

A plant outer mitochondrial membrane protein with high amino acid sequence identity to a chloroplast protein import receptor

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Abstract We have identified a novel protein on the outer membrane of *Arabidopsis thaliana* mitochondria. This protein displays 67% sequence identity with the 64 kDa translocase of the outer envelope membrane of chloroplasts (Toc). A mitochondrial localisation for this protein was determined by (i) its presence in the proteome of highly purified *Arabidopsis* mitochondria, (ii) Western blot analysis with antibodies to Toc64 from pea that indicate its presence in *Arabidopsis* and pea mitochondria, (iii) green fluorescent protein fusion proteins that indicate an exclusive mitochondrial localisation for this protein, and (iv) expression profiles in various tissue types and during development that are more similar to translocase of the outer mitochondrial membrane components than to chloroplastic Toc components. Thus *Arabidopsis* mitochondria contain a protein with high sequence identity to a plastid protein import receptor.

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1. Introduction

The combined action of a single translocase on the outer membrane (TOM) and two translocases on the inner membrane (TIM) are responsible for the import of the majority of proteins into mitochondria [1–3]. The TOM complex plays two roles in protein import into mitochondria. Firstly it is responsible for the recognition of mitochondrial precursors synthesised in the cytosol that are destined to be located in the mitochondrion [4,5]. Secondly it forms a channel in the outer membrane to facilitate the translocation of proteins across it [6,7]. Proteins that are imported into mitochondria are passed from TOM to either TIM17:23 or TIM22. TIM17:23 is responsible for the import of proteins that contain N-terminal targeting signals, termed the general import pathway [8,9], whilst TIM22 is responsible for the import of

proteins that contain internal targeting signals, termed the carrier import pathway [10,11].

The recognition of proteins destined to be located in the mitochondria is achieved by the receptor function of TOM. TOM20 and TOM70 act as the primary receptors, with TOM22 also having the ability to specifically recognise mitochondrial targeting information [2]. Isolation of the TOM complex from yeast, mammals and plants indicates that it is generally well conserved in structure [12–16]. However, the plant TOM complex appears to differ to that of mammals and yeast with respect to the receptor components. Firstly the *cis* receptor domain of TOM22 appears to be absent and thus it is difficult to envisage how this protein could have any receptor function [17,18]. Secondly despite several biochemical characterisations no protein equivalent to the TOM70 receptor has been reported [17,18]. Additionally no homologue with any significant sequence identity to TOM70 can be detected in the *Arabidopsis* genome [19]. The absence of the *cis* receptor domain of TOM22 has been proposed to be a result of the unique cell environment of plant cells where high targeting specificity between plastids and mitochondria is required [20]. The absence of TOM70 is more puzzling as a distinct carrier import pathway has been demonstrated in plants [21]. Although some carrier proteins in plants appear to contain N-terminal cleavable signals, this is only the case for 30–50% of the predicted carrier proteins present in the *Arabidopsis* genome [22]. Additionally carrier proteins lacking an N-terminal targeting signal, such as the oxoglutarate-malate carrier, are clearly imported via a carrier pathway into plant mitochondria [21].

Subcellular proteomic analysis is an alternative approach to define the protein complement of an organelle [23–25]. We have used various direct proteomic approaches to define the mitochondrial proteome of *Arabidopsis* [22,26–28]. Using this approach we identified a protein that was annotated as a Toc64 protein in the TIGR *Arabidopsis* protein dataset (release 4, April 2003). Due to the high purity of our mitochondrial preparations and the lack of other plastid translocase of the outer envelope membrane of chloroplasts (Toc) components we carried out further investigations to confirm the mitochondrial localisation of this protein.

2. Materials and methods

2.1. Plant materials

Soybean (*Glycine max* cv. Stevens) and pea (*Pisum sativum* cv.

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Abbreviations: TOM, translocase of the outer membrane of mitochondria; TIM, translocase of the inner membrane of mitochondria; Toc, translocase of the outer envelope membrane of chloroplasts; TPR, tetratricopeptide repeat

Greenfeast) plants were grown in a 28°C incubator, which was fitted with artificial lights of 600 $\mu\text{mol}/\text{m}^2/\text{s}$ set to a 16 h light and 8 h dark cycle. *Arabidopsis thaliana* (ecotype Columbia) were grown at 22°C under artificial lights at 150 $\mu\text{mol}/\text{m}^2/\text{s}$ set to 16 h light and 8 h dark period on solid medium [29]. *Arabidopsis* (ecotype Landsberg *erecta*) suspension cells were grown at 22°C in 250 ml flasks containing medium [30]. Soybean (*G. max* cv. Stevens) leaf tissue suspension cells were grown at 28°C in 250 ml flasks containing medium [31].

2.2. Cloning of cDNAs

The following polymerase chain reaction (PCR) primers were used to clone cDNAs: Toc64-I-fwd, ATGGCGACCAATAATGATTTTGG; Toc64-I-rev, TCAAATAAATGCAGCAAGGG; Toc64-III-fwd, ATGGCGTCTCAAGTGGC; Toc64-III-rev, TCACTGGAATTTTCTCAGTC; mtOM64-fwd, ATGTCAATACGCTTCTTTG, mtOM64-rev, TCATATGTGTTTTCGGAGTCTC, Toc159-fwd, ATGGACTCAAAGTCGGTTAC; Toc159-rev, CAACCATAGAGTCTCCTTAC; Toc34-fwd, CAGGTAAGGATTTGTGTCC; Toc34-rev, CAAAACCTCGTGTCCACAAC.

Primers used to clone cDNAs encoding other mitochondrial import components have been previously described [32].

2.3. Purification of plant mitochondria and chloroplasts

Mitochondria were purified from 7 day old soybean cotyledons according to the method of Day et al. [33]. Ten day old pea leaf chloroplasts were isolated following published procedures [34,35]. *Arabidopsis* mitochondria were purified from dark-grown cell culture according to Millar et al. [28].

2.4. Mass spectrometry

Q-TOF MS/MS was performed on an Applied Biosystems Q-STAR Pulsar (Q-TOF MS) using an IonSpray source. Aliquots of 50 μg of mitochondrial protein were acetone-precipitated and the protein pellets air-dried. A digestion solution consisting of 100 mM Tris–HCl pH 8.6 and 50 $\mu\text{g}/\text{ml}$ trypsin was added to a final volume of 45 μl and incubated for 16 h at 37°C. Peptide extracts were bound onto a micro-bore HPLC C18 column (Agilent) and eluted over 6 h with a linear acetonitrile gradient from 2 to 80% (v/v) in H_2O . Mass spectra and collision MS/MS data from elution runs were analysed with Analyst QS and BioAnalyst software (Applied Biosystems, Sydney, Australia).

2.5. Immunodetection of proteins

Mitochondrial proteins (50 μg) and chloroplast proteins (60 μg chlorophyll) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subsequently transferred to a Hybond[®]-C extra nitrocellulose membrane (Amersham Pharmacia Biotech, Sydney, Australia) using a semi-dry blotting apparatus. Chemiluminescence of secondary antibodies conjugated to horseradish peroxidase was detected using a LAS 1000 (Fuji, Tokyo, Japan). Production of antibodies to Toc64 [36], Toc34 [37], TOM20 [38] and uncoupling protein (UCP) [39] has been described previously. Antibodies to the intermembrane space protein cytochrome *c* and matrix-located HSP60 were purchased from StressGen (Victoria, BC, Canada) and BD BioScience (San Diego, CA, USA) respectively.

2.6. Transient expression of green fluorescent protein (GFP) constructs in vivo

Creation of GFP fusion constructs, biolistic transformation of soybean cell suspension cultures and visualisation of GFP were performed as previously described [40]. The sequence corresponding to the alternative oxidase (AOX) presequence, full-length Toc64-I, Toc64-III and mtOM64 was amplified with appropriate restriction sites by PCR using plasmid DNA as templates and primers listed as follows: AOXp(BamHI)-fwd, GCTACGGATCCAACAATGATGATGATGATGAGCC; AOXp(EcoRI)-rev, GTACGAATTCATCTCCACACACCGCC; Toc64-I(BamHI)-fwd, CGTACGGATCCAACAATGGCGACCAATATTGATTTTG; Toc64-I(EcoRI)-rev, GTACGAATTCAATAAATGCAGCAAGGGAATC; Toc64-III(BamHI)-fwd, CGTACGGATCCAACAATGGCGTCTCAAGCTGCG; Toc64-III(EcoRI)-rev, GTACGAATTCATGTGTTTTCGGAGTCTC; mtOM64(BamHI)-fwd, CGTACGGATCCAACAATGTGCAATACGCTTTC; mtOM64 (EcoRI)-rev, GTACGAATTCATGTGTTTTCGGAGTCTC.

2.7. Real-time PCR analysis of transcript abundance

Arabidopsis tissue (4, 6 and 10 day old cotyledons, 4, 6 and 10 day

old roots, 10 day old leaves, flowers and 7 day old suspension cells) was harvested and three independent RNA isolations were carried out using the RNeasy Plant Mini kit (Qiagen, Clifton Hill, Australia). Primers used for real-time PCR analysis were as follows: LC-Toc64-I-fwd, GATGTGGAAGGACGCGTTACC; LC-Toc64-I-rev, GCACTCTATCGAAAGCAATCG; LC-Toc64-III-fwd, GTCTCGCTGGGATTCTTATGC; LC-Toc64-III-rev, CAAATCCAGTCACATACCCTG; LC-mtOM64-fwd, GTCCACAATGGAAGAAGACTC; LC-mtOM64-rev, CAACAGAACTGCTGAGCCAC; LC-Toc159-fwd, CCAACCAACCCCTTCTACGC; LC-Toc159-rev, CACCCACCAAAATTCGGCTTC; LC-Toc34-fwd, GCAGTCCCCACCTGCTACTC; LC-Toc34-rev, GGATGGCCTCAGTCCTTCAG.

Primers used for real-time PCR analysis of other mitochondrial import components have been described previously [32].

3. Results

A proteomic and bioinformatic approach to identify components of the mitochondrial import apparatus confirmed the presence of 17 proteins that we previously predicted to be present based on sequence similarity to components of the yeast mitochondrial import apparatus [19,32]. In this analysis we identified two peptides that were derived from an *Arabidopsis* Toc64 homologue on chromosome 5, At5g09420 (Fig. 1A). This annotation is based on high sequence identity with the previously characterised Toc64 from pea [36]. No other Toc or translocase of the inner envelope membrane of chloroplasts components were identified in our proteomic analysis. We have previously demonstrated that a two Percoll gradient purification of *Arabidopsis* cell culture mitochondria yields an intact mitochondrial fraction with as little as 1–2% plastid contamination, as determined by measuring alkaline pyrophosphatase activity, a marker for plastids [41]. Three genes in *Arabidopsis* display high amino acid sequence identity with the previously identified pea Toc64: Toc64-III (At3g17960) displays the highest identity of 67%, Toc64-V (At5g09420) displays 52% identity, and Toc64-I (At1g08980) displays 50% identity (Fig. 1B). The Toc64-V protein had a predicted molecular mass of 65.7 kDa and hydropathy plot analysis indicated that it has a hydrophobic stretch of approximately 30 amino acids at the N-terminal end. Toc64 from pea is known to be anchored in the membrane by the N-terminal end [36]. A hydrophobic region at the N-terminal end is also predicted in Toc64-III, which has a predicted molecular mass of 64 kDa. Toc64-I differs from the other two Toc64 isoforms in that it contains 450 amino acids, has a predicted molecular mass of 45 kDa, and does not appear to have a hydrophobic region at the N-terminal end (Fig. 1B). Like pea Toc64 and *Arabidopsis* Toc64-III [36], Toc64-V is also predicted to contain three tetratricopeptide (TPR) domains. For clarity we will now refer to *Arabidopsis* Toc64-V as mtOM64 to indicate its mitochondrial location.

We carried out Western blot analysis with antibodies to pea Toc64, pea Toc34 and *Arabidopsis* TOM20 to further investigate the presence of mtOM64 in mitochondria. We used purified pea chloroplasts, pea mitochondria and *Arabidopsis* mitochondria. Antibodies to pea Toc64 yielded a strong band with all three samples (Fig. 2). A strong band was apparent in proteins separated from pea chloroplasts, but a distinct band was also detected in proteins separated from pea and *Arabidopsis* mitochondrial preparations. Immunoreactivity of the same organellar preparations with antibodies raised against Toc34 produced a distinct signal with pea chloroplasts as expected, but yielded a negative result in *Arabidopsis* mitochon-

A

Accession	Matched Peptide	Delta Mass	Mowse Score	Peptide MM
At5g09420	SLIFADDFELSDIPK	0.010	67	1821.93
	ATIQSLLKEDGILVIPTVADPPPR	-0.140	25	2542.32

B

<i>PsToc64</i>	1	~~~~~MRSMAS	PSQ...IMVI	LGGLAGLIV	ETKRTQWK	...EDFGAF	LIDKLLPP	PPAPPKAP	LSSIFAISD	IFDIECVST	FCHPQWVRH	BAASSTSAV	110
<i>AtToc64-III</i>		~~~~~MAS	QANN...LNVL	LGGLAGLIV	ETKRTQWK	...EDFGAF	LIDKLLPP	PPAPPKAP	LGLTFASD	VEDITGVYV	FCHPQWVRH	BAASSTSAV	
<i>AtToc64-V (mtOM64)</i>		MSNTLSLIC	NASPKVWV	ETVTAGTVI	LAETRRIR	ALREDFGAF	TDRELLPP	PPAPPAKOS	LSGLTFISD	APDVKDIFG	FCHPQWVRH	BAASSTSAV	
<i>AtToc64-I</i>		~~~~~	~~~~~	~~~~~	~~~~~	MATNIDFGAF	TDRELLPP	PPAPPAKOS	LSGLTFISD	APDVKDIFG	FCHPQWVRH	BAASSTSAV	

<i>PsToc64</i>	111	SLIFESGATC	IGFTVVDLA	YGISGKNKE	CTPTNPAPN	RVPGSSSGA	AVAVAAVND	FSLGIDTGG	VRVPAGVCG	LGRPSHGAV	SAVGIIPVST	SLDTVGWFAV	220
<i>AtToc64-III</i>		SLIFESGATC	IGFTVVDLA	YGISGKNKE	CTPTNPAPN	RVPGSSSGA	AVAVAAVND	FSLGIDTGG	VRVPAGVCG	LGRPSHGAV	SAVGIIPVST	SLDTVGWFAV	
<i>AtToc64-V (mtOM64)</i>		SLIFESGATC	IGFTVVDLA	YGISGKNKE	CTPTNPAPN	RVPGSSSGA	AVAVAAVND	FSLGIDTGG	VRVPAGVCG	LGRPSHGAV	SAVGIIPVST	SLDTVGWFAV	
<i>AtToc64-I</i>		SLIFESGATC	IGFTVVDLA	YGISGKNKE	CTPTNPAPN	RVPGSSSGA	AVAVAAVND	FSLGIDTGG	VRVPAGVCG	LGRPSHGAV	SAVGIIPVST	SLDTVGWFAV	

<i>PsToc64</i>	221	DPVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	330
<i>AtToc64-III</i>		DPVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	
<i>AtToc64-V (mtOM64)</i>		DPVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	
<i>AtToc64-I</i>		DPVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	

<i>PsToc64</i>	331	EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	440
<i>AtToc64-III</i>		EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	
<i>AtToc64-V (mtOM64)</i>		EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	
<i>AtToc64-I</i>		EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	

<i>PsToc64</i>	441	EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	550
<i>AtToc64-III</i>		EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	
<i>AtToc64-V (mtOM64)</i>		EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	
<i>AtToc64-I</i>		EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	

<i>PsToc64</i>	551	EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	608
<i>AtToc64-III</i>		EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	
<i>AtToc64-V (mtOM64)</i>		EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	
<i>AtToc64-I</i>		EFVTRRVGH	VLIOLPFAV	RNPROHIAA	DCFOLEIVL	DRISQVVRH	APKLE	QKQ	LKHNEED	SAVSSLMAC	SIQKNGVIR	...SSSKL	ANVMOLQRH	

Fig. 1. Proteomic identification of mtOM64 as a mitochondrial protein. A: Peptides of At5g09420 identified by MS/MS analysis of the *Arabidopsis* mitochondrial proteome. Delta mass differences between predicted peptide mass and the experimental mass are reported along with the Mowse score for matching to At5g09420. B: Sequence alignment of pea Toc64 sequence with homologous proteins in *Arabidopsis*. The *Arabidopsis* protein are designated according to their chromosome location. The mtOM64 peptides identified are underlined.

dria (Fig. 2). The very weak signal for Toc34 in pea mitochondria is probably because these mitochondria are only purified through a single Percoll gradient, while the *Arabidopsis* mitochondria are purified through two Percoll gradients. Immuno-

reactivity of the three preparations with an antibody raised to TOM20 from *Arabidopsis* produces a strong band in *Arabidopsis* mitochondria, a distinct but weaker band in pea mitochondria, and no band in pea chloroplasts.

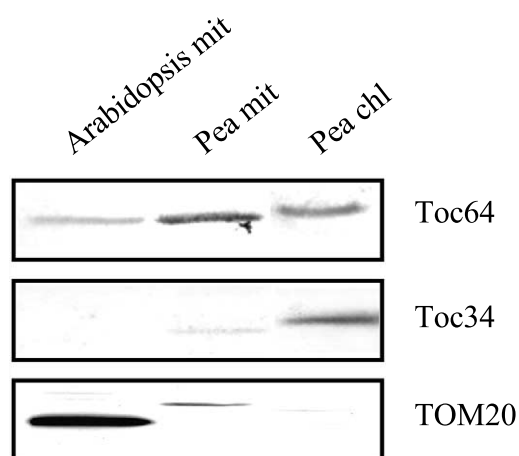


Fig. 2. Western blot analysis of pea and *Arabidopsis* mitochondria and pea chloroplasts. Isolated organelles were solubilised and proteins separated by SDS-PAGE and blotted to supported nitrocellulose and probed with antibodies raised against pea Toc64, pea Toc34 and *Arabidopsis* TOM20.

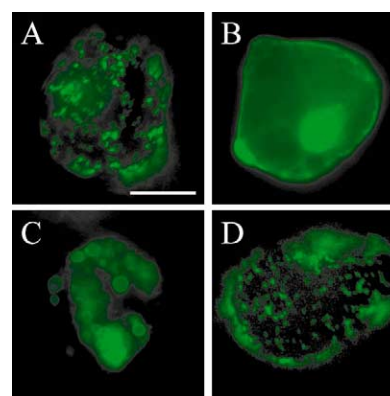


Fig. 3. Fluorescence patterns obtained with GFP attached to the C-terminal end of the coding regions for the three Toc64 homologues in *Arabidopsis*. A: The targeting signal of mitochondrial AOX placed in front of GFP. B: The full coding sequence of Toc64-I placed in front of GFP. C: The full coding sequence of Toc64-III placed in front of GFP. D: The full coding sequence of mtOM64 placed in front of GFP. Scale bar indicates 20 μm.

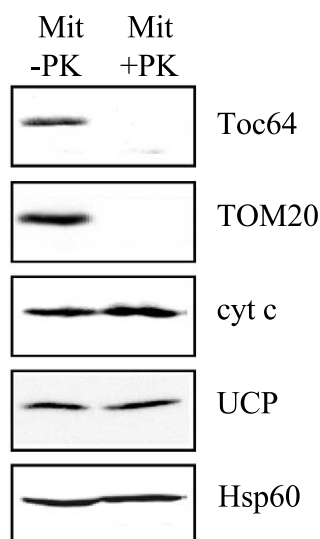


Fig. 4. Protease sensitivity of mtOM64. Western blot analysis of mitochondria treated with protease. Mitochondria were probed with antibodies to pea Toc64 (Toc64), *Arabidopsis* TOM20, cytochrome *c* (cyt *c*), UCP and heat shock protein 60 (Hsp60).

To define the subcellular location of the three Toc64 homologues in *Arabidopsis* we made gene fusions linking *GFP* to the 3' end of the cDNAs. Transient transformation of cultured soybean cells with these chimeric products was performed to determine subcellular localisation. We linked the

targeting signal of soybean AOX to GFP as a control for mitochondrial localisation (Fig. 3A). The subcellular localisation of Toc64-III and mtOM64 GFP chimeric products gave distinct but different fluorescence patterns. The Toc64-III fluorescence appeared in large organelles, 5 μ m in diameter and low in number (Fig. 3C). The mtOM64 fluorescence was observed in numerous small punctate organelles that were similar in distribution and number to those observed when the AOX targeting signal was linked to GFP (Fig. 3D). Toc64-I yielded a different fluorescence pattern and did not appear to be targeted to any organelle, rather it appeared to accumulate in the nucleus (Fig. 3B). On the basis of this result we concluded that mtOM64 was targeted to mitochondria, which is consistent with its detection in the mitochondrial fraction and Western blot analysis, while Toc64-III appears to be targeted to chloroplasts.

We also carried out Western blot analysis on protease-treated mitochondria with antibodies to TOM20 (outer membrane marker protein), cytochrome *c* (intermembrane space marker protein), the uncoupling protein (inner membrane marker protein), Hsp60 (matrix marker protein) and Toc64 (Fig. 4). TOM20 was digested upon protein kinase treatment with intact mitochondria, as was the cross-reacting band with the Toc64 antiserum. However, the intermembrane space, inner membrane and matrix marker proteins were not digested. On the basis of these results we propose that the mtOM64 protein is exposed on the outer mitochondrial membrane.

In order to determine if *mtOM64* displayed a similar ex-

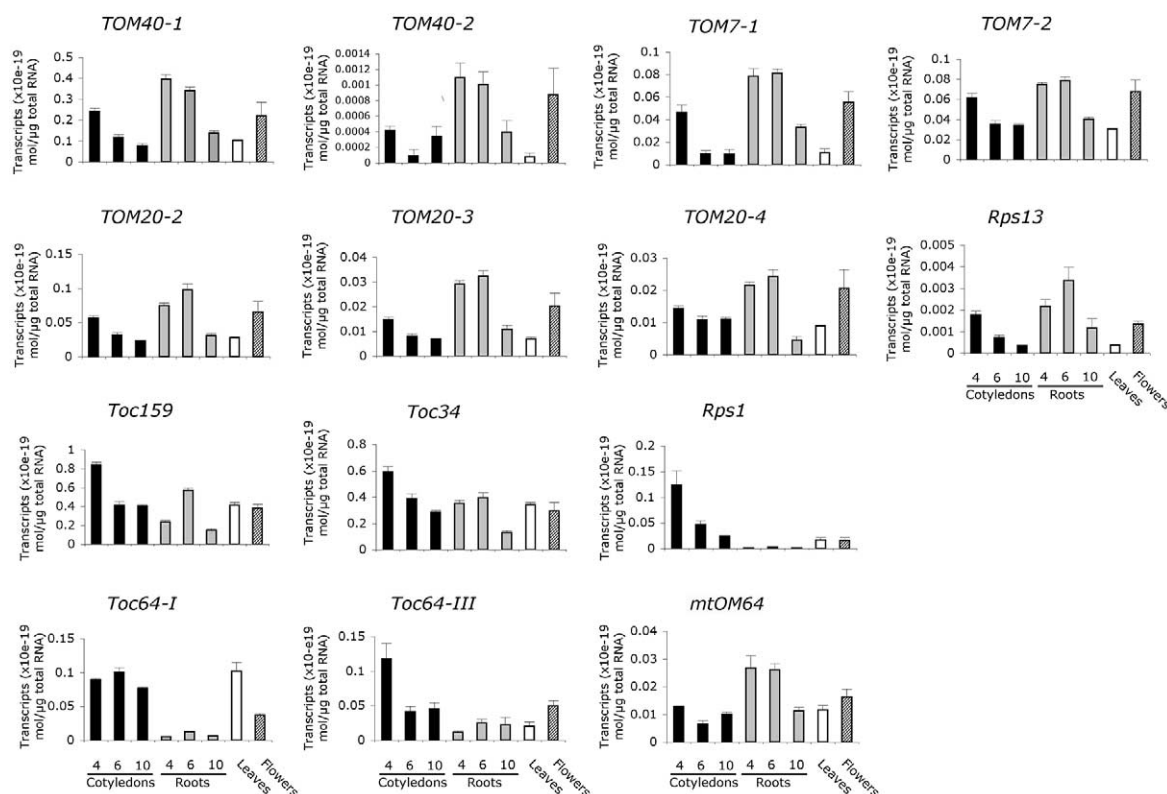


Fig. 5. Expression analysis of *TOM* and *Toc* genes. The expression profiles of *Toc64* homologues in various tissues were determined and compared to those of other import components of the chloroplastic and mitochondrial import apparatus. The expression profiles of *TOM40*, *TOM20* and *TOM7* genes are shown for mitochondria and the expression profiles of *Toc159* and *Toc34* are shown for plastid import components. Additionally the expression profile of a nuclear-encoded mitochondrial ribosomal protein (*Rps13*) and nuclear-encoded plastid ribosomal protein (*Rps1*) was determined.

pression profile to either TOM or Toc components we examined the expression of the three *Toc64* genes from *Arabidopsis* and compared them to the expression of *TOM7*, *TOM20* and *TOM40* which we had previously examined [32]. *Toc64-I* and *Toc64-III* showed high expression levels in green tissues, namely cotyledons and leaves (Fig. 5). In contrast *mtOM64* was most highly expressed in root tissue. Interestingly, *Toc64-I* and *Toc64-III* exhibited similar expression patterns to those measured for the plastid *Toc159* and *Toc34* components and *Rps1*, a nuclear gene encoding a plastid ribosomal protein. In contrast, the *mtOM64* expression profile was similar to those observed for mitochondrial located proteins, in particular *TOM40-2* and *TOM7-1*. Thus the transcript abundance profile of *mtOM64* had a similar expression profile to the mitochondrial rather than the chloroplastic import receptor apparatus.

4. Discussion

A sequence similarity-based view of the plant mitochondrial import apparatus yields an incomplete picture. Many components of the import apparatus are conserved across wide phylogenetic gaps but significant differences still exist between these processes in different eukaryotic lineages [19]. The experimental characterisation of the TOM complex from plant mitochondria has confirmed these differences exist. Within the TOM complex of plant mitochondria the apparent absence of TOM70 based on both sequence similarity and experimental studies has been regarded as a significant difference compared to the mitochondrial import apparatus of yeast, fungal and mammalian systems [18,19]. Furthermore, the essential import component TOM22 is also highly modified in plants, lacking the *cis* receptor domain. These plant-specific changes may be explained by the co-existence of mitochondria and plastids in plant cells [20]. Here we report the presence of a protein on the outer membrane of plant mitochondria, designated *mtOM64*, that displays high amino acid sequence identity to pea *Toc64* [36]. In chloroplasts, this protein is proposed to represent an early docking site for the guidance complex that has been characterised for protein import [36].

Although we detected and demonstrated that *mtOM64* was a mitochondrial protein there is no direct evidence that it plays a role in import of proteins into mitochondria. Attempts to inhibit protein import with antibodies raised to pea *Toc64* have been unsuccessful to date (data not shown). However, this lack of inhibition may be due to the antibody not recognising a critical domain for protein import or binding to it with low affinity and being displaced by precursor protein binding with higher affinity. Alternatively, as observed in yeast, the overlapping specificities of the TOM20 and TOM70 receptors may indicate that even blocking one receptor does not result in a detectable inhibition of import with radiochemical amounts of precursor proteins in vitro [1,3]. *mtOM64* has not been detected in the isolated plant TOM complex [18,19]. Again this may simply represent a technical limitation, as TOM70 is only weakly associated with the TOM complex in yeast, and even *Toc64-III* does not appear to be part of the core Toc complex in plastids, as it is not purified with the 500 kDa complex that contains *Toc159*, *Toc75* and *Toc34* [42,43]. *mtOM64* is the first N-terminally anchored protein to be identified on the plant outer mitochondrial membrane. N-terminally anchored import proteins have

been characterised in yeast and mammalian systems, but the TOM20 receptor in plants appears to be anchored by a C-terminal transmembrane region [14]. Analysis of the N-terminal region of *mtOM64* predicts a highly hydrophobic region in the first 30 amino acids followed by a putative helical region that contains several positive and negative amino acids. Although this structure is similar to what has been defined for targeting TOM20, TOM70 and an outer membrane protein of 45 kDa to yeast mitochondria [44], *mtOM64* was predicted to display significantly higher hydrophobicity in the N-terminal region (data not shown). GFP fusions in which GFP was placed in front of rather than behind the Toc proteins showed no organellar targeting of *mtOM64* (data not shown). This further supports the proposal that the targeting information is located at the N-terminal. In contrast, for *Toc64-I* no targeting to any subcellular structures was observed with GFP fused to either the N- or C-terminus (Fig. 3B, data not shown).

The finding of this protein on the mitochondrial surface further complicates the question of how the specificity of protein import into mitochondria and plastids is maintained. *mtOM64* is similar in sequence to *Toc64-III* and both contain predicted TPR motifs [36]. Several possible roles for *mtOM64* in import, if it is a receptor, can be proposed. First and foremost, it could substitute for the missing TOM70. Alternatively, with the finding that many proteins are dual targeted to mitochondria and plastids [45], it may function as a receptor for a subset of dual localised proteins. Analysis of many dual targeted proteins using targeting prediction programmes predicts them to be mostly plastid targeting [45,46], thus one means to achieve mitochondrial targeting of these proteins could be utilisation of a similar receptor in both locations. Distribution of the proteins between the two organelles may be achieved by regulation of the cytosolic guidance complex through phosphorylation or other as yet unknown mechanisms [47]. A recent proteomic characterisation of the chloroplastic outer envelope reported the presence of several mitochondrial TIM protein isoforms [48]. This suggests that a similar situation could exist in chloroplasts with these TIM-like proteins, which classically constitute part of the protein import apparatus of mitochondria, functioning in chloroplastic protein import. Thus the import machineries of mitochondria and plastids may have interchanged or co-opted members from each other to facilitate dual targeting or simply to co-ordinate targeting in the complex environment of plant cells.

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