v] agar). *metaxin* homozygote plants were then transplanted to soil and grown until flowering. Columbia-0 control plants were grown under the same conditions as *metaxin*.

Iodine Staining

For iodine staining of starch, plant tissue was boiled for 5 min in 80% (w/v) ethanol, washed in water, and then incubated for 5 min in 50% (v/v) Lugols's solution (Sigma-Aldrich). Plant tissue was then destained for 90 min in water.

T-DNA Insertion Lines

The following T-DNA insertion lines were obtained from SALK (Alonso et al., 2003), SAIL (Sessions et al., 2002), and GABI-KAT (Rosso et al., 2003) collections and genotyped to confirm homozygosity for the T-DNA insert: *tom20-2* (At1g27390): SALK_067986, SALK_134973; *tom20-3* (At3g27080): GABI_554C03, SAIL_88_A03; *tom20-4*: SALK_147093, SALK_004057; *mtOM64* (At5g09420): SALK_068772, SALK_089921; and *metaxin* (At2g19080): SALK_107629, SALK_039892.

Mitochondrial Isolation

Approximately 10 g (fresh weight) of aerial tissue from soil-grown plants or 20 g (fresh weight) of 14-d-old seedlings from liquid-grown (water culture) plants were used to isolate mitochondria as described previously (Day et al., 1985), with 10 mM L-Cys added to the grinding medium. Typically, 10 g of soil-grown and 20 g of liquid-grown plant tissue yielded ~2 mg of mitochondrial protein. For isolation of mitochondria for protein gel blot analysis, BSA was omitted from the final washes. All mitochondria were isolated from liquid-grown seedlings except for the *metaxin* genotypes, for which aerial tissue was used due to sterility of the *metaxin* null mutant.

Cytosol Isolation

Rosette tissue was homogenized in grinding buffer without BSA (Day et al., 1985), centrifuged at 2500*g* for 5 min, and then the supernatant centrifuged at 40,000*g* for 40 min. Crude cytosolic supernatant was centrifuged at 100,000*g* for 1 h at 4°C. The supernatant was concentrated in a >5 kD centrifugal filter unit (Millipore).

Immunodetection of Proteins, Pull-Down Assays, and Antibody Inhibition of Import

Mitochondrial or cytosolic proteins (50 μg) were resolved by SDS-PAGE, transferred to Hybond-C extra nitrocellulose membrane, and immunodetection performed as previously outlined (Murcha et al., 2005). Polyclonal antibodies were raised in rabbits against recombinant protein encoded by the predicted cytosolic regions of METAXIN, mtOM64, TOM20-2, and TOM20-4. TOM20-3 polyclonal antibody was obtained from Trevor Lithgow (University of Melbourne, Vic, Australia) (Taylor et al., 2003). Antibodies to HSP60 were obtained from Stress-Gen, and antibodies to COXII and cFBPase were obtained from Agrisera. Monoclonal antibodies against VDAC (PM035) and $E_1\alpha$ (PM030) were obtained from Tom Elthon (University of Nebraska, Lincoln, NE). Antibodies to AOX (Elthon et al., 1989), TIM17 (Murcha et al., 2005), and UCP (Considine et al., 2001) have been described previously. For the immunodetection experiments, mitochondrial proteins were isolated from the same water culture-grown seedling tissue that was used to prepare mitochondria for the in vitro import experiments, except for *metaxin* plants, for which tissue was always obtained from aerial rosettes.

IgG was purified for pull-down and inhibition studies using the Pierce Melon IgG purification kit according to the manufacturer's instructions (Pierce). For the pull-down assays, TOM20, mtOM64, and METAXIN were expressed in the wheat germ RTS (Roche), and on completion of the 24-h synthesis reaction, 1 μL of freshly synthesized radiolabeled precursor was incubated with 15 μL of freshly synthesized TOM20, mtOM64, or METAXIN and incubated at 24°C for 1 h. RTS lysate programmed to synthesize GUS was used as a control, antibody obtained from Sigma-Aldrich. At the end of the incubation period, the volume was adjusted to 300 μL in PBS with 1% (w/v) BSA, and 50 μL of Protein A Sepharose 4B conjugate (Sigma-Aldrich) was added to preclear the lysate for any nonspecific binding to the Protein A Sepharose (Sambrook et al., 1989). After incubation with gentle mixing for 1 h, the Protein A Sepharose was removed by centrifugation. Five microliters of the appropriate purified antibody was added and incubated for 1 h, followed by addition of 50 μL of Protein A Sepharose and incubation for a further 1 h. The beads were pelleted by centrifugation at 5000 rpm, washed in 200 μL of PBS with 1% (w/v) BSA, and repelleted. Products were analyzed by SDS-PAGE followed by exposure to a BAS-TR2040 plate for 48 h and imaged in a BAS2500 (Fuji). Four different sets of negative controls were performed to ensure that the antibody was only pulling down the target proteins and that interactions of the precursor protein with the test proteins was mediated by the mitochondrial targeting signal. First, AOX and F_Ad proteins were engineered that lacked the mitochondrial targeting signal, called AOXΔp and F_AdΔp, respectively (see below for details), to test if interaction was with this region of the protein. Second, the pull downs were performed in the absence of TOM20, METAXIN, mtOM64, or GUS to ensure that any radiolabeled precursor was not being pulled down directly with the antibody. Third, the nonmitochondrial protein GUS was used as a comparison. Finally, the specificity of the pull down was tested by protein gel blotting.

Yeast Two-Hybrid Interaction Assay

The Clontech Matchmaker two-hybrid system was used to determine interactions between the precursor proteins AOX, AOXΔp, F_Ad, F_AdΔp, PiC, GR, and TOM40 with TOM20-2, TOM20-3, TOM20-4, mtOM64, METAXIN, and At TOC64-III. The latter were cloned into the bait vector pGADT7-Rec[2] (Leu selection) and the precursor proteins cloned into the prey vector pGBKT7 (Trp selection) by recombination cloning according

to the manufacturer's instructions. Positive interactions were screened via two rounds of selection, first by growth on -His (with -Leu and -Trp) media and secondly by growth on -Ade media (with -Leu, -Trp, and -His); this higher stringency protocol reduces the rate of false positives (James et al., 1996). In the case of the AOX and F_Ad precursor proteins, the first 42 and 31 amino acids were removed as these regions had been previously shown to contain the mitochondrial targeting activity (Dessi et al., 2003; Lee and Whelan, 2004).

BN-PAGE

Mitochondrial membrane complexes were solubilized in 5% (v/v) digitonin and separated by first dimension BN-PAGE as described previously (Eubel et al., 2005). Proteins from the first dimension gel were transferred onto nitrocellulose membrane and immunodetection performed as described above.

Clones/Constructs

The cDNAs encoding the following proteins have been described previously: AOX (GenBank accession number X68702) (Whelan et al., 1995), F_Ad (GenBank accession number X79057) (Dessi et al., 1996), GR (At3g54660) (Chew et al., 2003a), PiC (GenBank accession number ABO16064) (Bathgate et al., 1989; Murcha et al., 2004), and RPS10 (At3g22300) (Adams et al., 2002). METAXIN (At2g19080), mtOM64 (At5g09420), At TOC64-III (At3g17970), TOM40 (At3g20000), and VDAC (At3g01280) were amplified from *Arabidopsis* cDNA as described previously (Murcha et al., 2003).

GFP Subcellular Localization

GFP subcellular localization was performed by cloning GFP-5 in frame with the N or C terminus of the cDNA clone and subsequent transformation of *Arabidopsis* suspension cells by biolistic transformation (Thirkettle-Watts et al., 2003; Lee and Whelan, 2004). RFP was fused to the targeting signal of soybean (*Glycine max*) alternative oxidase (AOX-RFP) as a mitochondrial control (Murcha et al., 2007). Fluorescence patterns were visualized after 48 h under an Olympus BX61 fluorescence microscope and imaged using CellR imaging software.

In Vitro Mitochondrial Protein Import

[^{35}S]-Met–labeled precursor proteins were synthesized using rabbit reticulocyte T_NT in vitro transcription/translation lysate (Promega) as described previously (Whelan et al., 1995). The use of equivalent quantities of mitochondria from different genotypes in import reactions was ensured by triplicate measurement of protein concentration with the Coomassie protein assay reagent (Pierce). Time-course analysis of precursor protein import into intact mitochondria isolated from wild-type or mutant plants was performed as described previously (Whelan et al., 1995), but with the addition of 1 mM GTP and 1 mM NADH to the import master mix. Briefly, 250 μg of mitochondria were added to 450 μL of ice-cold import master mix (0.3 M sucrose, 50 mM KCl, 10 mM MOPS, 5 mM KH_2PO_4, 0.1% [w/v] BSA, 1 mM $MgCl_2$, 1 mM Met, 0.2 mM ADP, 0.75 mM ATP, 5 mM succinate, 5 mM DTT, 1 mM GTP, and 1 mM NADH, pH 7.5) and incubated on ice for 3 min. Twenty-five microliters of radiolabeled precursor protein was added and the import reaction initiated by incubation at 26°C with gentle rocking. One hundred microliters containing 50 μg mitochondrial protein was removed at 2, 5, 10, and 20 min. Upon removal, each aliquot was mixed with 3.2 μg PK and incubated on ice for 30 min. Proteolysis was inhibited by the addition of 1 μL of 100 mM PMSF. Mitochondria were reisolated by centrifugation at 20,000*g* for 3 min at 4°C, the mitochondrial pellet resuspended in sample buffer, and the protein sample separated by SDS-PAGE gel and imported radiolabeled proteins detected as outlined previously (Murcha et al., 1999). PK protected mature radiolabeled protein was quantitated at each time point and normalized to the highest time point measurement for replicate experiments ($n = 3 \pm$ SE).

For import competition assays, competitor proteins were synthesized using the RTS wheat germ lysate in vitro transcription/translation system (Roche), according to the manufacturer's instructions. Competitor protein was used in the wheat germ lysate mix (consisting of wheat germ lysate resuspended in wheat germ reconstitution buffer) in which it was synthesized, without further purification from the wheat germ lysate mix. Five microliters of radiolabeled precursor protein was mixed with 15 μL of competitor protein (2 μg) in wheat germ lysate mix and preincubated at room temperature for 15 min. A control competition reaction was performed by preincubation of precursor protein with 15 μL (2 μg) of GUS in wheat germ lysate mix. Therefore, the same quantity of wheat germ lysate mix was added to both competitor and control competition import reactions. The expressed competitor protein was quantitated by separation of the proteins by SDS-PAGE and protein gel blotting with Anti-6-His antibodies, as all proteins expressed in this system contained a 6-His tag. The radiolabeled precursor/competitor solution was added to an import master mix containing mitochondria and incubated at 26°C for 10 min, after which the import reaction was stopped by incubation on ice and PK treatment. PK-protected mature radiolabeled protein was quantitated at each time point and normalized to the amount of protein imported after preincubation with GUS. To test for the effect of the wheat germ lysate mix upon mitochondrial protein import, AOX and F_Ad precursor proteins were preincubated for 15 min at room temperature with either 15 μL of wheat germ lysate mix or 15 μL of wheat germ reconstitution buffer alone, after which protein import was performed as described above. Mature imported radiolabeled protein was quantitated after import and normalized to the amount of protein imported in a control import reaction in which precursors had not been preincubated in wheat germ lysate mix or wheat germ reconstitution buffer.

PK Digestion of Mitochondrial Outer Membrane Proteins

Mitochondria were isolated from liquid grown plants as described above and 500 μg of mitochondria incubated with 0 to 32 μg PK in a final volume of 300 μL of 1× wash buffer (Day et al., 1985) on ice for 15 min. Proteolysis was inhibited by the addition of 2 μL of 100 mM PMSF. Mitochondria were reisolated by centrifugation at 20,000*g* for 5 min and resuspended in SDS-PAGE sample buffer. Fifty micrograms was separated in each lane by SDS-PAGE and immunodetection with specific antibodies performed.

Two-Dimensional IEF/SDS-PAGE

Mitochondria for two-dimensional IEF/SDS PAGE separation were further purified by centrifugation on a 45% Percoll gradient in 1× wash buffer minus BSA (Day et al., 1985). The mitochondrial fraction was washed twice in 1× wash buffer minus BSA, then pelleted by centrifugation at 31,000*g* for 15 min. Mitochondrial proteins were separated by two-dimensional IEF/SDS-PAGE as described previously (Ito et al., 2006).

Proteomic Analysis of Mitochondrial Proteins by Mass Spectrometry

Trypsin digestion and tandem mass spectrometry identification of proteins from two-dimensional PAGE gels was performed as described previously (Chew et al., 2003a).

Transcript Analysis

Quantitative RT-PCR was performed as per the manufacturer's instructions using the Roche LC480 and LightCycler480 SYBR Green I Master (Roche)

in a total volume of 10 μL. Gene-specific primers were used with cDNA pools synthesized from wild-type and *metaxin* rosette leaf total RNA as described previously (Murcha et al., 2003). Primers for measurement of transcript abundance are listed in Supplemental Table 1 online. Transcript abundance for each amplicon was normalized to the wild-type sample.

Phylogenetic Analysis

The phylogenetic relationship was inferred using the neighbor-joining method. Sequence alignment was performed using ClustalW (see Supplemental Figure 5 online). The bootstrap consensus tree inferred from 5000 replicates was taken to represent the relatedness of the sequences analyzed. Branches corresponding to partitions reproduced in <50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (5000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There were a total of 263 positions in the final data set. Phylogenetic analyses were conducted in MEGA4.

Accession Numbers

Sequence data from this article can be found in the National Center for Biotechnology Information and Arabidopsis Genome Initiative databases under the following accession numbers: TOM20-1, NP_189343 and At3g27070; TOM20-2, NP_174059 and At1g27390; TOM20-3, NP_189344 and At3g27080; TOM20-4, NP_198909 and At5g40930; MEXAXIN, NP_565446 and At2g19080; mtOM64, NP_196504 and At5g09420; Pic, X57566; AOX, X68702; F_Ad, X79057; GR, X27456; TOM40, NP_188634 and At3g2000; VDAC, Q9SMX3 and At5g15090; ACT2, NM_112764 and At3g18780; UBC, ABF59034 and At5g25760.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Analysis of the Expression of TOM20-1.

Supplemental Figure 2. Null Mutations in Any of the *TOM20* Genes Do Not Lead to Deficiencies in Mitochondrial Protein Import.

Supplemental Figure 3. Effect upon Protein Import by Components of the Wheat Germ Lysate Expression System.

Supplemental Figure 4. *metaxin* Mutants Are Depleted in *METAXIN* Transcript.

Supplemental Figure 5. ClustalW Protein Sequence Alignment of Plant METAXIN, Animal METAXIN1, and Fungal SAM37 Protein Sequences.

Supplemental Figure 6. The Mitochondrial Proteome Is Not Significantly Altered by Lesions in *METAXIN*, *mtOM64*, or Any *TOM20* Isoform.

Supplemental Figure 7. Negative and Positive Controls for Yeast Two-Hybrid Assay.

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