Plant mitochondria contain the protein translocase subunits TatB and TatC

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Summary statement
This paper provides evidence that plant mitochondria contain two subunits of a twin arginine translocation pathway indicating the presence of an active Tat pathway.
Abstract
Twin-arginine translocation pathways have been well characterized in bacteria and chloroplasts. However, genes encoding for a TatC protein are found in almost all plant mitochondrial genomes. For the first time it could be demonstrated that this mitochondrial encoded TatC is a functional gene which is translated into a protein in the model plant Arabidopsis thaliana. An inner membrane localized TatB like subunit was also identified, which is nuclear encoded, and is essential for plant growth and development. Indicating that plants potentially require a Tat pathway for mitochondrial biogenesis.

Keywords: mitochondria, protein translocation, molecular evolution, protein import, plant molecular biology
Introduction

The twin-arginine translocation (Tat) pathway differs from most other protein translocating systems in that it transports fully folded proteins (Lee et al., 2006). It is named after its targeting signal which contains a pair of adjacent arginine residues (the twin arginines). Identified in all domains of life Tat pathways play essential roles in a number of different cellular processes including: bacterial pathogen virulence, cell separation, phosphate and iron metabolism, photosynthetic and respiratory metabolism (Berks, 2015, Palmer and Berks, 2012).

The minimal Tat pathway which is found mostly in archaea and gram-positive bacteria consists of two subunits: TatA (possessing one transmembrane helix (TMH)) and TatC (possessing six TMHs) (Barnett et al., 2008). In general, most Tat systems, including the best studied *Escherichia coli* Tat system, contain a second functionally distinct member of the TatA family called TatB (Sargent et al., 1999). Outside of prokaryotes a well-established Tat pathway has also been identified and characterized inside chloroplasts (Robinson and Klosgen, 1994, Cline and Henry, 1996). It is essential for the assembly of photosystem II and the cytochrome b\(_6\)f complex in thylakoid membranes (Molik et al., 2001). Genes for Tat pathway components have also been identified in a number of mitochondrial genomes. Including jakobids, whose mitochondria encode for a minimal Tat system of TatA and C (Jacob et al., 2004). TatC like genes are also found in the mitochondrial genomes of higher plants (Unseld et al., 1997). Despite the conservation of Tat pathways across phyla, it was thought to have been completely lost from all opisthokont lineages which includes Fungi, Metazoa, and relatives. So far only two known exceptions have been described, the choanoflagellate *Monosiga brevicollis*, (Burger et al., 2003) which is interesting as it is thought to be one of the oldest relatives to animals, and the other is a member of the homoscleromorph sponges Oscarellidae, (Wang and Lavrov, 2007) both of which have a mitochondrionally encoded TatC. However, since most mitochondrial genomes only encode a TatC like protein, no function has ever been attributed to these proteins.

Here we present data that demonstrate that in *Arabidopsis thaliana* the mitochondrial encoded TatC is not a pseudogene. It is also demonstrated that Arabidopsis mitochondria contain a second member of a Tat pathway, a TatB like protein which is an essential gene. This mitochondrial TatB like protein has been long sought after and strengthens the evidence that plant mitochondria contain a Tat pathway in contrast to mitochondria from other eukaryotic lineages.
Results

Plant mitochondria contain a TatC protein

It has been known for more than 20 years that the *Arabidopsis thaliana* mitochondrial genome contains a gene encoding for a TatC like protein (although it is also known as either orfX or Mttb) (Sunkel et al., 1994, Unseld et al., 1997). However, no functional data has ever been ascribed to it. One of the main reasons for this is that the mtTatC (mitochondrially encoded TatC) has no classical start codon in Arabidopsis and has been thought of as a pseudogene. There are however several pieces of evidence to suggest that mtTatC is a functional gene that encodes a protein. The mtTatC transcript in Arabidopsis is edited at 36 individual sites (Bentolila et al., 2013). There are also several studies demonstrating that the Arabidopsis mtTatC is an expressed gene (van der Merwe and Dubery, 2007, Gutierrez-Marcos et al., 2007, Sunkel et al., 1994, Wang et al., 2012). To gather further evidence that mtTatC is a functional gene we searched every plant mitochondrial genome in the NCBI organelle genomes database (http://www.ncbi.nlm.nih.gov/genome/organelle/) for the presence or absence of a TatC like gene. In all we searched 124 plant mitochondrial genomes which are displayed phylogenetically in Figure 1A. Out of 124 plant mitochondrial genomes 102 of them contain a TatC gene (Figure 1A) of which 90 contain classical start codons (Figure 1A). These 102 mitochondrial genomes contained all sequenced higher plant species (Figure 1A). The only major difference was observed in the Chlorophyte lineages (green algae) which is split in two. One half containing mainly the class Chlorophyceae and having no TatC gene and the other half containing mainly the class Trebouxiophyceae which contain a TatC gene (Figure 1A). What can be determined is that the majority of plant mitochondrial genomes contain a TatC gene with a classical start codon. We believe that this conservation of a mitochondrial TatC must mean it has some function within plant mitochondria otherwise like almost all animal, fungi and Chlorophyceae mitochondrial genomes it would have been lost. Therefore, we first aimed to prove that the Arabidopsis mtTatC gene is translated into a protein.

To this end we raised an antibody against the peptide VREEGWTSGMRESGIEKKNKSSPPPRTW which corresponds to amino acids 253 to 281 of the Arabidopsis mtTatC. The collected whole serum was then affinity purified against the peptide to obtain a purified antibody. This antibody detected a band of approximately 27 kDa in isolated Arabidopsis mitochondria that was not recognized by the preimmune serum (Figure 1B). To confirm the specificity of the antibody we pre-incubated it with the peptide which abrogated binding of the 27 kDa protein (Figure 1B). While the protein size of 27 kDa
is slightly lower than the predicted size of 33 kDa it is a well-known phenomenon that TatC and other hydrophobic proteins run at different molecular masses than predicted (Jakob et al., 2009, Mori et al., 2001). From these results it can be concluded that the antibody is specific for the Arabidopsis mtTatC and that it is in fact a functional gene.

_The Arabidopsis genome encodes a second TatB like protein_

Since all known Tat pathways contain at least TatC and TatA but the majority contain TatA, TatB and TatC subunits, for plant mitochondria to have a functional Tat pathway other subunits are required. To rectify this situation, we sought to identify any possible TatA or TatB encoding genes in the Arabidopsis genome which may be targeted to mitochondria. We first excluded the chloroplast targeted cpTatA and cpTatB as being dual targeted to mitochondria, by using antibodies to the _Pisum sativum_ (pea) chloroplast TatA (Mori et al., 1999) and TatB (Mori et al., 2001) proteins on isolated chloroplastic and mitochondrial fractions from pea (Figure 1C). As expected cpTatA and cpTatB were only detected in the chloroplastic fractions (Figure 1C). BLAST (Altschul et al., 1990) searches of the Arabidopsis genome using either _E. coli_ TatA or TatB sequences, the Arabidopsis chloroplastic targeted TatA or B sequences, or the mitochondrial encoded sequences of TatA from jakobids were also performed. The only TatA or TatB like sequences recovered by this approach were the known chloroplast Tat subunits.

Therefore, we tried another approach and used the program Phyre2 in the back phyre mode (Kelley et al., 2015). This utilizes the known structures of proteins to search genomes for similar proteins. So we used the known structures of the _E. coli_ TatA (PDB: 2NM7) (Zhang et al., 2014b) and TatB (2MI2) (Zhang et al., 2014c) to search the Arabidopsis nuclear genome (Supplementary Table 1). While both the known cpTatA and cpTatB proteins were identified with high confidence scores (Phyre2 confidence scores are a representation of the probability that the match between the sequence and the template is a true homology) a third unknown protein was also identified with similar confidence scores with the locus At5g43680 (Supplementary Table 1). In fact, At5g43680 came back as the highest ranked protein when using the structure for EcTatB as the search reference.

Using the protein sequence of At5g43680 we performed BLAST searches of higher plant genomes. In all cases a protein similar to At5g43680 could be identified. To try and confirm that what we had identified was a TatB or TatA subunit, phylogenetic analysis was utilized using TatA and TatB subunits from a variety of bacterial and jakobid species along with the chloroplastic TatA and TatB subunits from plant species (Figure 2). We observed
that the At5g43680 and related proteins grouped most closely with the TatB proteins from bacteria indicating that At5g43680 is a possible TatB like protein (Figure 2). Although some of the bootstrap values are low we believe the tree is accurate. We also tested Bayesian homology using MrBayes (Ronquist et al., 2012) and an almost identical tree was obtained (data not shown). Secondly when BLAST searches excluding plant species with At5g43680 were carried out the only hits were bacterial TatB proteins (data not shown). Therefore, we believe that At5g43680 is a TatB like protein. This is also supported by basic sequence comparisons as At5g43680 shows a 14% identity and 29% similarity to EcTatB in comparison to 10% identity and 19% similarity to EcTatA. Furthermore, a sequence alignment between EcTatB and At5g43680 showed that it contains the conserved glutamate residue at amino acid position 8 at the start of the transmembrane helix (TMH) and also the invariant glycine between the TMH and the second alpha helix (APH) (Figure 3A). One major difference however is the length, At5g43680 is 61 amino acids longer than EcTatB. This difference is found predominantly in the C-terminus.

To try and get a better understanding of the possible function of At5g43680 we built a model of its 3D structure. This was carried out using the Phyre2 in the intensive mode which returned a model fitting the identified structure of EcTatB (Figure 3B) (Zhang et al., 2014c, Kelley et al., 2015). The model is based on the first 104 amino acids of EcTatB and the first 125 amino acids of At5g43680 as the C-termini of both proteins are thought to be unstructured. EcTatB contains four alpha helices which adopt an L-shape conformation (Figure 3B) (Zhang et al., 2014c). The model we obtained for At5g43680 has an almost identical structure containing four alpha helices in an L-shape conformation, further supporting the phylogenetic analysis that it is a TatB like protein. To determine the quality of our model we calculated an overall RMSD of 1.866 and a Q-score of 0.501 when compared to EcTatB using Chimera (Pettersen et al., 2004). This indicates that our model for At5g43880 is very close the structure of EcTatB also evidenced by the superimposed structures in Figure 3B. All this data indicate that At5g43680 is most likely a TatB like protein.

**At5g43680 is an inner mitochondrial membrane protein**

As yet little or no data is available about the localization of At5g43680. We only know that several peptides of this protein have been identified in whole protein fractions in the pep2pro proteomics database (Baerenfaller et al., 2011), indicating that it is an expressed protein. To determine the subcellular localization of At5g43680 we first performed **in vivo**
GFP targeting analysis. Firstly, we cloned the full length coding sequence for At5g43680 in frame with a C-terminally located GFP tag. Tobacco leaves were infiltrated with agrobacterium carrying the At5g43680-GFP construct. Analysis of the GFP expression of protoplasts isolated from the transformed tobacco leaves displayed a GFP signal which overlapped with mitochondria as visualized using mitotracker (Figure 3C). Since At5g43680 colocalized with mitochondria we decided to rename it as AtmtTatB.

To test the AtmtTatB-GFP localization results, in vitro import assays into isolated Arabidopsis mitochondria using radio-labeled precursor proteins were performed. When AtmtTatB was incubated with isolated mitochondria a protease resistant product was observed after incubation with proteinase K of the same size as the precursor protein (Figure 4A, Lanes 1-3), indicating that AtmtTatB is imported into mitochondria but does not contain a cleavable presequence. Pretreatment of the mitochondria with the ionophore valinomycin prior to import abolished the appearance of the protease resistant band of AtmtTatB (Figure 4A, Lanes 4 and 5). Valinomycin dissipates the mitochondrial membrane potential which is required for the import of proteins into or across the inner membrane, indicating that AtmtTatB is imported into the matrix or inner membrane of plant mitochondria. When the outer membrane was removed prior to the addition of proteinase K, AtmtTatB remained protease resistant indicating that if it is located in the inner mitochondrial membrane the majority is facing towards the matrix (Figure 4A, Lanes 6-9). Finally, the addition of 1% Triton X-100 prior to protease treatment demonstrated that when total mitochondria were ruptured AtmtTatB is accessible to the added protease (Figure 4A, Lane 10). The Glycine max AOX (Alternative oxidase 1a) and Arabidopsis Tim23 (Translocase of the inner membrane protein of 23 kDa) proteins were used as controls in the import assays as both are associated with the mitochondrial inner membrane. AOX was imported in a membrane potential dependent manner and processed to a 32 kDa mature protein which was protease resistant even when the outer membrane was ruptured (Figure 4A, Lanes 1-9). Tim23 was also imported in a membrane potential dependent manner and was shown to be protease resistant in intact mitochondria but produced its characteristic smaller membrane protected fragment after outer membrane rupture and protease treatment (Figure 4A). Both proteins were also fully digested by protease K when the mitochondria were ruptured by 1% Triton X-100 treatment (Figure 4A, Lane 10). These experiments demonstrated that the AtmtTatB is targeted to and imported into mitochondria and is most likely located either within the mitochondrial inner membrane or mitochondrial matrix.
To further test the mitochondrial location of AtmtTatB we raised an antibody against the full length protein. Immuno blotting against Arabidopsis mitochondria using the purified AtmtTatB antibody detected a band of 30 kDa that was absent when the preimmune serum was used (Figure 4B). Incubation of the antibody with the antigen prior to western blotting abrogated binding of the antibody to this 30 kDa protein indicating that the antibody is specific (Figure 4B). Using the antibody against mitochondria and ruptured mitochondria treated with proteinase K indicated that AtmtTatB is resistant to protease treatment even when the outer membrane is ruptured (Figure 4C). Supporting the import results that AtmtTatB is located either in the inner mitochondrial membrane or the matrix. To verify AtmtTatB is located in the membrane, mitochondrial membrane fractions were extracted with carbonate. Like the membrane control CoxII, AtmtTatB was located in the pellet membrane fraction as opposed to the soluble fraction which contained subunit H of the glycine decarboxylase (Figure 4D). Putting these results together we could demonstrate that AtmtTatB is located within the inner mitochondrial membrane most likely with its C-terminus facing the matrix. A similar orientation of the EcTatB protein within the cytoplasmic membrane has been described previously (Koch et al., 2012).

AtmtTatB is an essential gene

Since AtmtTatB is nuclear encoded it is possible to test T-DNA insertion lines for its physiological role to be inferred. We identified two potential insertion lines in CSHL_GT11254 and SALK_003481 and characterized them. The T-DNA insertion for CSHL_GT11254 was found to be directly downstream of the ATG start codon and the T-DNA insertion for SALK_003481 was found to be located in exon five (Figure 5). We attempted to identify homozygous plants using PCR, however for both lines we never obtained homozygous plants, only heterozygous and wild-type plants were found. When we examined the siliques of self-fertilized heterozygous plants we observed that one quarter of all embryos were aborted (displayed by the white embryos) (Figure 5). This corroborates the observation that offspring of heterozygous plants produced heterozygous to wild type ratios of 2:1 (Figure 5). To confirm these results, we PCR screened the progeny of a self-fertilized heterozygous plants. After screening 140 plants from the line CSHL_GT11254 we obtained 95 heterozygous plants and 45 wildtype plants which gives a ratio of 2.1:1. After screening 157 plants from the line SALK_003481 we obtained 106 heterozygous plants and 51 wildtype plants which gives a ratio of 2.1:1. These results are almost identical to the predicted results of a heterozygous to wildtype ratio of 2:1 as predicted by Mendelian
Mitochondrial TatC and TatB proteins are located in the same 1500 kDa complex

In other organisms the TatB and TatC subunits are normally found in a stable complex termed the TatBC complex (Cline and Mori, 2001, Bolhuis et al., 2001). To determine if the mtTatB and mtTatC proteins are in a stable complex of similar molecular weights we used two dimensional BN-SDS-PAGE followed by immuno blotting. Using the controls of Tom40 from the TOM complex (300 kDa), COXII from complex IV (240 kDa) and Qcr7 from complex III (500 and 1500 kDa), we could determine that mtTatB is located in one complex with the molecular weight of 1500 kDa and mtTatC was located in complexes of molecular weights of 1500 kDa and 150 kDa (Figure 6). As the supercomplex of complexes I + III also runs at 1500 kDa it could be interpreted that mtTatB and C are part of those complexes. However extensive work on identifying the subunits of plant mitochondrial complexes I + III has never identified either mtTatB or C (Meyer et al., 2008, Peters et al., 2013, Klodmann and Braun, 2011, Klodmann et al., 2010), which means it is unlikely they are part of those complexes. The fact that both mtTatB and mtTatC are both found in a high molecular weight complex of the same size is a good indication that they form a stable complex together in a similar manner to that of other organisms.

Mitochondrial TatC and TatB proteins are associated with a lack of Bcs1

In yeast and mammalian mitochondria the protein Bcs1 is responsible for the insertion of the Rieske Fe/S (Rip) protein into complex III, (Wagener et al., 2011) which has also been hypothesized as a substrate for a potential mitochondrial Tat pathway (Hinsley et al., 2001, Pett and Lavrov, 2013). By sorting a selection of organisms based on their mitochondrial evolution in the same manner as before by using the protein sequences of COX1 and COB and then over laying this with first the structural arrangement of that organisms Bcs1 and then also including whether or not that organism contains a mitochondrial TatC and a mitochondrial TatB like protein (Figure 7). It can be observed that the majority of organisms which contain a complete Bcs1 like protein which encompasses both the Bcs1 and AAA ATPase domains, do not contain either a mitochondrial encoded TatC gene or mitochondrial TatB like gene. The opposite can then also be observed that the majority of organisms which encode for a mitochondrial TatC and TatB like genes do not contain a protein with a Bcs1 domain but only contain the AAA ATPase domain. This is in agreement with what was...
previously reported (Pett and Lavrov, 2013). The only two exceptions being *Naegleria gruberi* and *Chlamydomonas reinhardtii*. This observation indicates that organisms that have evolved a complete Bcs1 protein, i.e. the AAA ATPase and Bcs1 domains have been able to lose their mitochondrial Tat pathway as it is no longer required for Rip insertion. On the other side organisms which do not contain a complete Bcs1 protein have therefore retained their mitochondrial Tat pathway, potentially required for Rip insertion. A similar theory was also proposed by (Pett and Lavrov, 2013).

The Arabidopsis Rip protein requires a Tat signal and a ΔpH membrane potential for proper assembly

It has been previously demonstrated that some mitochondrial Rip proteins contain Tat-like targeting signals immediately preceding the transmembrane domain (Hinsley et al., 2001, Pett and Lavrov, 2013). To test if the Tat-like targeting signal is functional we analyzed the import and assembly of a mutated Rip protein. We mutated the potential Arabidopsis Rip Tat signal of KR to QQ, glutamines were chosen because substitutions with glutamines had been previously shown to completely abolish Tat pathway export in bacteria (Kreutzenbeck et al., 2007). It was observed using *in vitro* import assays into isolated Arabidopsis mitochondria that the Rip-KR imports and assembles into complex III and the super complex of I + III very efficiently as seen by the increase in radioactive signal over time (Figure 8A). However, in contrast the Rip-QQ displays a far weaker signal in the complexes indicating that it is not assembled efficiently in Arabidopsis mitochondria (Figure 8A). Interestingly we noticed that in the Rip-QQ samples the signal peaked at 60 mins and subsequently decreased in the further time points while Rip-KR continued to increase (Figure 8A). This suggests that exchanging the Rip KR Tat signal to QQ disrupts the stable assembly into complex III. In order to test if both the Rip-KR and Rip-QQ proteins are imported and stable within Arabidopsis mitochondria we repeated the experiment but used SDS-PAGE instead of BN-PAGE. Both proteins were imported and processed to the mature form in a time dependent manner with similar efficiencies (Figure 8B). Importantly we also checked that both Rip-KR and Rip-QQ were inserted into the inner membrane. This was confirmed by extracting the membranes using 0.1 M Na₂CO₃ (Supplementary Figure 1). Both Rip-KR and Rip-QQ were located within the membrane fractions (Supplementary Figure 1). From these results we conclude that the Rip-QQ was imported and stable within Arabidopsis mitochondria but cannot be efficiently assembled into complex III, indicating that the insertion of the Rip protein into complex III requires a functional Tat pathway.
To further investigate whether the Arabidopsis mitochondrial Rip protein is inserted by a Tat pathway, we analyzed the assembly in the presence of nigericin. Nigericin is an ionophore which dissipates the ΔpH of the membrane potential but does not affect the electrical component of the membrane potential (Yuan and Cline, 1994). Import and assembly of mitochondrial proteins has been previously demonstrated to rely heavily on the electrical component of the membrane potential and not the ΔpH (Martin et al., 1991, Pfanner and Neupert, 1985). Therefore, we first incubated wildtype Rip-KR with mitochondria under normal import conditions for a pulse time of 7.5 minutes. After the pulse period the mitochondria were re-isolated and washed to remove un-imported protein. The reaction was then split into two, one containing normal import buffer and the other containing in addition to normal import buffer, 2 µM nigericin. Assembly into complex III was analyzed by BN-PAGE (Figure 8C). Only the reactions not containing nigericin showed a clear assembly of Rip-KR into complex III and the super complex of I+III indicated by the increase in radioactive signal over time (Figure 8C). The reactions incubated in the presence of nigericin did not show an increase in complex III and super complex I+III intensity (Figure 8C). The fact that Arabidopsis Rip requires the ΔpH is another indication that it is inserted into complex III by a Tat pathway. As originally the Tat pathway was called the ΔpH pathway as many of its substrates in both bacteria and chloroplasts are inhibited by nigericin in a similar manner as what was seen for the Arabidopsis chloroplast Rip protein (Molik et al., 2001).

Discussion

Since the first plant mitochondrial genome sequences were obtained it has been hypothesized that plant mitochondria contained a Tat pathway. However only ever one subunit, TatC was ever identified and was mostly thought of as a pseudogene. Especially in Arabidopsis as the mtTatC did not contain a classical start codon. But even with a functional TatC gene plant mitochondria were still missing other subunits to complete a functional Tat pathway.

The identification of a plant mitochondrial TatB like protein opens up a new whole field of research in plant mitochondrial biology. Interestingly mtTatB lacks a cleavable transit peptide but is still correctly targeted to the mitochondria. Most likely this would mean that mtTatB is imported by the carrier import pathway. The carrier import pathway is specialized in the import of inner membrane proteins the majority of which lack cleavable presequences (Sirrenberg et al., 1996). Targeting signals of the carrier import pathway a thought to be
mediated by the transmembrane domains of substrate proteins (Endres et al., 1999). Therefore, the mitochondrial targeting signal for mtTatB is most likely its N-terminal transmembrane domain. Several questions however, still require answering such as: where is mtTatA, does the mitochondria Tat pathway require a TatA subunit, why are mtTatB proteins much longer than other TatB proteins, what are the substrates for the mitochondrial Tat pathway and why have plant mitochondria retained a Tat pathway while other eukaryotes have replaced it?

To answer the first question, as yet we could not identify any protein within Arabidopsis which is an obvious target for being a mtTatA protein. This may be related to the second question of why mtTatB is much longer than other TatB proteins. It may be possible that mtTatB has gained some sort of dual functionality and performs both the roles of TatA and TatB. This though will require extensive biochemical testing to confirm. So far in our hands attempts to try and complement *E. coli* Tat mutants with the mitochondrial subunits have not worked. Attempts to also over express full length mtTatC in *E. coli* have also failed therefore limiting any *in vitro* reconstitution assays. As for the substrates of the mitochondrial Tat pathway, this study has identified that the Rip protein from complex III as a good candidate. Rip is an interesting candidate as in the mitochondria of yeast and humans it undergoes a unique import and assembly process. Firstly, Rip is synthesized on cytosolic ribosomes and imported in a post translational manner through the outer and inner membrane by the general import pathway utilizing the translocation complexes TOM and the TIM17:23 (Hartl et al., 1986). Secondly, after reaching the matrix Rip has its targeting signal removed in two steps. Thirdly, Rip then has its iron sulfur cluster inserted and the C-terminus is fully folded (Kispal et al., 1997, Kispal et al., 1999). This is all thought to happen in the matrix and at this stage Rip is found as a soluble intermediate (Hartl et al., 1986). Fourthly, Rip is chaperoned by the protein Mzm1 to Bcs1 in the inner membrane where Rip is then inserted into the membrane and the fully folded C-terminus is passed back through the inner membrane to the intermembrane space (Cui et al., 2012, Wagener et al., 2011). Fifthly, Rip is the last protein inserted into the so-called pre-Bc1 complex forming the complete and functional complex (Wagener et al., 2011).

Up until recently it was also thought that in plant mitochondria a similar pathway existed. However as stated before the closest related protein to human or yeast Bcs1 in plant mitochondria is located in the outer membrane and plays no role in the assembly of the bc1 complex (Zhang et al., 2014a). As for Rip assembly in plant mitochondria it is most probable that of the five steps outlined before the first four are exactly the same. Arabidopsis Rip is a
nuclear encoded gene, which its protein product is synthesized in the cytosol and post translationally imported into mitochondria. Similar to yeast AtRip contains a cleavable presequence and one transmembrane domain therefore most likely also uses the TOM complex and TIM17:23 complexes for translocation through the outer and inner membranes. The plant mitochondrial matrix also contains all the required proteins for iron sulfur biogenesis and also contains a homolog of Mzm1 (Balk and Pilon, 2011). So plant mitochondrial Rip probably also must have a soluble intermediate in the matrix while its iron sulfur cluster is assembled. Therefore, the only divergent part in Rip assembly in plant mitochondria is the use of a Tat pathway for membrane insertion and passing the fully folded C-terminus back into the intermembrane space. This is fully consistent with our observations that mutation of the AtRip Tat targeting signal and also that AtRip requires a ΔpH for assembly. Rip proteins of chloroplasts and bacteria have also been demonstrated previously as requiring a Tat pathway for proper insertion (Molik et al., 2001, Aldridge et al., 2008, Bachmann et al., 2006, De Buck et al., 2007). These observations are strengthened by the correlation of mitochondrial Tat proteins with the absence of a complete Bcs1 proteins in a variety of organisms.

It could be argued that AtRip could also be inserted back into the inner membrane by way of the Oxa pathway however this seems unlikely as the Oxa pathway has never been demonstrated to translocate fully folded proteins. This can also be countered by the fact that yeast and humans do not use Oxa for Rip assembly. If this was possible why have Bcs1? It may also be argued that AtRip could be laterally inserted from the TIM17:23 complex into the inner membrane. In this case AtRip would not have a soluble intermediate and the C-terminus would never reach the matrix. The problem here is that all the iron sulfur cluster biogenesis machinery and also the Mzm1 chaperone which is specific for Rip are located within the mitochondrial matrix. Therefore, if the C-terminal domain of AtRip never gets to the matrix there is no known mechanism to insert its iron sulfur cluster. Therefore, after excluding all other possibilities and looking at the data presented in this paper it is most likely that AtRip uses a Tat pathway for assembly into complex III.

The data presented here demonstrate that plant mitochondria contain the translocation subunits TatB and TatC. Since the TatB subunit is an essential gene it can be extrapolated that this potential Tat pathway is required for plant mitochondrial biogenesis. However, further work is required to determine if this potential mitochondrial Tat pathway requires a TatA subunit or somehow functions with only TatB and TatC subunits. We have also sought to demonstrate that the AtRip protein is a substrate of this potential mitochondrial Tat
pathway. AtRip requires the ΔpH and a Tat like targeting signal for proper assembly into complex III. Functional analysis of viable mtTatB mutants (e.g. RNAi or antisense knockdowns) will prove invaluable in identifying more possible substrates and also the functional role of the potential plant mitochondrial Tat pathway.

As to why plant mitochondria have retained a Tat pathway compared to other eukaryotes is a difficult question to answer. The most obvious explanation is that Bcs1 evolved in eukaryotes later after the split of plants from other eukaryotes. There is some evidence to support this with the base animal Monosiga brevicollis containing both mitochondrial TatC and TatB like proteins and also lacking a complete Bcs1 protein, indicating the mitochondrial Tat pathway was lost rather late in opisthokont evolution. It is also interesting to note that a selection of related green algae also appears to lack both a complete Bcs1 and mitochondrial Tat subunits (e.g. Chlamydomonas reinhardtii). How these organisms assemble their mitochondrial Rip is another interesting question.

Materials and methods

GFP subcellular localization

The Agrobacterium tumefaciens strain AGL1 was transformed with the full coding sequence of AtmtTatB (At5g43680) fused to GFP in the vector pK7FWG2 (Karimi et al., 2002) and used to infiltrate 4 - 6 week old Nicotiana benthamiana leaves as described previously (Schweiger et al., 2012). Protoplasts were prepared as outlined in (Koop et al., 1996) except cell walls were digested for 90 mins at 40 rpm in 1% cellulase R10 and 0.3% macerase R10 after vacuum infiltration. Mitotracker (Life Technologies) was added to the protoplast suspensions to a final concentration of 500 nM. Fluorescence was observed with a confocal laser scanning microscope at 20°C (Leica, Type: TCS SP5).

T-DNA insertion lines

The following T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Centre (NASC) and Cold Spring Harbor Laboratory (CSHL) respectively: SALK_003481 and CSHL_GT11254 (Alonso et al., 2003, Sundaresan et al., 1995). T-DNA insertions were genotyped by PCR and insertion sites were confirmed by sequencing.
Mitochondrial isolation

Mitochondria for in vitro import and assays and Western blot analysis were harvested from 14 day old Arabidopsis thaliana seedlings grown in liquid culture as previously described (Lister et al., 2007). Typically between 2 - 4 mg of mitochondrial protein was obtained from each preparation.

In vitro import studies

For in vitro import studies the full coding sequence of AtmtTatB (AT5G43680) was cloned into the destination vector pDest14 (Invitrogen). The clones for AOX (X68702), Tim23 (At1g72750) and Rip (At5g13430) have been described previously (Murcha et al., 2003, Whelan et al., 1995, Carrie et al., 2015). [35S]Met-labeled precursor proteins were synthesized using the Flexi Rabbit Reticulocyte Lysate (Promega) as previously outlined (Chang et al., 2014). In vitro mitochondrial imports were then performed using isolated mitochondria as previously described in (Whelan et al., 1995, Lister et al., 2007). All in vitro imports were obtained using radiography and images were scanned using a Typhoon scanner (GE Healthcare).

Mitochondrial fractionation

Outer membrane-ruptured mitochondria were prepared by osmotic shock as previously described (Murcha et al., 2005). Carbonate extraction of mitochondrial membrane proteins was carried out as previously described (Tan et al., 2012).

BN-PAGE and immunoblotting

BN-PAGE was performed as in (Eubel and Millar, 2009) using 5% digitonin (Serva). Immunodetection of proteins was performed as described previously (Murcha et al., 2005). Unless specified the equivalent of 50 µg of protein was used in each lane. For the production of the antibody against AtmtTatC the peptide VREEGWTSGMRESGIEKKNKSSPPPRTW which corresponds to amino acids 253 to 281 of the Arabidopsis TatC was produced by Ganaxxon bioscience (Germany) and injected into 2 New Zealand white rabbits as per standard protocols (Cooper and Paterson, 2008). For the antibody against AtmtTatB the full protein sequence of AtmtTatB was expressed in E. coli fused to a six his tag. The protein was purified using denatured IMAC followed by electro-elution. The final protein was concentrated to approximately 2 mg/ml and then sent to Pineda Antikörper-service.
(Germany) for antibody production. Both antibodies were affinity purified using their respective antigens. The antibodies CoxII (A S04 052) and GDC-H (A S05 074) were purchased from Agrisera (Sweden). Antibodies against Tom40 and Qcr7 have been previously published (Kuhn et al., 2011, Carrie et al., 2009).

**Phylogenetic analysis**

For the phylogenetic tree in Figure 1 the protein sequences of Cytochrome c 1 (COX1) and Cytochrome b (COB) were concatenated prior to analysis. Protein sequences were obtained from the NCBI organelle genomes database. The protein sequences were first aligned using MEGA5 (Tamura et al., 2011) with the Muscle algorithm (Edgar, 2004). The evolutionary history was then inferred using Maximum Likelihood method based on the Whelan and Goldman model (Whelan and Goldman, 2001). The bootstrap consensus tree was inferred from 1000 replicates with the percentage of replicate trees in which the associated proteins clustered together in the bootstrap test shown next to branches. For the phylogenetic tree of the TatA and B sequences in Figure 2 the process was exactly the same. For the phylogenetic tree in Figure 6 the procedure and use of COX1 and COB sequences was exactly the same. For species and sequence information see Supplementary Table 2 for all phylogenetic trees.
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Competing interests

None

Author contributions

CC carried out all experimental work. SW carried out the modeling of mtTatB. Both CC and JS planned and designed all experiments and all authors co-wrote the manuscript.
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Figure 1. Presence or absence of TatC like genes in plant mitochondrial genomes and confirmation in Arabidopsis thaliana that TatC is an expressed protein. A) The evolutionary history of plant mitochondria was inferred using the Maximum Likelihood method on concatenated protein sequences of Cytochrome c oxidase I (Cox1) and Cytochrome b (COB). Displayed is the consensus tree and bootstrap values after 1000 replicates. Blue dots represent TatC genes with a classical start codons and red dots represent TatC genes without a classical start codon. B) Immunoblotting of Arabidopsis mitochondria separated by SDS-PAGE labeled with either pre-immune or purified antibody raised against AtmtTatC. Labeling of the TatC protein was abrogated by pre-incubation of the antibody with the TatC peptide. C) Western blot analysis of mitochondrial and chloroplast fractions from pea. Antibodies to CpTatA and cpTatB were tested for mitochondrial localization. Controls for mitochondria were the Voltage dependent anion channel protein, VDAC, the Rieske iron sulfur cluster protein from complex III, Rip and the translocase of the outer membrane protein of 40 kDa, Tom40. To control for chloroplasts, the light harvesting II complex protein was used, LHCII.
Figure 2. Phylogenetic analysis of At5g43680. The evolutionary history of TatA, TatB and At5g43680 like proteins was inferred using the maximum Likelihood method. Displayed is a bootstrap consensus tree inferred from 100 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Red text indicate the proteins identified by using At5g43680 in BLAST searches and are annotated as TatAB as it is unknown whether they are TatA or TatB proteins. Purple text indicate bacterial TatB proteins. Brown text indicates bacterial TatA proteins. Light blue text indicates TatA proteins from the mitochondrial genomes of jakobids. Green text indicates TatA and TatB proteins which are located in chloroplasts or are from cyanobacteria.
See Supplementary Table 2 for accession numbers of protein sequences used and also for species names abbreviations.
Figure 3. Multiple sequence alignment between At5g43680 and EcTatB and a structural model for At5g43680 and GFP localization. A) Sequence alignment of the EcoTatB and At5g43680. Indicated are the transmembrane domains and aliphatic alpha helices along with the conserved Glutamate at residue 8 and the conserved Glycine at the end of the transmembrane domain. Numbers indicate the amino acid number. Black amino acids are identical and grey amino acids are similar. B) Comparison of the structure of the *E. coli* TatB protein (EcoTatB) and the model for At5g43680 protein from Arabidopsis. Indicated are the transmembrane domains (TMH) and the three aliphatic alpha helices (APH, α3 and α4). C) Protoplasts of transformed tobacco leaves expressing At5g43680 tagged to the N-terminus of GFP monitored by confocal laser scanning microscopy. Also shown are mitotracker false colored purple for mitochondrial localization and the chlorophyll auto-fluorescence (red channel) indicating the location of the chloroplasts.
Figure 4. At5g43680 is an essential inner mitochondrial membrane localized TatB protein. A) *In vitro* import of radio labeled AtmtTatB into isolated mitochondria. The control proteins AOX and Tim23 are also shown. Lanes contain: 1 precursor protein alone, 2 precursor protein incubated with mitochondria under conditions that support import into mitochondria, 3 as lane 2 but with Proteinase K added after incubation of the precursor with mitochondria, 4 and 5 as lanes 2 and 3 but with valinomycin added to the import assay before the addition of precursor protein, lanes 6-9 as lanes 2-5 except that the mitochondrial outer membrane was ruptured after the incubation period with precursor protein but before the addition of Proteinase K, 10 is the same as lane 3 except mitochondria were first treated with Triton X-100 before Proteinase K treatment. Mit: mitochondria, Mit*OM: mitochondria with the outer membrane ruptured, Pk: Proteinase K, Val: valinomycin, Tx100: Triton X100, p: precursor protein band, m: mature protein band, m*: inner membrane protected fragment of Tim23.B) Immuno blotting of Arabidopsis mitochondria separated by SDS-PAGE labeled with either pre-immune serum or the antibody raised against AtmtTatB. Labeling of the AtmtTatB protein was abrogated by pre-incubation of the antibody with the AtmtTatB antigen used in antibody production. C) Sub-mitochondrial localization of AtmtTatB analyzed by protease protection. Proteinase K (Pk) was applied to mitochondria (Mit) or
mitoplasts (Mit*OM, hypertonically swollen mitochondria). Antibodies to the translocase of the outer membrane of 20 kDa protein (Tom20), Cytochrome c oxidase subunit II (CoxII) and the H protein of glycine decarboxylase (GDC-H) were used as controls. D) Membrane association of AtmtTatB. Mitochondria were subjected to carbonate extraction using Na$_2$CO$_3$ (pH 11) and separated into membrane (M) and soluble (S) fractions. Again CoxII and GDC-H were used as membrane and soluble controls respectively.
**Figure 5. mtTatB is an essential gene.** Gene structure of AtmtTatB displaying the locations of the T-DNA insertions and pictures of open siliques displaying aborted seeds from heterozygous plants. Siliques from wild type plants from both lines are shown for comparison. +: viable seed, -: aborted seed, Ls - Landsberg erecta, Col-0 - Columbia 0.
Figure 6. Mitochondrial TatB and TatC are located in a complex of 1500 kDa. A) BN-SDS-PAGE stained with coomassie. B) BN-SDS-PAGE analyzed by immuno blotting using antibodies against the indicated proteins. Tom40 – translocase of the outer membrane of 40 kDa, CoxII – cytochrome c oxidase subunit II, Qcr7 – ubiquinol:cytochrome c reductase
subunit 7, mtTatB and mtTatC. The molecular weights in kDa is given for the known complexes.
Figure 7. Mitochondrial TatC and TatB proteins are associated with a lack of Bcs1. The evolutionary history of mitochondria from a variety of organisms was inferred using the Maximum Likelihood method on concatenated protein sequences of Cytochrome c oxidase 1 (Cox1) and Cytochrome b (COB). Displayed is the consensus tree and bootstrap values after 1000 replicates. The arrangement of the structural domains of the closest related protein to Bcs1 is shown and was determined by the SMART database. Also indicated is the presence or absence of mitochondrial TatC and TatB genes.
Figure 8. Arabidopsis mitochondrial Rieske Fe/S protein requires a Tat signal and a ΔpH for assembly. A) [35S]Met-labeled Arabidopsis mitochondrial Rieske Fe/S proteins with Rip-KR or with Rip-QQ signals were incubated with isolated mitochondria in conditions that support import. After certain time points the mitochondria were analyzed by BN-PAGE and analyzed by autoradiography. The location of complex III and the super complex formed between complexes I and III are indicated. B) Displays the same import experiment as B however the proteins were separated by SDS-PAGE to display import and not assembly. p: precursor protein band, m: mature protein band. C) Wildtype AtRip-KR was incubated with isolated mitochondria in normal import conditions for a pulse period of 7.5 minutes. After the pulse period the mitochondria were re-isolated and washed of un-imported protein and the reaction was split in two. One half of the mitochondria were incubated in normal import buffer while the second half contained 2 μM nigericin (Nig). Samples were then collected at the indicated time points and analyzed by BN-PAGE.
Supplementary figure and tables

Supplementary Figure 1. Membrane insertion of AtRip-KR and AtRip-QQ.

\[^{35}\text{S}\text{-labeled Arabidopsis mitochondrial Rieske Fe/S proteins with Rip-KR or with Rip-QQ signals were incubated with isolated mitochondria in conditions that support import. After import mitochondrial membranes were extracted using 0.1 M Na}_2\text{CO}_3\]. Both membrane and soluble fractions were then separated by SDS-PAGE.
Supplementary table 1. BackPhyre results of Arabidopsis genome searching with the structures of TatA and TatB. Results are displayed in ranking order according the Phyre program. Displayed are the GI accession numbers and the NCBI description, the confidence of the prediction, as well as the sequence percentage identity to the bait. Also shown are the respective AT accession numbers as well as the TAIR10 description.

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Supplementary table 2. Species and accession numbers for all proteins used in phylogenetic analysis for Figures 1,2 and 6.

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