A Transcriptomic and Proteomic Characterization of the Arabidopsis Mitochondrial Protein Import Apparatus and Its Response to Mitochondrial Dysfunction[1][w]

Ryan Lister, Orinda Chew, May-Nee Lee, Joshua L. Heazlewood, Rachel Clifton, Karen L. Parker, A. Harvey Millar, and James Whelan*

Plant Molecular Biology Group, School of Biomedical and Chemical Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, Western Australia 6009, Australia

Mitochondria import hundreds of cytosolically synthesized proteins via the mitochondrial protein import apparatus. Expression analysis in various organs of 19 components of the Arabidopsis mitochondrial protein import apparatus encoded by 31 genes showed that although many were present in small multigene families, often only one member was prominently expressed. This was supported by comparison of real-time reverse transcriptase-polymerase chain reaction and microarray experimental data with expressed sequence tag numbers and massive parallel signature sequence data. Mass spectrometric analysis of purified mitochondria identified 17 import components, their mitochondrial sub-compartment, and verified the presence of TIM8, TIM13, TIM17, TIM23, TIM44, TIM50, and METAXIN proteins for the first time, to our knowledge. Mass spectrometry-detected isoforms correlated with the most abundant gene transcript measured by expression data. Treatment of Arabidopsis cell culture with mitochondrial electron transport chain inhibitors rotenone and antimycin A resulted in a significant increase in transcript levels of import components, with a greater increase observed for the minor isoforms. The increase was observed 12 h after treatment, indicating that it was likely a secondary response. Microarray analysis of rotenone-treated cells indicated the up-regulation of gene sets involved in mitochondrial chaperone activity, protein degradation, respiratory chain assembly, and division. The rate of protein import into isolated mitochondria from rotenone-treated cells was halved, even though rotenone had no direct effect on protein import when added to mitochondria isolated from untreated cells. These findings suggest that transcription of import component genes is induced when mitochondrial function is limited and that minor gene isoforms display a greater response than the predominant isoforms.

The majority of the thousand or more proteins that are present in mitochondria are required to be imported from nuclear-encoded cytosolically synthesized precursors (Emanuelsson et al., 2000; Werhahn and Braun, 2002; Heazlewood et al., 2003; Taylor et al., 2003c). The import of these proteins is achieved by the mitochondrial protein import apparatus, comprising a multisubunit translocase on both the outer and inner membranes, a variety of chaperone proteins present in the cytosol and mitochondria, and a number of peptidases that remove the "transient" targeting information present on many, but not all, mitochondrial precursor proteins (Neupert, 1997; Pfanner and Geissler, 2001). Despite the fact that mitochondrial targeting signals contain little primary sequence similarity, the mitochondrial protein im-

port apparatus specifically recognizes and imports up to 1,000 proteins (Sjoling and Glaser, 1998; Zhang et al., 2001).

The mitochondrial protein import apparatus has been studied intensively in yeast (*Saccharomyces cerevisiae*) using biochemical and genetic approaches. A single translocase operates on the outer mitochondrial membrane (TOM), which contains seven proteins with two primary receptors, TOM20 and TOM70. With few exceptions, these receptors recognize all precursor proteins studied so far and transfer them to the central TOM40 pore via TOM22, which can also act as a receptor for a small number of proteins. Two translocases on the inner mitochondrial membrane (TIM), called TIM17:23 and TIM22, function in the general and carrier import pathways, respectively (Pfanner and Geissler, 2001). Although not clearly understood mechanistically, TOM40 mediates the transfer of proteins to either of these TIM complexes and plays some role in sorting between the TIM complexes (Ahting et al., 2001; Gabriel et al., 2003). The TIM17:23 complex also contains a 50-kD protein, TIM50, and binds the matrix-located TIM44 (Milisav et al., 2001; Geissler et al., 2002; Yamamoto et al., 2002; Mokranjac et al., 2003). The TIM17:23 complex is responsible for the import of precursor proteins that contain N-terminal targeting signals that are generally removed after import by the mito-

chondrial processing peptidase (MPP; Neupert, 1997; Pfanner and Geissler, 2001). The TIM22 complex contains at least three other inner membrane proteins (TIM54, 18, and 12) that together with the small TIM proteins of the intermembrane space (TIM8, 9, 10, and 13) are responsible for the import of carrier proteins into mitochondria (Bauer et al., 1999; Kerscher et al., 2000; Koehler et al., 2000; Rehling et al., 2003a). A third less well-characterized translocase of the inner membrane, Oxa1p, consists of a homooligomeric complex that appears to be related to the YidC protein of the secretory apparatus in *Escherichia coli* (Luirink et al., 2001; Yen et al., 2001; Nargang et al., 2002). Sixteen of the 33 import components are essential for viability in yeast and constitute over one-third of the 40 mitochondrial proteins thought to be essential for yeast viability (Rehling et al., 2003b), whereas TIM23 also has been implicated in programmed cell death in yeast (Lohret et al., 1997).

In plants, biochemical approaches have characterized the TOM and MPP components of the plant import apparatus (Braun and Schmitz, 1995; Glaser and Dessi, 1999; Werhahn et al., 2001). Unlike yeast, the plant MPP is an integral component of the cytochrome bc_1 complex, although a specific matrix activity also appears to exist (Braun and Schmitz, 1995; Szigyarto et al., 1998). The plant TOM complex differs slightly from the yeast complex because it lacks any apparent ortholog of the TOM70 receptor (Jansch et al., 1998; Werhahn et al., 2001), whereas the plant TOM22 homolog, known as TOM9, does not contain the cis-receptor domain that is present in yeast and mammalian systems (Mascasev et al., 2000). Genetic approaches indicate that an *Oxa1p* ortholog from Arabidopsis can complement a yeast mutant for this protein (Sakamoto et al., 2000). In addition, modifications to the *TIM17* and *TIM23* genes of Arabidopsis allows complementation of yeast mutants (Murcha et al., 2003). A bioinformatics approach using all the known yeast components of the mitochondrial protein import apparatus identified 27 Arabidopsis orthologs of the 33 components present in yeast (Lister et al., 2003). Overall, it was notable that many of the components of the carrier import pathway were not identifiable from the genome of this model plant, i.e. TOM70, TIM12, 18, and 54. In addition, many of the components identified were encoded in small multigene families and did not contain many of the motifs that either have been shown to be important for function in yeast or that are conserved between yeast and mammalian systems.

Protein import into mitochondria can be affected by organ, developmental, and diurnal factors (Dessi and Whelan, 1997; Dudley et al., 1997; Murcha et al., 1999). It also has been demonstrated recently that protein import into mitochondria is altered under conditions of environmental stress that also inhibited important mitochondrial functions (Taylor et al., 2003a). An analysis of the effects of oxidative stress and respiratory inhibitors on the Arabidopsis mitochondrial proteome found changes in protein abundance and also documented losses of mitochondrial function (Sweetlove et al., 2002). Using an array which contained over 11,000 genes from Arabidopsis, it was observed that changes in gene expression after antimycin A treatment were similar to those observed with diverse biotic and abiotic stresses. It was proposed that mitochondria may be an important point in mediating responses to a variety of stresses (Yu et al., 2001). If mitochondria undergo alterations to adapt to such stresses, newly synthesized proteins in the cytosol may need to be imported into the mitochondria. However, the mechanisms by which mitochondrial protein import is modulated either by development, organ type, imposed stress or mitochondrial dysfunction have not yet been elucidated.

Therefore, to gain a better understanding of the structure of the plant mitochondrial protein import apparatus and how it may change under conditions that inhibit mitochondrial function, we conducted expression analysis of the 31 genes encoding the Arabidopsis import components identified by homology to the yeast import machinery. We analyzed their expression in various organs and looked for the presence of the proteins in mitochondria and submitochondrial compartments isolated from Arabidopsis cell cultures. Arabidopsis cell cultures were treated with the mitochondrial electron transport chain inhibitors rotenone and antimycin A, and transcript abundance was measured over time by quantitative real-time PCR. Transcriptomic analysis and the rate of protein import into isolated mitochondria were investigated after rotenone treatment.

RESULTS

Experimental Definition of the Mitochondrial Protein Import Apparatus

Gene Expression of Components of the Mitochondrial Protein Import Apparatus

Bioinformatic approaches have identified 19 putative components of the Arabidopsis mitochondrial protein import machinery encoded by 31 genes (Lister et al., 2002). To investigate the expression patterns of the putative protein import machinery genes, quantitative real-time reverse transcriptase (RT)-PCR analysis was used to measure transcript abundance in Arabidopsis cotyledons, roots, leaves, and flowers (Fig. 1). Initially, we measured the transcript abundance of ribosomal proteins that are required in the three cellular compartments where translation takes place. Transcripts of *RPS13* and *RPS15A*, nuclear-encoded ribosomal proteins that reside in the mitochondria and cytosol, respectively, were found in all Arabidopsis organs examined. In cotyledons, the transcript abundance of these two genes was highest at 4 d after germination and de-
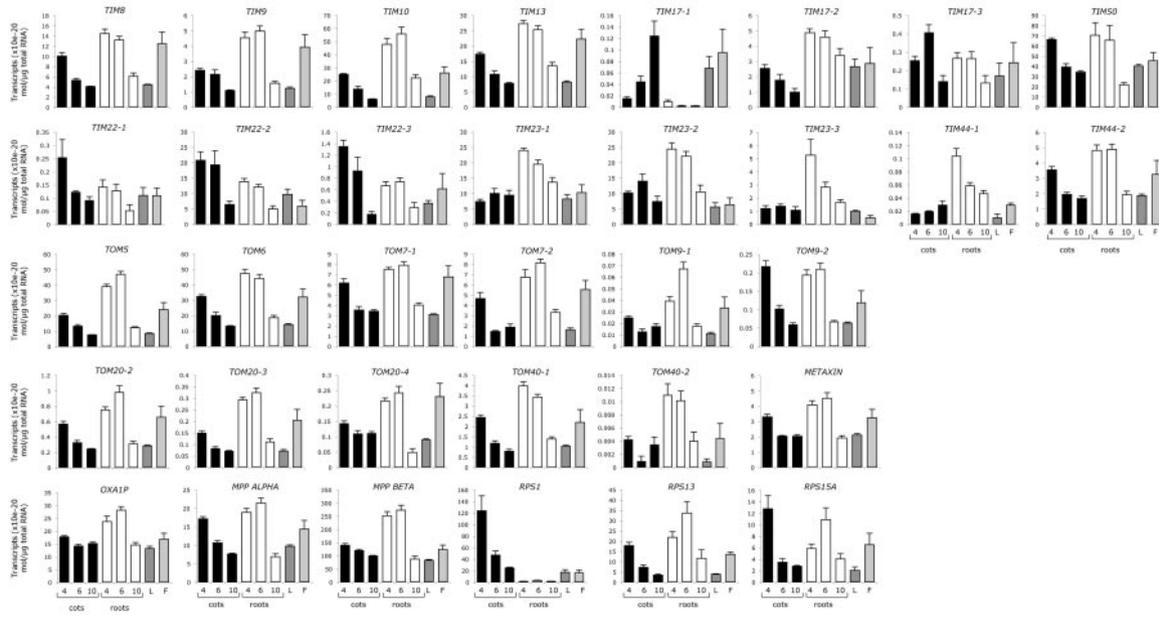
Figure 1. Transcript abundance of the cDNAs encoding the protein import machinery. Quantitative real-time RT-PCR analysis of the cDNAs encoding the Arabidopsis mitochondrial protein import machinery was performed on Arabidopsis cotyledons (C; 4, 6, and 10 d after germination), roots (R; 4, 6, and 10 d after germination), leaves (L; 10 d after germination), and flowers (F).

creased as the plant aged. In roots, the transcript abundance increased to peak at 6 d, then decreased. Message levels of the nuclear-encoded chloroplast ribosomal protein, *RPS1*, were similar to the cytosolic and mitochondrial *RPS* genes in cotyledons, leaves, and flowers, whereas the transcript abundance in roots was very low, as expected.

Message levels for the 31 genes encoding the plant mitochondrial protein import machinery were measured. With only one exception, transcripts for all genes were found in all samples examined, indicating that all were expressed. The exception was *TOM20-1*, which displayed expression below reliable detection levels. In roots, transcript abundance of the TIM and TOM components predominantly peaked at 4 and 6 d after germination, then decreased significantly by 10 d. Interestingly, like the chloroplast *RPS1*, *TIM17-1* message was present at very low levels in roots compared with other organs, possibly indicating that it was not utilized in nongreen organs. As in roots, message levels in cotyledons for most components were generally highest at 4 to 6 d but significantly decreased by 10 d. However, *TIM23-1*, *TIM23-3*, *TIM44-1*, and *TOM20-4* displayed a more constant transcript abundance over cotyledon development. *TIM17-1* again showed an expression profile differing from the general pattern in cotyledons, increasing over development. All genes were expressed in leaves and flowers. Included in this analysis was the Arabidopsis homolog of METAXIN (At2g19080), the outer membrane receptor for animal mitochondrial protein import (Armstrong et al., 1997). The Arabidopsis homolog displays 23% amino

acid identity to human (*Homo sapiens*) and fruitfly (*Drosophila melanogaster*) METAXIN proteins. Previously uncharacterized in plants, the similarity of the Arabidopsis *METAXIN* expression pattern to that of other import components provides preliminary evidence of it being the plant homolog of the METAXIN protein import receptor in animals.

Quantitative real-time RT-PCR was also used to compare the absolute transcript abundance for each import component in Arabidopsis cell culture (Fig. 2). Most import components displayed similar transcript levels, except for the low quantity of *TIM44*, *TOM9*, *TOM20*, *TOM40*, and *METAXIN*. In yeast, it has been calculated that TOM complexes are approximately 4 times more abundant than TIM complexes (Dekker et al., 1998). This stoichiometry was not observed in the transcript abundance of TOM and TIM import components in Arabidopsis cell culture (Fig. 2). Therefore, the low message abundance of these components may indicate other means of regulation, such as RNA stability, translation efficiency, and/or protein turnover.

Many import components are encoded in multiple gene families (Fig. 2, rectangles) that generally display high sequence similarity (Lister et al., 2003). The gene families each possess a predominant isoform that had the highest transcript abundance in cell culture (Fig. 2, arrows). The predominance of specific isoforms was also evident in whole plants as supported by a comparison of the message abundance in cell culture with the levels measured in Arabidopsis cotyledons, roots, leaves, and flowers (data not shown). With the exception of *TOM7*, the majority of
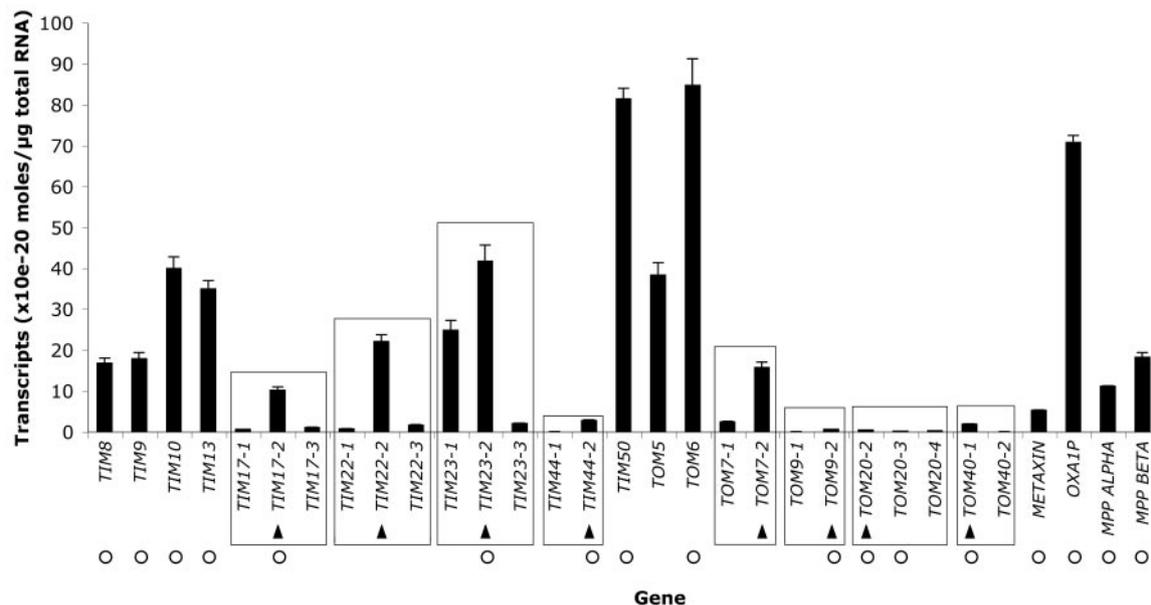
Figure 2. Absolute transcript abundance of the cDNAs encoding the protein import machinery. Quantitative real-time RT-PCR analysis of Arabidopsis cell culture was used to measure the absolute transcript abundance for cDNAs encoding the Arabidopsis mitochondrial protein import machinery. Multiple gene families are encompassed by a rectangle. An arrow indicates the isoform with the highest transcript abundance in each gene family. A white circle indicates the protein isoform found by mass spectrometry.

these organs displayed the same predominant isoforms as observed in the cell culture.

Additional support for the presence of predominant isoforms was obtained by analysis of the massively parallel signature sequence (MPSS) project data (Brenner et al., 2000), expressed sequence tag (EST) numbers, and our microarray analysis of Arabidopsis cell culture, all of which generally showed similar expression patterns to the real-time RT-PCR data (Fig. 3). Expression data were present for several different organs for both real-time RT-PCR (cell culture, cotyledon, root, leaf, and floral) and MPSS (callus, shoot, root, silique, and floral) experiments. However, because growth conditions and sample particulars varied between the two different approaches, the data were adjusted as indicated in "Materials and Methods." As seen in the *TOM20* transcript abundance in Arabidopsis cell culture (Fig. 2), the comparison of real-time RT-PCR, microarray, MPSS, and EST data did not reveal a distinct predominant isoform but indicated that they were present at similar levels. The predominant isoform within the gene families of *TIM17*, *TIM22*, *TIM23*, and *TOM40* was the same regardless of the technique used, whereas for *TIM44*, *TOM7*, and *TOM9*, there was a significant discrepancy in the isoform that predominated using one technique. For *TOM9*, real-time RT-PCR, microarray, and MPSS data were similar, but EST numbers did not agree. For *TOM7*, real-time RT-PCR data did not agree with the other approaches. Real-time RT-PCR and microarray data for *TIM44* indicated that *TIM44-1* message levels were very low, whereas *TIM44-2* transcript abun-

dance was 100-fold higher. This was not in agreement with MPSS and EST data, which indicated that both *TIM44* genes had similar transcript levels. *TOM40-2* was not represented by ESTs or MPSS data, perhaps because its transcript appears to be present at very low levels. Unfortunately, probe pairs to detect *TIM22-1* and *TIM22-3* were not present on the Affymetrix ATH1 GeneChip (Affymetrix, Santa Clara, CA).

Detection of Proteins for Components of the Mitochondrial Protein Import Apparatus

Highly enriched mitochondrial preparations were isolated from Arabidopsis cell culture and fractionated into four sub-compartments: inner membrane, outer membrane, matrix, and intermembrane space. Although the purity of the mitochondrial preparation is approximately 98% (Millar et al., 2001), the sub-fractionation procedure is an enrichment process rather than a high-purity separation; hence, the intermembrane space is nearly 20% contaminated by matrix, the outer membrane contains 20% to 50% inner membrane, and the inner membrane contains a significant amount of still-attached outer membrane portions. Protein aliquots from whole mitochondria and the sub-fractions were trypsin digested to identify peptides from import components by an liquid chromatography tandem mass spectrometry (MS/MS) strategy of shotgun sequencing of peptides (Heazlewood et al., 2003). Peptides from 17 different import components were identified in the mitochondria and sub-fraction aliquots (Werhahn and Braun, 2002). In
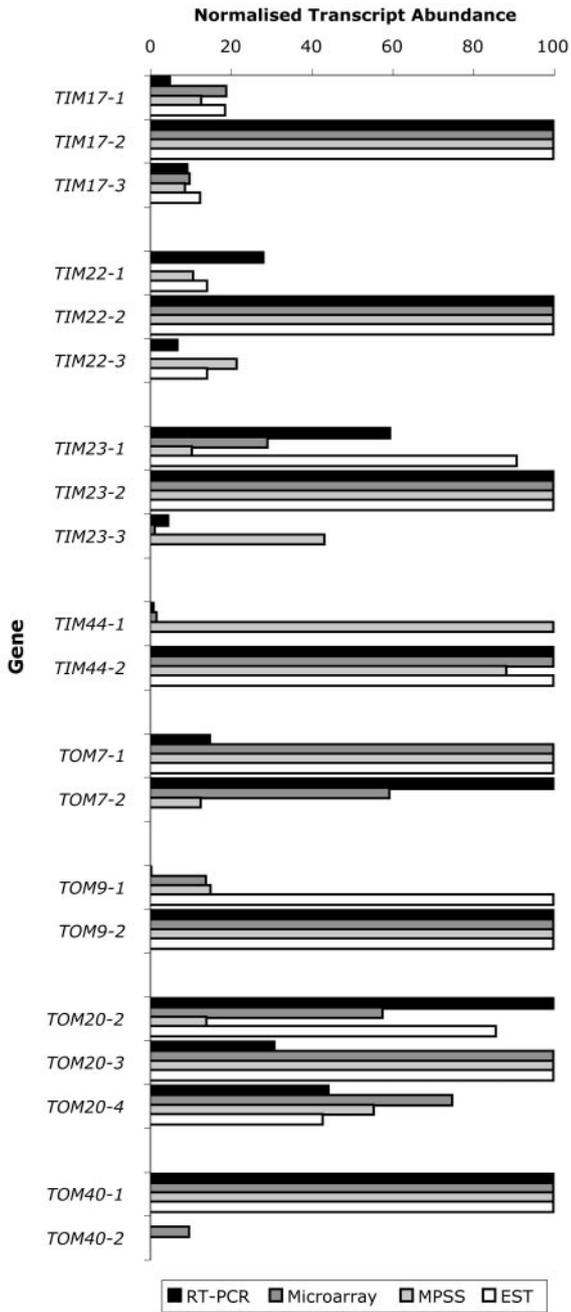
Figure 3. Comparison of quantitative real-time RT-PCR, microarray, MPSS, and EST data. For each experimental approach, the transcript abundance of every gene was averaged over the different tissue types, then within each gene family, these average message levels of the different isoforms were normalized, with the most abundant isoform given the arbitrary value of 100. EST numbers and microarray signal values (from control arrays) within each gene family were normalized against the isoform with the highest transcript abundance, allowing comparison of EST and microarray values in each gene family with real-time RT-PCR and MPSS data.

all cases of import components encoded in multiple gene families, the peptides identified were from the predominant isoform apparent from the cell culture transcript analysis (Fig. 2, peptide presence indicated

by white circle). Notably, with TIM44, the proteomic identification of TIM44-2 matches the expression data we obtained by real-time PCR and microarray analysis. This reveals a close correlation between the highly expressed members of the gene families and the presence of the encoded protein in the mitochondria. Subfractionation of the mitochondria assisted in the identification of six import components that were clearly too low in abundance to be readily detected in whole mitochondrial isolates. Enrichment of intermembrane space proteins, which normally constitute approximately 5% of the total mitochondrial protein content (Sweetlove et al., 2001), enabled identification of TIM8, TIM9, and TIM10, known in yeast to function in the intermembrane space. Inner membrane enrichment allowed identification of TIM17-2 and TIM44-2, whereas TOM20-2 was only identified after enrichment of outer membranes.

The identification of import components from the mitochondrial sub-compartments in which they are found in yeast reinforces the hypothesis that they are the authentic plant mitochondrial protein import components. The small TIM proteins (8, 9, 10, and 13) were readily identified in the intermembrane space but were largely absent from other compartments. The outer membrane-enriched fractions contained the three key TOM complex subunits, but we did not identify the small TIMs or the TIM translocase subunits in this fraction. The inner membrane TIM subunits (TIM17-2, TIM23-1, TIM44-2, and TIM50) were found in the enriched inner membrane and whole mitochondrial samples but were not identified in the soluble fractions investigated. The MPP subunits were identified in all fractions, which, in our opinion, does not suggest localization outside the inner membrane but rather their high abundance in mitochondrial samples.

The import components directly identified in mitochondrial samples encompass nearly the entire plant mitochondrial protein import apparatus as characterized by comparison with yeast and mammalian systems. Specific peptides from both TOM20-2 and TOM20-3 were sequenced in our analysis, which correlates with the similar transcript abundance of both isoforms of this family observed in the real-time RT-PCR, microarray, MPSS, and EST data. This also correlates with the dominant isoforms of TOM20 detected by Werhahn et al. (2003) in the purified TOM complex from Arabidopsis. The absence of two of the small TOM components (TOM5 and TOM7) may be explained by the relative paucity of suitable peptides generated by trypsin digestion of small proteins (data not shown). However, TOM7-1 and TOM5 have been identified in purified TOM complex from Arabidopsis mitochondria (Werhahn et al., 2003). The data presented in Table I constitute the first experimental identification of the Arabidopsis TIM8, TIM13, TIM17, TIM23, TIM44, TIM50, and METAXIN proteins.

Table I. *Import component peptides identified by mass spectrometry of trypsin-digested mitochondria*

Component	Locus	Peptides Found	Mowse Score	Found in				
				Mitochondria	Outer Membrane	Intermembrane Space	Inner Membrane	Matrix
TIM8	At5g50810	1	35			x		
TIM9	At3g46560	1	22			x		
TIM10	At2g29530	2	70			x		
TIM13	At1g61570	2	129	x		x		
TIM17-2	At2g37410	1	13				x	
TIM23-2	At1g72750	2	87	x			x	
TIM44-2	At2g36070	1	50				x	
TIM50	At1g55900	2	70	x			x	
TOM6	At1g49410	1	60	x				
TOM9-2	At5g43970	1	50	x				
TOM20-2	At1g27390	1	70		x		x	
TOM20-3	At3g27080	2	61	x	x		x	
TOM40-1	At3g20000	3	99	x	x		x	
METAXIN	At2g19080	2	132	x			x	
Oxa1p	At5g62050	1	32	x				
MPPα	At3g16480	>10	na[a]	x	x	x	x	x
MPPβ	At3g02090	>10	na	x	x	x	x	x

[a] na, Not available.

The Gene Expression of Components of the Mitochondrial Protein Import Apparatus Responds to Mitochondrial Dysfunction

We investigated if gene expression of components of the mitochondrial protein import apparatus responded to changes in mitochondrial activity. To assess this, we added inhibitors to the mitochondrial electron transport chain and assessed changes in gene expression at 1, 3, and 12 h after addition. Gene expression was measured in this period to assess if changes in expression occurred immediately, i.e. 1 to 3 h, or were a secondary affect, i.e. 12 h. Mitochondria were chemically stressed with 40 μM rotenone or 5 μM antimycin A, compounds that inhibit the action of complexes I and III of the mitochondrial electron transport chain, respectively. Both chemicals were added at concentrations intended to perturb mitochondrial function and did not result in cell death, as determined by vital stains and oxygen consumption assays (data not shown). Cell samples were taken 0, 1, 3, and 12 h after treatment for quantitative real-time PCR analysis. In addition, cells treated with rotenone were collected after 12 h for analysis using full-genome Affymetrix GeneChip ATH1 microarrays.

Table II lists groups of genes identified in the microarray analysis with altered transcript abundance after rotenone treatment, fold induction, and microarray signal value listed for each. These genes encode proteins involved in diverse mitochondrial activities: mitochondrial protein import components, molecular chaperones, proteins involved in respiratory chain complex assembly, protein degradation, and mitochondrial division. Twelve import component genes were up-regulated more than 1.5-fold after rotenone treatment, including those encoding pore-forming subunits (*TIM17*, *TIM23*, and *TOM40*) and outer membrane receptor components (*TOM20* and *METAXIN*). The concurrent increase in transcript abundance of eight of 10 genes encoding mitochondrial molecular chaperone proteins suggests a requirement for the folding and assembly of proteins in the mitochondria. Also up-regulated were genes encoding Arabidopsis proteins similar to yeast proteins involved in the correct assembly of mitochondrial respiratory chain complexes: BCS required for the assembly of a functional cytochrome bc_1 complex (Nobrega et al., 1992); COX15, 17, 19, SCO1, and SURFEIT 1 required for the assembly of the cytochrome oxidase complex (Schulze and Rodel, 1988; Nobrega et al., 1992; Glerum et al., 1996, 1997; Nijtmans et al., 2001; Balandin and Castresana, 2002); and an m-AAA protease required for the assembly of ATP synthase (Arlt et al., 1996; Kolodziejczak et al., 2002). A zinc metalloprotease (At3g19170) involved in the rapid degradation of mitochondrial presequences after they have been removed by MPP was up-regulated 3.4-fold (Stahl et al., 2002). In addition, genes possibly involved in mitochondrial division (*ADL2a* and *FIS1*) were up-regulated (Mozdy et al., 2000; Arimura and Tsutsumi, 2002).

Quantitative real-time RT-PCR analysis of the transcript abundance of import components in cells treated with antimycin A or rotenone at 0, 1, 3, and 12 h was performed to confirm the microarray data and determine whether the changes observed in the microarray analysis were primary or secondary responses to the chemical treatments (Fig. 4). Shown are the import components that displayed significant changes in transcript abundance in comparison with the control (fold change > 1.5). In addition, the mi-

Table II. *Microarray measurements of changes in transcript abundance after rotenone treatment*

Gene	Locus	Fold Change	Microarray Signal	
			Control	Rotenone
Import components				
TIM10	At2g29530	1.6	1,244	1,990
TIM17-1	At1g20350	3.9	583	2,259
TIM17-2	At2g37410	1.6	3,081	5,049
TIM17-3	At5g11690	−1.6	309	193
TIM23-1	At1g17530	2.4	556	1,323
TIM23-3	At3g04800	5.6	24	137
TIM44-2	At2g36070	1.5	415	625
TIM50	At1g55900	1.7	894	1,562
TOM20-4	At5g40930	1.7	1,046	1,768
TOM40-2	At1g50400	5.3	160	850
TOM7-2	At1g64220	5.8	161	933
METAXIN	At2g19080	1.9	1,247	2,348
Oxa1p	At5g62050	1.6	1,013	1,591
Mitochondrial molecular chaperones				
CPN10	At1g23100	4.6	128	588
HSP60-2	At2g33210	2.0	2,187	4,322
HSP60-3a	At3g13860	1.6	1,281	2,050
HSP60-3b	At3g23990	2.1	1,225	2,608
HSP60-3c	At3g13470	−1.8	1,159	644
HSP70-4	At4g37910	2.2	3,659	8,026
HSP70-5	At5g09590	1.9	1,795	3,451
HSP90-3	At4g24190	−2.5	4,753	1,901
GRPE	At5g55200	4.2	916	3,846
MGE	At5g17710	1.9	847	1,573
Respiratory chain assembly				
BCS1a	At3g50930	12.2	124	1,516
BCS1b	At3g50940	2.5	90	231
BCS1c	At5g17760	1.5	960	1,469
COX15	At5g56090	3.2	706	2,228
COX17	At1g53030	1.9	352	668
COX17	At3g15352	2.2	245	532
COX19	At1g69750	1.8	1,379	2,483
SCO1	At3g08950	2.2	648	1,409
SURFEIT1	At3g17910	1.8	730	1,291
m-AAA protease	At1g07510	1.5	1,150	1,712
Mitochondrial division				
ADL2a	At4g33650	1.8	380	684
Fis1 homolog	At3g57090	2.8	925	2,590
Fis1 homolog	At5g12390	2.0	116	232
Mitochondrial proteases				
Lon protease	At5g26860	2.3	301	693
Lon protease	At3g05780	3.7	13	49
ClpC1	At5g50920	1.9	1,769	3,362
ClpX2	At5g49840	1.7	912	1,550
DegP10	At5g36950	2.1	221	465
FtsH protease	At1g06430	3.7	420	1,554
PreP	At3g19170	3.4	894	3,041

croarray fold change is indicated for comparison with the fold change measured by real-time RT-PCR. Probe pairs to detect *TIM22-1* were not present on the Affymetrix ATH1 GeneChip, so real-time RT-PCR was used to quantify the changes in transcript abundance. The fold changes in transcript abundance as measured by real-time RT-PCR were very similar in most cases to the fold changes observed in the microarray analysis (Dekker et al., 1998). The transcript abundance changes of *TIM10*, *METAXIN*, and *Oxa1p*

observed by microarray analysis were not supported by the quantitative real-time RT-PCR analysis. Of the TIM components, seven genes showed significant up-regulation after rotenone treatment, whereas only *TIM17-3* showed a decrease in transcript abundance (Fig. 4A). Moreover, except for *TIM22-1*, the highest fold change was observed after 12 h, indicating that this was likely to be a secondary response. *TIM22-1* transcript abundance doubled only 1 h after rotenone treatment, possibly indicating an early response of

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Figure 4. Changes in import component transcript abundance over time after treatment with mitochondrial electron transport chain inhibitors. Transcript abundance of import components were measured by quantitative real-time PCR at 0, 1, 3, and 12 h after treatment of Arabidopsis cell culture with 5 μM antimycin A or 40 μM rotenone. The fold change in transcripts of TIM components (A) and TOM components (B), which changed significantly in comparison with the transcript abundance in control cells over the same period. The fold change measured by microarray analysis after 12 h of rotenone treatment is shown (M). C, Comparison of the increase in transcript abundance of minor (indicated in black) and predominant (indicated in white) import component isoforms after 12 h of rotenone treatment as measured by quantitative real-time PCR.
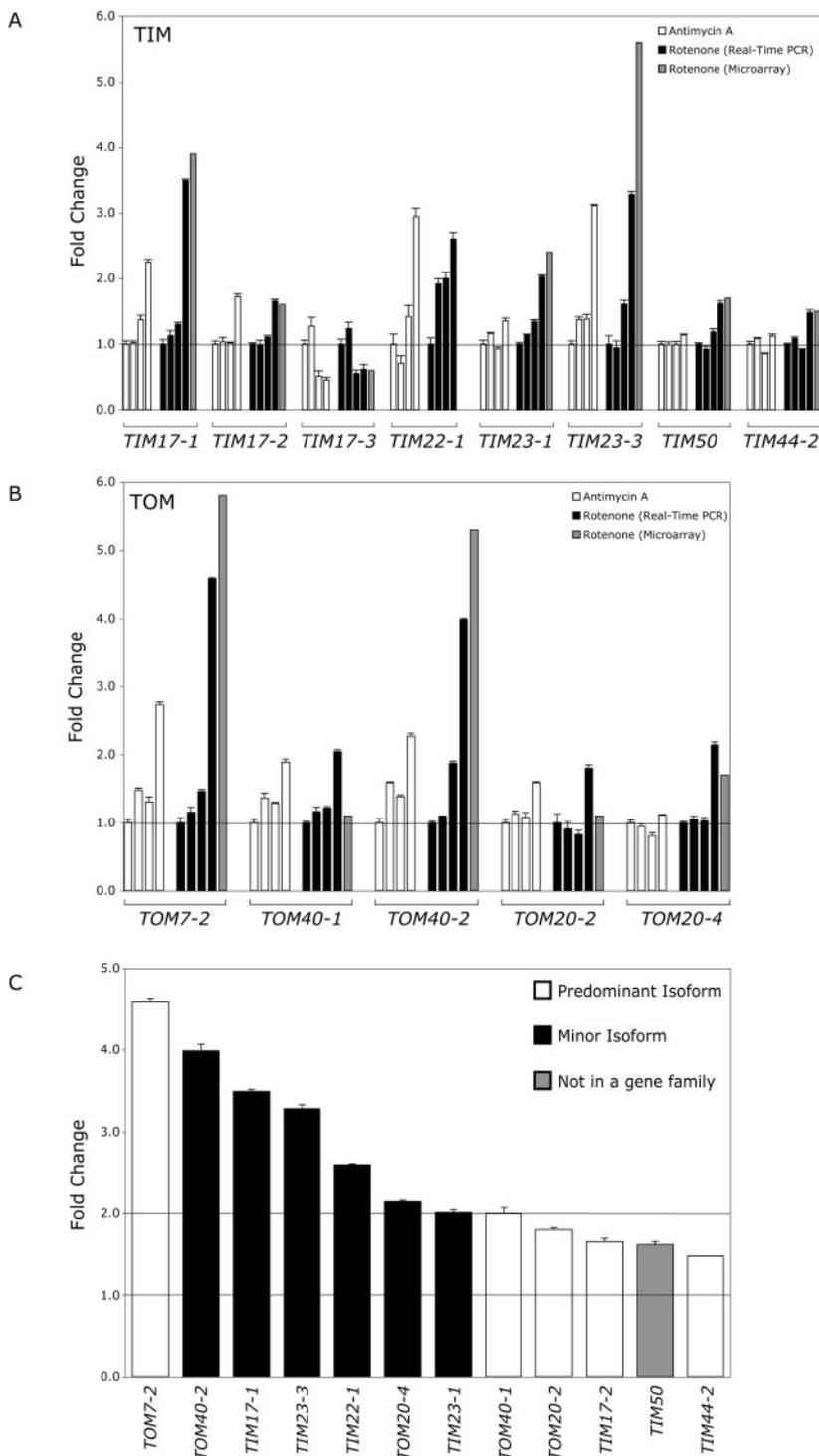
the carrier import pathway. The message levels of the small TIM components (8, 9, 10, and 13) did not change after inhibitor treatment, but they may not be rate limiting in the carrier import pathway. The five TOM components changed primarily 12 h after treatment (Fig. 4B). Comparison of the fold changes of the import components 12 h after rotenone treatment revealed an interesting trend (Fig. 4C). The fold change in up-regulated minor import component isoforms was greater than that of the induced predominant isoforms (white), perhaps indicating a specialized role for the minor import component isoforms as stress-responsive genes. In addition, the highest inductions of the minor isoforms involved the pore-forming import components TOM40, TIM17, and TIM23.

The microarray gene expression profiles after rotenone treatment of cell culture indicates an increase in the expression of genes encoding mitochondrial protein import components and mitochondrial biogenesis in general. The increase of several mitochondrial proteases may be required to replace real damaged components of mitochondria because of the rotenone treatment or the perception of damage because of the partial inactivation of electron transport capacity. To test if rotenone treatment of cells affects protein import in vitro, protein import assays were performed with mitochondria isolated from control and rotenone-treated cells (Fig. 5). Import of the Arabidopsis nuclear-encoded mitochondrial protein RPS10 was lower in mitochondria isolated from Arabidopsis cell culture 12 h after rotenone treatment (Fig. 5A). Time course experiments indicated that the rate of import into mitochondria isolated from rotenone-treated cells was approximately 50% of the control rate (Fig. 5B). Addition of rotenone to mitochondria isolated from untreated cells with 40 μM rotenone immediately before import did not result in a significant change in the rate of protein import (Fig. 5C), indicating import per se was not being directly inhibited by rotenone.

DISCUSSION

Molecular or genetic approaches are required to characterize the majority of components of the mitochondrial protein import apparatus because of their scarcity. This is especially true for the translocases of the inner membrane, where the abundant respiratory chain complexes mean that even in yeast, the identification of these components was achieved largely by genetic means (Rehling et al., 2001). Subsequent tagging and knockout and mutational approaches have allowed the determination of biochemical mechanisms. The identification of these components in yeast also has greatly facilitated efforts in plant and mammalian systems in the identification and study of components of the mitochondrial protein import apparatus. This in silico approach shows that although there are many similarities, some major differences also exist, and even when orthologs can be identified in Arabidopsis for components, close analysis indicates that they either differ structurally to their yeast counterpart or lack motifs or residues shown to be essential for function in yeast (Rapaport et al., 2001; Gabriel et al., 2003; Taylor et al., 2003b). Therefore, to identify which genes are expressed in Arabidopsis whose products comprise the mitochondrial protein import apparatus and how either expression changes with mitochondrial function, we carried out detailed analysis of the expression patterns of all genes identified by homology to the yeast import components. Transcriptomic analysis was used to investigate additional changes in components required for mitochondrial biogenesis, and a proteomic approach to identify many of the import apparatus proteins actually present in mitochondria.

A comprehensive expression analysis of all the genes identified in the Arabidopsis genome involved in the mitochondrial protein import apparatus indicates that they are all expressed in all organs examined, except for *TOM20-1*. Notable differences to the general pattern observed was evident for low *TIM17-1* expression in roots and the high level of *TIM23-3* and *TIM44-1* message levels in roots compared with other organs. This indicates, at least for some members of the small gene families, that specialization in expression is evident. Examination of the actual transcript levels indicates that a 10-fold difference in the level of message for some components is observed despite the fact that protein levels would be expected to be the same in a functioning complex (Dekker et al., 1998; Moro et al., 1999). The transcript abundance of *TIM44* was almost 10-fold
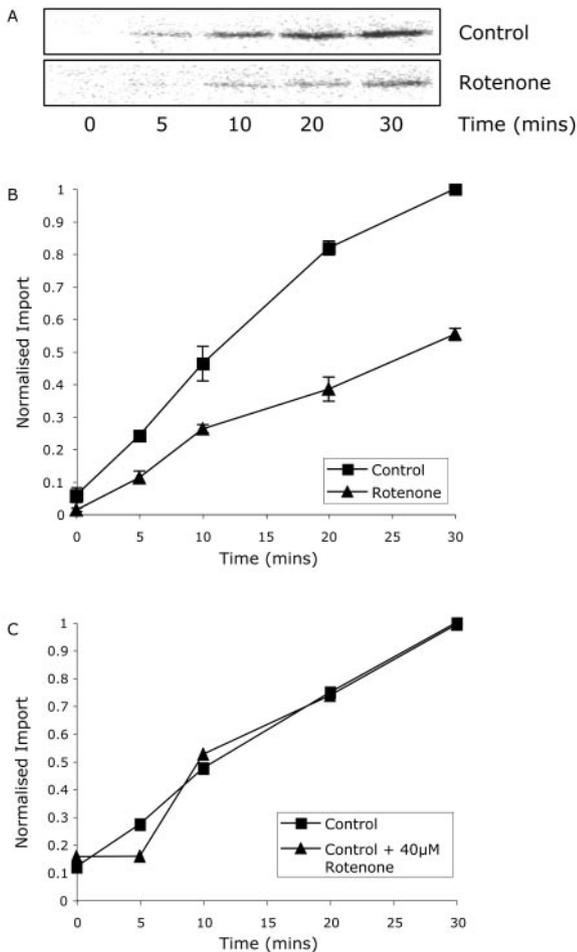
Figure 5. The rate of mitochondrial protein import is decreased in rotenone-treated mitochondria. A, In vitro import of RPS10 into mitochondria isolated from control or rotenone-treated cell culture. B, Rate of import into mitochondria isolated from control or rotenone-treated cell culture. C, Rate of import in the presence or absence of 40 μM rotenone of mitochondria isolated from cell culture.

lower than *TIM50* and *TIM23*, whereas the levels of *TOM9*, *TOM20*, and *TOM40* were almost 10-fold lower than *TOM6*. However, within the gene families themselves, there was a good correlation between the member of each gene family with the highest transcript level and the protein isoforms detected in purified mitochondria. Therefore, although many import components are encoded by small multigene families, it appears that largely one member functions to form the mitochondrial protein import apparatus.

The general question of why some components are encoded by small gene families is pertinent given that expression of some isoforms is extremely low. To investigate this, we treated cells with the mitochondrial poisons rotenone and antimycin A and analyzed the transcript abundance of the import components. It was evident that on treatment with both compounds, the expression of many import components was up-regulated significantly. The minor isoforms displayed the greater induction by these treatments, most notably for the pore-forming subunits TOM40, TIM17, TIM23, and TIM22. Rotenone appeared to have a slightly greater effect than antimycin A, with higher stimulation observed for *TIM17-1*, *TIM23-1*, *TIM50*, *TIM44-2*, *TOM7-2*, *TOM40-2*, and *TOM20-4*. We observed that isolated mitochondria from rotenone-treated cells imported proteins at 50% of the rate of mitochondria isolated from control cells. Thus, rotenone treatment may be causing mitochondrial damage and protein turnover that require replacement through mitochondrial biogenesis. Thus, the signal driving the up-regulation of expression of genes for components of the mitochondrial import apparatus is the decreased rate of import, which is still evident after 12 h. We propose that the up-regulation of gene expression observed is an attempt to overcome this decreased rate of import.

Diverse changes upon inhibition of complex I have been reported previously (Dutilleul et al., 2003). It has been demonstrated that cytoplasmic male-sterile mutant plants impaired in complex I function lost diurnal patterns of alternative oxidase activity, had altered antioxidant enzyme expression and activity, and showed higher tolerance to ozone levels and tobacco mosaic virus. Recently, the association of the terminal enzyme in ascorbate biosynthesis, L-galactono-1,4-lactone dehydrogenase, with complex I has been discovered (Millar et al., 2003). Treatment with rotenone impeded ascorbate biosynthesis, demonstrating a link between complex I activity and synthesis of a key cellular antioxidant. The reduction of ascorbate levels may result in damage to proteins by reactive oxygen species, necessitating replacement of the damaged components. Such damage to the protein import machinery may be the mechanism by which the rate of protein import into rotenone-treated mitochondria is reduced. With the results presented here, this indicates that perturbation of complex I activity may have widespread effects on gene expression and, thus, could be an important point for retrograde signaling between the mitochondrion and the nucleus.

The presence of multigene families is not unique to nuclear-encoded proteins destined for mitochondria several of the chloroplast import components are encoded in small gene families (Jackson-Constan and Keegstra, 2001; Davila-Aponte et al., 2003). In the case of the plant mitochondrial protein import components, it is likely that some components encoded by gene families will have overlapping but distinct roles. It is possible that the multiple forms of TOM20 compensate for the lack of a receptor domain of TOM9 and the apparent lack of TOM70 in plants (Werhahn et al., 2001; Werhahn and Braun, 2002). We have shown previously that the different isoforms of TIM17 and TIM23 differ in their ability to complement knockout mutants for the orthologous subunits in yeast, which further suggests some specialization of function between different isoforms (Murcha et al., 2003). In addition, we have demonstrated here that the various isoforms display differential induction upon inhibitor treatment that also may account for common changes in the mitochondrial proteome observed upon oxidative stress and inhibitor treatments (Sweetlove et al., 2002) and changes in import of various precursor proteins observed under environmental stress (Taylor et al., 2003a).

Overall, our investigations showed that with few exceptions, there is one prominent expressed isoform for each import component in Arabidopsis, which is confirmed by the protein detected in mitochondria. This suggests a similar import apparatus in the majority of organs. The expression of the additional isoforms was induced upon respiratory inhibitor treatment. The induction of genes encoding components of the mitochondrial protein import apparatus may allow recovery from such treatments. It also may contribute to differences observed in mitochondrial proteomes and to protein import upon chemical oxidant and environmental stress treatments. Thus, various members of the gene families may produce an import apparatus with different characteristics to that observed under normal conditions.

MATERIALS AND METHODS

Plant Growth

Arabidopsis plants were grown in media (Gamborg et al., 1968) at 22°C with a 16-h-light (100 μE m^{-2} s^{-1}) and 8-h-dark photoperiod. Arabidopsis suspension cell culture was maintained as described (Sweetlove et al., 2002).

Chemical Treatment of Arabidopsis Cell Culture

Arabidopsis cell cultures were treated by the addition of 12 μL of 50 mM antimycin A (final concentration 5 μM) or 480 μL of 10 mM rotenone (final concentration 40 μM) to 120 mL of suspension cell culture 4 d after subculturing.

Cloning and Expression Analysis of Components of the Mitochondrial Protein Import Apparatus

Cloning and transcript abundance for components of the mitochondrial protein import apparatus were carried out as previously described (Murcha et al., 2003). Primers for amplification of the cDNAs and determination of transcript abundance are listed in the supplemental material, available in the online version of this article at http://www.plantphysiol.org.

To compare the expression patterns of the various genes obtained from quantitative RT-PCR, MPSS, and microarrays, the data were normalized as follows. For each experimental approach, the transcript abundance of every gene was averaged over the different organ types, then within each gene family, these average message levels of the different isoforms were normalized, with the most abundant isoform given the arbitrary value of 100. This enabled comparison of the average transcript abundance of different isoforms within a gene family in all organs analyzed and between real-time RT-PCR and MPSS data. Within each gene family, microarray signal values and EST numbers were normalized against the isoform with the highest transcript abundance, allowing comparison of microarray data and EST numbers in each gene family with real-time RT-PCR and MPSS data.

Microarray Analysis

Microarray analysis of the changes in transcript abundance in Arabidopsis cell culture was performed using Affymetrix GeneChip Arabidopsis ATH1 Genome Arrays (catalog no. 510690, Affymetrix). Total RNA was isolated from Arabidopsis cell culture 12 h after treatment with 40 μM rotenone using the RNeasy Plant mini protocol (Qiagen, Clifton Hill, Victoria, Australia). The high quality of the total RNA was verified by using both an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA) and spectrophotometric analysis of the A_{260} to A_{280} ratio. Six flasks of Arabidopsis cell culture were treated with rotenone and combined into two separate pools. Total RNA was isolated from each of the two pools; after cRNA sample preparation, each was analyzed on separate GeneChips, resulting in two biological replicates of rotenone-treated cells. Six untreated control cell culture flasks were prepared similarly, resulting in two biological replicates of control treated cells. Double-stranded cDNA synthesis, biotin-labeled cRNA target synthesis, target hybridization, washing, staining, and scanning were performed exactly as described in the Affymetrix GeneChip Expression Analysis Technical Manual, using the kits, chemicals, and reagents precisely as outlined. Control Oligo B2 and Biotinylated Hybridization Controls (Affymetrix) were included in the hybridization. Before hybridization to an ATH1 GeneChip, the cRNA target quality was assessed by hybridization of an aliquot of the prepared cRNA to a Test3 array (catalog no. 510599, Affymetrix). Hybridization was performed in an Affymetrix GeneChip hybridization Oven 640. Washing and staining were performed using an Affymetrix Fluidics Station 400. Scanning was performed with an Agilent GeneArray Scanner G2500A. GeneChip scans were initially analyzed using the Affymetrix Microarray Suite 5.1 software, from which PivotData tables were exported. Raw data from the PivotData Tables were analyzed in GeneSpring software version 6 (Silicon Genetics, Redwood City, CA), using the parameters suggested by Silicon Genetics for analysis of Affymetrix Microarrays. All quoted changes in transcript abundance between control and rotenone-treated cells were significant, with Student's t test P values < 0.05.

Isolation and Fractionation of Arabidopsis Mitochondria

For protein identification, Arabidopsis cell culture mitochondria were isolated from cultures 7 d after subculturing as described (Sweetlove et al., 2002). Sub-fractionation of mitochondria into inner membrane, matrix, outer membrane, and intermembrane space was performed as described previously (Lister et al., 2002) based on the methods outlined by Sweetlove et al. (2001). For in vitro import experiments, Arabidopsis cell culture mitochondria were isolated after 12 h of chemical treatment.

Trypsin Digestion and Liquid Chromatography-MS/MS Analysis of Whole Mitochondrial Protein Extracts

Mitochondrial protein extracts were acetone precipitated at $-20°C$ overnight. Fifty micrograms of protein was digested with 5 μg of trypsin (Roche,

Sydney) overnight at 37°C in 100 mM Tris (pH 8.5). Resulting tryptic peptides were differentially separated over 10 h using a 0.3- $\times$ 150-mm Zorbax C18 column (Agilent, Sydney) and injected directly into Q-Star Pulsar I MS/MS (Applied Biosystems, Sydney) via an electrospray source. Peptides were automatically selected by Analyst QS (Applied Biosystems) for MS/MS analysis and fragmented with N_2. Mass spectra and collision MS/MS data were analyzed and matched to predicted gene products with BioAnalyst and ProID software (Applied Biosystems), using mass accuracy cutoffs of peptide mass $\pm$ 0.15 and MS/MS $\pm$ 0.05. Collision-induced dissociations were also analyzed by Mascot (Matrix Science, London) for independent matching.

[^{35}S]-Met-Labeled Precursor Proteins

Arabidopsis ribosomal protein S10 (RPS10) was amplified using primers designed to sequences obtained from cDNA to the RPS gene (At3g22300; Adams et al., 2002). [^{35}S]-Met labeled precursor proteins were synthesized using the rabbit reticulocyte T_NT in vitro transcription/translation kit (Promega, Madison, WI) as described previously (Whelan et al., 1995).

In Vitro Import into Mitochondria

Import of precursor proteins was carried out in import master mix using intact mitochondria isolated from control or rotenone-treated Arabidopsis cell culture as described previously (Whelan et al., 1995). Rotenone treatment of mitochondria was performed by addition of 10 mM rotenone to a final concentration of 40 μM in the final import reaction. Protein import, protease treatment of mitochondria, separation, and detection of imported proteins using phosphor imaging was performed as previously outlined (Murcha et al., 1999). The use of equivalent amounts of control and rotenone-treated mitochondria was ensured by measuring protein concentration with the Coomassie Protein Assay Reagent as per the manufacturer's instructions (Pierce, Rockford, IL). Three independent cell culture treatments, mitochondrial isolations, and in vitro import reactions were performed.

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