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Review

The plant mitochondrial protein import apparatus — The differences make it interesting☆

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ABSTRACT

Background: Mitochondria play essential roles in the life and death of almost all eukaryotic cells, ranging from single-celled to multi-cellular organisms that display tissue and developmental differentiation. As mitochondria only arose once in evolution, much can be learned from studying single celled model systems such as yeast and applying this knowledge to other organisms. However, two billion years of evolution have also resulted in substantial divergence in mitochondrial function between eukaryotic organisms.
Scope of Review: Here we review our current understanding of the mechanisms of mitochondrial protein import between plants and yeast (*Saccharomyces cerevisiae*) and identify a high level of conservation for the essential subunits of plant mitochondrial import apparatus. Furthermore, we investigate examples whereby divergence and acquisition of functions have arisen and highlight the emerging examples of interactions between the import apparatus and components of the respiratory chain.
Major conclusions: After more than three decades of research into the components and mechanisms of mitochondrial protein import of plants and yeast, the differences between these systems are examined. Specifically, expansions of the small gene families that encode the mitochondrial protein import apparatus in plants are detailed, and their essential role in seed viability is revealed.
General significance: These findings point to the essential role of the inner mitochondrial protein translocases in Arabidopsis, establishing their necessity for seed viability and the crucial role of mitochondrial biogenesis during germination. This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

1. Introduction

Mitochondria are membrane bound organelles that play essential roles in metabolism, energy production and biosynthesis of a variety of compounds in almost all eukaryotic cells. They are endosymbiotic in origin, and over time the majority of genes in the endosymbiont were lost or transferred to the host nucleus [1]. Thus, the majority of the 1000+ proteins located in mitochondria are encoded by nuclear genes, translated in the cytosol and imported into mitochondria [2]. *Saccharomyces cerevisiae* (yeast) has long been established as the pre-eminent model for the study of mitochondrial protein import [3]. However, many of the components involved in mitochondrial protein import are also well conserved across different species and whilst they have not been characterized in plants to the extent they have been in yeast, the presence of orthologous genes and complexes is well established across plants, animals, fungi [4–6] and even protists to a slightly lesser extent [7,8]. Fig. 1 summarizes the various pathways and complexes involved in protein import in plant mitochondria.

A multi-subunit protein complex on the mitochondrial outer membrane, termed the Translocase of the Outer Membrane (TOM), recognizes mitochondrial precursor proteins and passes them to one of two inner membrane multi-subunit protein complexes, termed the Translocases of the Inner Membrane (TIM) (Fig. 1). One of these is TIM17:23, which is responsible for the import of proteins via the general import pathway, i.e. for proteins that contain N-terminal targeting signals (Fig. 1). Alternatively, TIM22 is responsible for the import of proteins via the carrier import pathway, which is specific for the import of inner membrane proteins containing internal mitochondrial targeting signals (Fig. 1). Along with the Sorting and Assembly Machinery (SAM) complex on the outer membrane and the Mitochondrial Intermembrane space Assembly (MIA) in the intermembrane space,

Abbreviations: TOM, Translocase of the Outer Mitochondrial membrane; OM, Outer Membrane; SAM, Sorting and assembly machinery; TIM, Translocase of the Inner Mitochondrial membrane MIA, Mitochondrial Inter membrane space import and Assembly; ERV, Essential for Respiration and Vegetative growth; MPP, Mitochondrial Processing Peptidase; PreP, Presequence Protease

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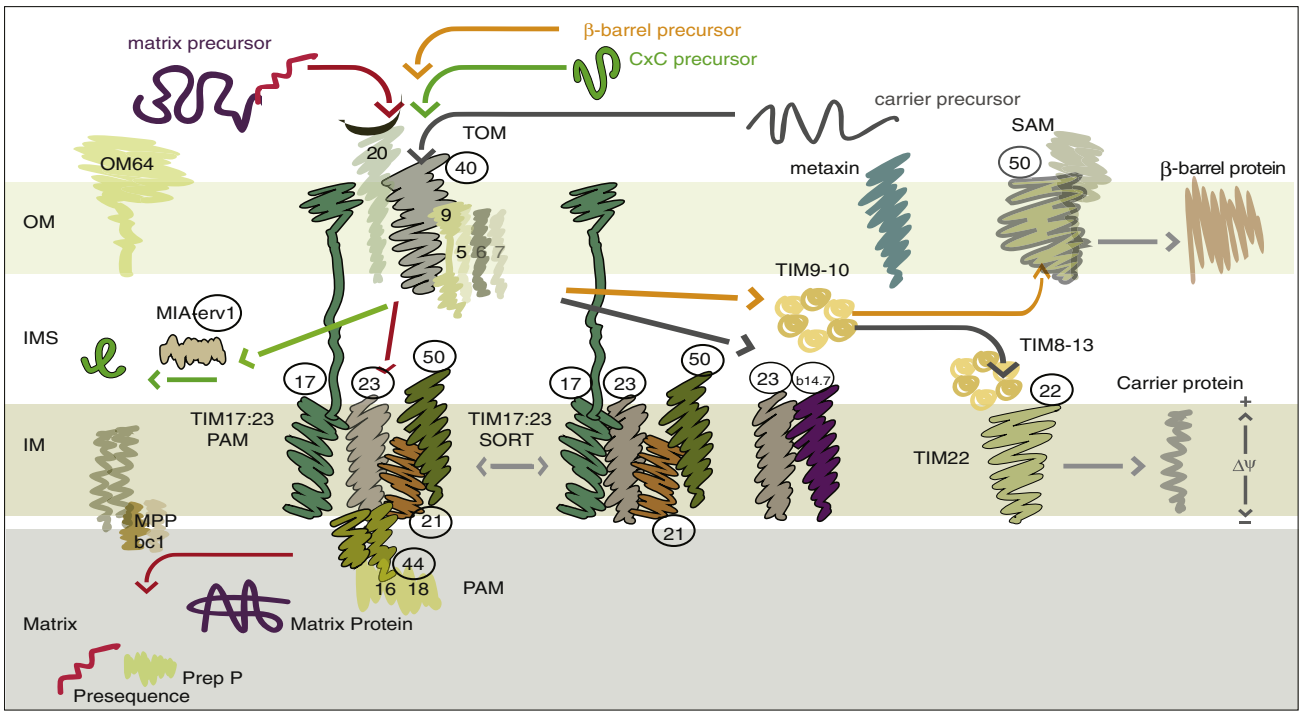

Fig. 1. Diagrammatic representation of protein import pathways and components in plant mitochondria. Targeting signals on cytosolically located precursor proteins determine the pathway of import. All precursors (containing a cleavable presequence, Twin CxC, β-barrel or carrier type proteins) pass through the Translocase of the Outer Membrane (TOM). The pathways diverge in the InterMembrane Space (IMS). Insertion of β-barrel proteins into the outer membrane is carried out via the Sorting and Assembly Machinery (SAM). Carrier import pathway proteins translocate into the inner membrane via small soluble Tim proteins through the TIM22 channel located in the inner membrane. Twin CxC proteins located within the IMS, are imported via the MIA-Erv1 pathway. For the majority of mitochondrial proteins that contain a cleavable presequence, translocation is carried out through the TIM17:23 complex with the Presequence Assisted Motor (PAM) complex or inserted directly into the inner membrane. The TIM17:23 complex can exist as 2 forms, PAM and SORT. In addition Tim23 interacts with the Complex I subunit B14.7. Following translocation, the presequence is removed via Mitochondrial Processing Peptidase (MPP), integrated into the cytochrome bc1 complex. The presequence is further processed via a matrix located Presequence Peptidase (PrepP). The essential subunits required for seed viability are circled. A membrane potential is required for translocation across the inner membrane (ΔΨ). Abbreviations: TOM = Translocase of the Outer Membrane, TIM = Translocase of the Inner membrane, IMS = Intermembrane Space, SAM = Sorting and Assembly Machinery, PrepP = Presequence Peptidase, MPP = Mitochondrial Processing Peptidase. The subunits known to be essential for plant viability are bolded. SAM50 is bolded in grey as no experimental verification of it essential function has been reported in plants.

these complexes are responsible for the import of the majority of proteins into the mitochondria [3,9]. In the last few years, studies on the mechanisms of protein import into mitochondria have revealed interactions between the protein import complexes with other multi-subunit protein complexes. Many of the studies revealing these interactions have been carried out in yeast [10,11]. Additionally, there have been emerging reports of interactions between the TIM17:23 complex and respiratory chain in both yeast and plants, although the biological implications of these interactions are not yet known [12,13].

Whilst most mitochondrial import components are highly conserved between yeast and plants, there are several areas of divergence that have arisen. These include the presence of plant specific mitochondrial import components, as well as the expansion of the gene families encoding these components, resulting in sub-functionalisation and/or neo-functionalisation. Studies characterizing the function of mitochondrial import components in yeast have revealed that several are essential for viability [14]. Interestingly, studies in plants have revealed a requirement for mitochondrial import components very early during germination in Arabidopsis (*Arabidopsis thaliana* — At) and rice (*Oryza Sativa*), when organelle biogenesis is actively occurring [15–17]. This early requirement is also supported by the findings that knocking-out certain import components, such as AtTIM50, results in embryo lethality [18]. To date, over 400 genes have been defined as essential in Arabidopsis [18]. In this review, the plant mitochondrial import components are explored and several inner membrane components are shown to be essential, whereby knocking out these genes results in an embryo lethal phenotype in Arabidopsis.

2. Outer membrane

The recognition of mitochondrial preproteins and the commencement of translocation occur via protein complexes on the outer membrane, namely the TOM complex and the SAM complex. Characterization of the mitochondrial protein import machinery on the outer membrane reveals that whilst the overall process is conserved in both yeast and plants, there are important mechanistic differences [6].

2.1. The TOM complex

The TOM complex consists of the cytosolic facing receptor subunits Tom20 and Tom70, the 'convergent' receptor Tom22 and the pore forming Tom40 channel. Preprotein recognition generally occurs via the N-terminal presequence, present on ~70% of mitochondrial proteins that ranges from 6 to 90 amino acids [19]. Tom20 exhibits substrate specificity with N-terminal presequences containing proteins whilst Tom70 binds proteins with internal non-cleavable targeting signals [20,21]. Tom40, a β-barrel protein, constitutes the translocation pore for almost all mitochondrial proteins and was the first mitochondrial membrane protein shown to be essential for yeast viability. Similarly, in Arabidopsis, T-DNA insertional knock-out lines for At*Tom40-1*, the highest expressed isoform [22] are not viable, suggesting that whilst there are two isoforms, At*Tom40-2* cannot compensate for the loss of At*Tom40-1*.

No direct orthologs to yeast and mammalian Tom20 and Tom70 can be identified in plants. Instead, the plant Tom20 receptor is likely to have an independent genetic origin, as it is anchored to the membrane via the C-terminus (the reverse of yeast, mammalian and fungal systems) and displays a unique bidendate binding site for precursor proteins [23] (Fig. 1). Given these significant differences, this cautions the use of mitochondria or precursor proteins from other systems to study protein import into plant mitochondria, as the recognition mechanism for precursor proteins, whilst similar, arose independently. It is not yet known whether this took place before or after the endosymbiotic event that gave rise to plastids in plants cells. The removal of all three isoforms of Tom20 in Arabidopsis resulted in a decrease in protein import ability to around 70% of that seen in wild-type plants, yet only small phenotypic differences were observed under normal or optimal growing conditions [24].

Following transfer from the Tom20 and 70 receptors, preproteins are subsequently transferred to Tom22 [25]. In yeast, Tom22 is essential for viability [26] and initiates the transfer of preproteins to the Tom40 channel [21,25]. Tom22 has also been implicated in the assembly of the TOM complex itself and for further transfer of preproteins into the IMS to the TIM17:23 complex via its extended intermembrane space domain [27–29]. The plant ortholog to Tom22 is termed Tom9, being significantly shorter, as it lacks the cytosolic domain of Tom22, and is highly conserved in a variety of plant species [5,30,31]. It has been suggested that Tom22 was present prior to the divergence event and that plants selectively either lost the receptor domain of Tom22 or acquired a novel Tom9 to functionally replace Tom22. Furthermore it has been suggested that the absence of the cytosolic domain may prevent the mis-targeting of chloroplast precursor proteins to plant mitochondria [5,30,31]. In Arabidopsis, two orthologs exist for At*Tom9*, both identified in the outer membrane TOM complex [32], though no further characterization has been carried out. Given the essential nature of Tom22 it would be of interest to establish if AtTom9 has equivalent functions to yeast Tom22 in terms of viability.

Additional small non-essential Tom5, 6 and 7 proteins have also been identified to be associated with the TOM40 complex in yeast. These small proteins have been shown to be involved in regulating the assembly of the complex, maintaining its stability and function to transfer precursor proteins to the Tom40 insertion pore [33–36]. Arabidopsis also contains small Toms [37], with AtTom7 identified as part of the TOM40 complex proteome [32] and AtTom5 and 6 identified by proteomic analysis of enriched outer membrane proteome fractions [38]. However apart from Tom7, the small size of these proteins makes it difficult to determine if they are orthologous or analogous.

Whilst Tom70 appears to be absent in plants, a novel plant specific outer membrane receptor has been identified termed Outer Membrane protein 64 (OM64), a paralog of the Toc64 protein import receptor found in plastids [5,24]. BN-PAGE analysis, did not find OM64 to associate with any protein complex, though does not rule out the possibility of transient interactions with the TOM40 complex [24]. Whilst functional studies have shown that the depletion of OM64 affects the import of selected mitochondrial proteins, mutants exhibited minimal phenotypic abnormalities [24]. Thus, whilst TOM20 and OM64 proteins may be unique in plants, removal of each is clearly not deleterious [24]. However, the generation of a quadruple knock-out line, consisting of deletions of all three Tom20 isoforms and OM64 has been shown to result in an embryo lethal phenotype in Arabidopsis, indicating an essential role for at least one of these at a time and the inability of putative additional receptors to compensate for the combined loss of all Tom20 and OM64 subunits [6].

2.2. The SAM complex

In yeast, the SAM complex is composed of several subunits termed Sam50, Sam35 and Sam37/metaxin, involved in the assembly of β-barrel proteins into the outer membrane. Sam50 is integrated in the outer membrane whilst Sam35 and 37 are peripheral subunits, with cytosolic domains facing the cytosol that bind and release substrates respectively [39,40]. Additional components that have been shown to interact with the SAM complex are Mdm10, Mdm12, Mmm1 and Mmm2, all previously identified in maintaining mitochondrial distribution and morphology [41–44], have been determined to specifically interact with SAM complex and involved in the assembly of Tom40 [45–48]. Plants contain orthologs to Sam50, and Sam37/metaxin only, with AtSam50 confirmed to be located in the outer membrane [38] and AtSam37/metaxin implicated in the import of β-barrel proteins VDAC and TOM40. Furthermore T-DNA insertional knock-out of metaxin in plants exhibited severe phenotypic abnormalities with decreased import ability of all precursor proteins tested suggesting that whilst not essential, AtSam37 plays a vital role in mitochondrial biogenesis in Arabidopsis [24].

2.3. The ERMES complex

An important development with regard to the TOM and SAM complexes is their functional connections with a newly identified ERMES network (Endoplasmic Reticulum Mitochondrial Encounter Structure). The ERMES complex was first identified by a genetic screen in yeast and was found to tether the ER and mitochondria and proposed to aid in the exchange of phospholipids and calcium [49]. This complex is composed of four proteins, Mmm2 and Mdm10 being located on the mitochondria, Mdm12 forming a cytosolic bridge and Mmm1 located on the ER [49], components previously identified to associate with the TOM and SAM complexes. Therefore, Mdm10 has a dual-location in both SAM and ERMES complexes [46,47,50] Furthermore, interactions of Mdm10 have also been reported with TOM subunits and mutants of Mdm10, Mdm12, Mmm1 and Mmm2 exhibit defects in β-barrel and Tom22 assembly [45–48]. These multiple interactions with protein complexes and the many phenotypes exhibited by ERMES mutants [51] have resulted in difficulty dissecting the specific functional roles of these proteins. The ERMES complex has yet to be identified in plants. Homology based searches against the Arabidopsis genome identifies candidate orthologs that exhibit some, albeit low identity (29–42%). Furthermore, bioinformatic analysis of Mdm10, Mdm10, Mmm1 and Mmm2 has identified conserved SMP domains (synaptotagmin-like mitochondrial and lipid-binding proteins) within subunits of the ERMES complex, which extends to Arabidopsis proteins [52,53]. Direct biochemical approaches are required to identify the subunits of this complex in plants. A study analyzing the outer mitochondrial proteome of mitochondria identified 42 mitochondrial outer membrane proteins from Arabidopsis, several of which had not been previously characterized [38]. Furthermore, transient over-expression of several of these proteins linked to GFP resulted in aberrations to mitochondrial morphology [38], and thus provides useful targets for further characterization to determine if they play a role in β-barrel assembly and/or ER networks.

2.4. The MINOS complex

The SAM and TOM complex have also been linked to a complex of the inner membrane termed Mitochondrial Inner Organising system (MINOS). This complex is composed of six subunits, Fcj1, Mio10, Aim5, Aim13, Aim37 and Mio27 initially identified as mutants that exhibit altered cristae morphology [54–58]. The main role of the MINOS complex has been proposed to be in the maintenance of the inner membrane architecture, but specific roles in biogenesis have also been uncovered. Fcj1 is involved in the import of cysteine rich proteins located in the IMS [54]. MINOS has also been shown to interact independently with the TOM and the SAM complex and is involved in the import of β-barrel proteins [55] and SOD1 variants in the intermembrane space [59]. Whilst MINOS subunits show strong conservation between yeast,

fungi and mammals [60] candidate orthologs only exhibit 27–32% identity in Arabidopsis and thus it is unclear if this complex exists in plants.

These newly discovered complexes involving TOM, SAM, ERMES and MINOS collectively involved in mitochondrial biogenesis, morphology and communication are proposed to be part of a larger network termed ERMIONE (ER-mitochondria organizing network) [61]. Spanning multiple membranes and with multiple functions, these complexes highlight the complex and dynamic nature of the mitochondrial import machinery and opens an exciting new area for research in plant mitochondria. However, it is likely that many would show embryo lethal phenotypes in plants, the use of biochemical approaches, combined with inducible genetic knock-downs or over-expression will be required to identify and characterize these complexes in plants.

3. Intermembrane space

The mitochondrial intermembrane space (IMS) contains the "tiny Tims", which are small soluble cysteine-rich proteins Tim 8, 9, 10 and 13 that form a hexameric ring like structure (Fig. 1). Tiny Tims are involved in the import of the carrier type proteins inserted into inner membrane or β-barrel proteins, designed for insertion back into the outer membrane [9]. Import of the cysteine-rich tiny Tims requires the MIA machinery consisting of Mia40 and Erv1 (Fig. 1), which are responsible for the oxidative folding and maturation of IMS proteins through the formation of disulphide bonds between cysteine residues in the disulphide relay system [62].

3.1. The tiny TIMs

Tim8, 9, 10 and 13, belong to a highly conserved protein family identified in a wide range of eukaryotic lineages [5]. In yeast, both Tim9 and 10 are essential for yeast viability and form a hexameric chaperone complex directing carrier precursor proteins from the TOM complex to the inner membrane TIM22 complex [63–65]. Tim8 and Tim13 form a similar chaperone complex, though are not essential for yeast viability [65]. The Tiny Tim complexes in yeast appear to show differential substrate specificity, with Tim9-Tim10 being cross-linked to carrier proteins such as the ATP/ADP carrier, outer membrane translocation pore Tom40, and the inner membrane Tim17 and 22 [66–68]. Additionally, yeast Tim8–Tim13 has so far only been identified to be involved in the import of Tim23 and the aspartate–glutamate carriers [65,69].

In plants, there are orthlogs to all of the yeast tiny Tims [37]. Direct biochemical reconstitution of IMS depleted mitochondria revealed that Tim9 and 10 are associated with carrier import assembly in plants [70]. Additionally, Tim9 and Tim10 could stimulate the import and assembly of carrier type proteins containing N-terminal cleavable presequences (a plant specific feature unique to some carrier proteins) to a greater extent than those carriers with only internal targeting signals [71]. The stimulation of carrier import in plants using enriched biochemical fractions has never been reported in yeast, presumably as it cannot be achieved technically. Thus, whilst genetic approaches show the essential role of Tim9–Tim10 in yeast the direct biochemical complementation in plants points to a mechanistic differences of how this pathway operates [70,72]. Taken together, the common occurrence of the N-terminal extension of plant carrier proteins that seem to enhance the efficiency of insertion into the inner membrane, and the absence of the yeast specific Tim12, Tim18 and Tim54 components, suggests that the carrier import pathway may display diversity in higher eukaryotes.

3.2. The Mia40-Erv1 pathway

The disulphide relay system involves the import of proteins located in the IMS that are rich in cysteine residues with twin CX(9)C or CX(3)C motifs [73]. This pathway consists of only two proteins, Mia40 and Erv1, both of which are essential for yeast viability [74–77]. Mia40 acts to transfer precursor proteins from the outer membrane to the IMS (Fig. 1), upon which the cysteine residues are oxidised by Mia40, which is subsequently oxidized by Erv1 [62]. Plants contain orthologs to the yeast Mia40 and Erv1 proteins, but important differences appear to exist between yeast and plants with respect to function. Mia40 in plants is not an essential protein, and is also located both in the mitochondria and peroxisomes [78,79]. Whereas Erv1 is essential in plants, as in yeast [78], however, the predicted Erv1 protein differs to that of yeast Erv1 and appears more similar to yeast Erv2 and that of parasitic organisms like Trypanosomes and Plasmodium [5,80]. Thus, whilst some organisms lack a gene encoding Mia40 (Trypanosomes and Plasmodium), and others have the genes (plants), it is apparent that the function differs with respect to oxidative protein folding upon import into mitochondria [81]. Furthermore, the dual-location of Mia40 has only recently been shown in higher plants, such as Arabidopsis and rice, whilst this was not seen in Physcomitrella [78,79]. Moreover, this dual-targeting ability also appears to have been acquired alongside the dual-targeting of the Mia40 substrate proteins superoxide dismutase (CSD1) and copper chaperone for SOD1 (Ccs1) in Arabidopsis [79]. This suggests that in plants, Mia40 function has extended its role to peroxisomes as well and may also be involved in the fatty acid oxidation in the peroxisome [78].

4. Inner membrane

In plants, analyses of the inner membrane components show that they are more conserved with the yeast inner membrane components compared to the outer membrane components, with most showing strong orthology to their yeast counterparts [5]. Whilst this is the case in terms of orthology, the expansion of gene families encoding inner membrane components combined with novel associations with respiratory chain complexes, means that the inner membrane translocases in plants may have undergone both neo- and sub-functionalisation resulting in several notable differences.

4.1. The inner membrane translocases

It has been shown in yeast that all mitochondrial precursor proteins targeted to the inner membrane or the matrix require to pass via the channel complexes TIM17:23 or TIM22 [3]. Precursors are either directly transferred from the outer membrane TOM40 complex via a dynamic interaction with TIM17:23 complex or co-chaperoned via the tiny Tims to the TIM22 complex [82]. The TIM17:23 complex, which is also conserved in plants, is responsible for the import of the majority of the mitochondrial proteome, and exists as two functionally and structurally distinct forms [9]. The initial form termed, TIM23-SORT, contains the channel forming proteins Tim23, and Tim17, along with its associated proteins Tim50 and Tim21. All components of TIM23-SORT are fundamental subunits responsible for the direct translocation of precursor proteins from the TOM40 complex through to the matrix or for insertion into the inner membrane [82]. The second form of the TIM17:23 complex in yeast, termed TIM23-PAM, functions in the transfer of proteins through the inner membrane to the matrix [39,83]. This complex also contains Tim17, Tim23, and Tim50 along with the Presequence translocase Associated Motor (PAM) complex, comprising of mtHSP70, Tim44 and its associated co-chaperones; Pam16, Pam17 and Pam18 [39,83]. This complex drives the translocation through the inner membrane via ATPase activity and cyclic binding of the presequence to promote transfer towards the matrix [84]. Associated chaperones Pam16, 17 and 18 are thought to modulate mtHSP70 activity and association with the TIM17:23 channel [85,86]. Based on the high level of orthology between these yeast inner membrane proteins their Arabidopsis counterparts, with the exception of Pam17, which is absent in plants and brown algae [5], it is very likely that these pathways are also functionally conserved in plants (Fig. 1).

Interestingly, it has been shown that genes encoding these plant mitochondrial inner membrane translocases are highly expressed during seed development and germination, when mitochondrial biogenesis is actively occurring [17,87]. It was also revealed that many of the genes showing this high expression during seed germination are essential for viability in Arabidopsis, whereby a loss-of-function resulted in embryo lethal phenotypes [87]. Thus, the conservation and divergence of the plant inner membrane translocases are discussed below, particularly revealing their essential role for seed viability.

4.2. The PReprotein and amino acid transporter family — plant specific expansion

Various studies, from yeast to higher eukaryotes, have shown the presence of Preprotein and Amino Acid Transporter (PRAT) domain-containing proteins [88,89]. PRATs are thought to have evolved from a single eubacterial ancestral LivH amino acid permease, characterized by four transmembrane domains and a conserved motif of [G/A]X_2[F/Y]X_{10}RX_3Dx_6[G/A/S]GX_3G, where X is any amino acid [89]. In Arabidopsis, this family consists of 16 members encoded by 17 genes [88,90]. The PRAT family of proteins in plants has clearly expanded in number, where 17 genes encode PRAT proteins in Arabidopsis, compared to just three in yeast [88] and brown algae — Ectocarpus [91]. Ten of the 17 Arabidopsis PRAT genes encode proteins that are located in mitochondria, whilst the other proteins are located in plastids, and have proposed roles in metabolite transport [88,92]. For the mitochondrial PRATs; three genes encode Tim17, three genes encode Tim23 and two genes encode Tim22 [88]. Additionally, there are 4 genes encoding other PRAT proteins in Arabidopsis that are yet to be characterized [88]. One such PRAT protein termed B14.7, was initially identified as a Complex I subunit in Arabidopsis, having orthologs in bovine and *Neurospora crassa* [93–95]. Interestingly, it was recently shown that this PRAT is also found in the TIM17:23 complex [13]. The function of the remaining mitochondrial PRAT proteins is unknown and challenges lie ahead in identifying the role of each of these specific protein isoforms in plant mitochondria. However, examination of the expression, predicted structure and experimental data on these PRAT proteins reveals a complex picture in terms of regulation (sub-functionalisation) and function (neo-functionalisation).

4.3. Tim17

With 17 PRAT family members in Arabidopsis, it has been proposed that a large expansion of these occurred in plants, pointing to the acquisition of new functions in higher eukaryotes and possible additional plant specific functions [6]. For example with regard to the Tim17 subfamily, both AtTim17-1 and AtTim17-2 contain a plant specific C-terminal extension not evident in AtTim17-3 [90]. This extension, in particular, for AtTim17-2, links the inner and outer membrane and has been shown to exhibit substrate binding specificities [96]. AtTim17-2 was only able to complement a *tim17* yeast deletion strain upon removal of the C-terminal extension [96]. Another factor that may provide insight into the specialised functionalisation of these three isoforms is their transcript expression profiles. The expression profiles of At*Tim17-1,* At*Tim17-2* and At*Tim17-3* appear to vary across tissue types, development and in response to environmental stress. The expression of At*Tim17-1* appears low in many tissue and developmental stages, unlike At*Tim17-2,* which is the most abundant isoform across development [22]. Notably, At*Tim17-1* does show some expression during seed development [97] and has been linked with the mitochondrial stress response where up to 30-fold induction was seen in response to ultraviolet light, high salt and high temperature stress [98,99]. The induction of At*Tim17-1* transcript following stress may alter mitochondrial import ability, as stress treatments have also altered the import ability of several precursor proteins in isolated mitochondria [100]. At*Tim17-3* on the other hand shows little expression specificity and moderate expression levels compared to At*Tim17-2* [6,22]. Furthermore, AtTim17-3 is most homologous to yeast Tim17 (50% identity) and whilst AtTim17-1 (48% identity) and AtTim17-2 (40% identity) contain a PRAT domain, AtTim17-3 contains a degenerate PRAT domain [88] and may represent a redundant isoform.

Given these differences in the Arabidopsis *Tim17* genes, we obtained knock-out lines for all 3 isoforms to gain an insight into their specificity and function (Table 1). Firstly, it was seen that At*tim17-1* knock-out plants showed faster germination rates compared to wild-type (Col-0) plants (Table 1), which supports a role for AtTim17-1 during germination and is also consistent with the increased transcript expression seen during seed development (Wang and Murcha — unpublished data). In contrast, no altered phenotype was observed in At*tim17-3* knock-out plants (Table 1), supporting the proposal that it is a redundant isoform. Lastly, it has been shown that At*Tim17-2* is the most highly expressed isoform, both at the transcript and protein levels (Wang and Murcha — unpublished data), and that At*Tim17-2* transcript expression peaks during seed germination [87]. Interestingly, screening of knock-out lines for At*tim17-2* (GK-561E03) failed to identify homozygous genotypes and subsequent self-fertilisation and screening of heterozygous progeny failed to identify any homozygous lines (Fig. 2). Closer inspection of silique development in the heterozygous germplasm (At*Tim17-2* (−/+)) revealed that embryos aborted early in seed development, indicating that At*Tim17-2* is an essential gene in Arabidopsis (Fig. 2; Table 1). Ratios of aborted/viable seeds were determined using 20 siliques from three individual plants (n = 20) and the percentage of defective seeds was shown to be 26.5 ± 2%, consistent with an embryo lethal phenotype (Fig. 2). Thus, whilst AtTim17-2 is not the most orthologous isoform to its yeast counterpart, it is the only essential Tim17 isoform in plants and contains a plant specific C-terminal extension that may also contribute to this essential role.

4.4. Tim23

There are also three isoforms for Tim23 in Arabidopsis, one of which contains a PRAT domain (AtTim23-3) whilst AtTim23-1 and AtTim23-2, contain a degenerate PRAT domain [88]. The three isoforms exhibit 70–92% sequence identity between each other [88]. Additionally, all three genes for Tim23 show different levels of orthology to the single Tim23 protein in yeast, with AtTim23-1, AtTim23-2 and AtTim23-3 exhibiting 40%, 39% and 41% amino acid sequence identity, respectively. Examination of transcript expression for each of these isoforms shows that all are most highly expressed during germination compared to other stages over development [87]. However, like At*Tim17-2*, At*Tim23-2* is the most highly expressed isoform at the transcript level compared to At*Tim23-1* and At*Tim23-3* [87]. As there is 92% amino acid sequence identity between AtTim23-1 and AtTim23-2, isoform specific antibodies have not been produced to date. Thus, when Tim23 protein abundance was examined by immunodetection with isolated mitochondria, it was shown that AtTim23-1 and/or AtTim23-2 protein is present in isolated mitochondria from 2 week old plants, whilst AtTim23-3 was not detected [13]. This study also characterized two T-DNA insertion mutants lines of At*tim23-2*, and found that one of these lines was an over-expressing line, showing a ~2-fold increase in AtTim23-2 protein abundance compared to wild-type (Col-0) [13]. Notably, the other T-DNA insertion line in the At*Tim23-2* coding region had no effect on growth, possibly due to compensation by the other isoform(s) [13].

Thus, in order to examine this more closely, we cross fertilised homozygous knock-out lines of At*Tim23-1* with At*Tim23-2*, At*Tim23-2* with At*Tim23-3* and At*Tim23-1* with At*Tim23-3* (Table 1). Interestingly, it was seen that these crosses failed to produce any double homozygous lines. Closer investigation of lines containing a homozygous genotype of one At*Tim23* gene and a heterozygous genotype of the other gene revealed defective seeds within the silique (Fig. 2). The defective seeds appear to have been aborted in the later stages of embryo development due to the presence of an intact seed coat, indicated by the red arrows (Fig. 2). The high expression of all At*Tim23* genes previously reported

Table 1
Summary of T-DNA insertional knock-out lines for mitochondrial import components in Arabidopsis. T-DNA insertional knock-out lines were screened for homozygosity and examined for growth defects. Where multiple isoforms exist for each component double knock-out lines were created to determine lethality of multiple gene inactivation. Abbreviations: Tom = Translocases of the Outer Membrane, Tim = Translocase of the Inner Membrane, NdufA11 = NADH dehydrogenase [ubiquinone] 1 subunit 11 (Human), B14.7 = NADH dehydrogenase [ubiquinone] 1 subunit 14.7 (Bovine), HM = Homozygous, Het = Heterozygous, bp = base pair, Ref = Reference, At = *Arabidopsis thaliana*.

Name	Vital in yeast	Gene name (AGI)	Line details	Insert location	Genotype	Altered Phenotype	Details	Ref
Tom20	Yes	AtTom20–2 (At1g27390)	SALK_067986	+1255 bp intron	HM	–	Non–essential gene	[24]
			SALK_134973	+126 bp intron	HM	–		[24]
		AtTom20–3 (At3g27080)	GK–554C03	+971 bp intron	HM	–	Non–essential gene	[24]
			SAIL_88_A08	+733 bp exon	HM	–		[24]
		AtTom20–4 (At5g40930)	SALK_147093	+579 bp exon	HM	–	Non–essential gene	[24]
			SALK_004057	+422 bp intron	HM	–		[24]
Tom40	Yes	AtTom40–1 (At3g20000)	SALK_128170	+1796 bp exon	Het	Defective embryo	essential gene	unpublished data
		AtTom40–2 (At5g40930)	SALK_004057	+422 bp intron	HM	–	Non–essential gene	unpublished data
OM64	NA	OM64 At5g09420	SALK_068772		HM	–	Non–essential gene	[24]
			SALK_089921		HM	–		[24]
Sam37/Metaxin	No	Sam37/Metaxin At2g19080	SALK_107629		HM	Delayed growth	Non–essential gene	[24]
			SALK_039892		HM			[24]
Tim23	Yes	AtTim23–1 (At1g17530)	SALK_030470	+699 bp 3'utr	HM	–	Attim23–2::Attim23–3 not viable	
			SALK_107963	+656 bp 5' utr	HM	–	Essential gene	
		AtTim23–2 (At1g72750)	SALK_143656	–135 bp 5' utr	HM	Delayed growth	Over–expressor	[13]
			GK–689C11	+43 bp exon	HM		Knock–out	[13]
							Attim23–1::Attim23–3 not viable	
							Essential gene	
		AtTim23–3 (At3g04800)	SAIL_1151_B01	+548 bp exon	HM	–	Attim23–1::Attim23–2 not viable	
			SALK_129386	+415 bp exon	HM	–	Essential gene	
Tim17	Yes	AtTim17–1 (At1g20350)	SALK_091528	–400 bp 5'utr	HM	Faster germination	Non–essential gene	unpublished data
			SALK_092885	+360 bp exon	HM	Faster germination		
		AtTim17–2 (At2g37410)	GK–561E03	+160 bp exon	Het	Defective embryo	Essential gene	
		AtTim17–3 (At5g11690)	SALK_048425	+543 bp 3'utr	HM	–	Non–essential gene	
			SALK_125567	+471 bp 3'utr	HM	–		
Tim22	Yes	AtTim22–1 (At1g18320)	GK–848H04	+71 bp exon	HM	–	Non–essential gene not expressed	
		AtTim22–2 (At3g10110)	SAIL_623_F03	+60 bp exon	HM	Sterile plant	Essential gene	
Tim50	Yes	AtTim50 (At1g55900)	SALK_000523	+2211 bp intron	HM	Delayed growth	Early termination	
			SALK_059376	+1177 bp intron	HM	–	Knock–down	[112]
							Essential gene	
Tim44	Yes	AtTim44–1 (At2g20510)	SALK_015319	+34 bp exon	HM	–	Attim44–1::Attim44–2 not viable	
							Essential gene	
		AtTim44–2 (At2g36070)	SALK_146901	–277 bp 5'utr	HM	–	Attim44–1::Attim44–2 not viable	
							Essential gene	
Tim21	No	AtTim21 (At4g00026)	sd3–2	intron	HM	seedling lethal	Essential gene	[111]
NdufsA11/B14.7	NA	AtB14.7 (At2g42210)	SAIL_434_E06	+619 bp intron	Het	Defective pollen	Essential gene	

during early germination [87] also supports these findings suggesting a crucial role of Tim23 for embryo viability. However, unlike the single essential gene in yeast, in plants we show that the removal of any two genes encoding Tim23 results in an embryo lethal phenotype (Fig. 2; Table 1), suggesting that each isoform may exhibit some specialized function in plants and at least 2 isoforms are essential for seed viability.

4.5. Tim22

The TIM22 complex is responsible for translocation of carrier type membrane proteins through and into the inner membrane containing internal targeting signals (Fig. 1). In yeast, the TIM22 complex comprises of the essential translocation channel protein Tim22 and all of its accessory proteins — Tim54, Tim18 and Tim12, which are essential for yeast viability [101–104]. A recently identified Tim18-associated protein, SDH3, has also been found to be located within the respiratory Complex II in yeast and thus is involved in both biogenesis and electron transfer [11]. In plants, homologs to yeast Tim54, 18 and 12 are not identified in any plant genome database [105] and it is expected that additional plant specific proteins are likely involved to replace the essential functions of Tim54 and 18. Whilst there has been little characterisation of the TIM22 complex in plants, preliminary data suggests that Tim22 may also associate with respiratory complexes, as it has been identified to be associated with Complex I by MS analysis of the mitochondrial complex proteome [94,106]. This is also supported by the observation that the expression pattern of *Tim22* genes in Arabidopsis shows similarity to that seen for genes encoding respiratory chain components, i.e. increasing in expression over the course of germination and remaining high in vegetative tissues, rather than peaking in expression early during seed development and germination as seen for genes encoding proteins in TIM17:23 [87].

In Arabidopsis, two genes encode Tim22 and both are 100% identical, even up to 500 bp upstream of the ATG start codon, suggesting a very recent gene duplication event. Therefore, in order to identify T-DNA insertional knock-out lines encoding Tim22, all available lines for each gene, At*Tim22-1* (At1g18320) and At*Tim22-2* (At3g10110), were obtained and screened using primers specific for each gene at −500 bp from the start codon (Fig. 3; Table 1). One homozygous line for each gene was identified, GK-848H04 for At*tim22-1* (At1g18320) and SAIL_623_F03 for At*tim22-2* (At3g10110) (Table 1). Whilst the homozygous line for At1g18320 did not exhibit any obvious deleterious phenotype (data not shown), the homozygous plant for the insertional knock-out within the At3g10110 gene resulted in a sterile plant with defects in silique maturation and seed production (Fig. 3). This phenotype is also consistent with the identification of this gene as being required for female gametophyte development and function in

Arabidopsis [107]. Thus, the At*Tim22-2* gene encoded by At3g10110 is also essential for seed viability in Arabidopsis.

4.6. Tim21

In Arabidopsis, AtTim21 is encoded by a single gene At4g00026, shown to interact with the TIM17:23 complex and respiratory complex III [13], similar to the function of yeast Tim21 as a core component of TIM17:23 and transiently interacting with the TOM and respiratory components [108,109]. In yeast, the Tim21 is non-essential [110] yet Arabidopsis knock-outs of AtTim21 (*sh3*) has been shown to result in a dwarf phenotype resulting in seedling lethality [111]. The Arabidopsis genome also encodes two additional Tim21-like proteins (At2g40800 and At3g56430) proteins 108–165 AA longer than AtTim21 (At4g00026). The absence of these Tim21-like proteins in yeast suggests possible specialised functions and further analysis would be required to determine if they have a role in mitochondrial protein import.

4.7. Tim50

Tim50 recognizes the targeting peptide within the presequence in the IMS domain, transferring it from the TOM complex to the TIM17:23 channel (Fig. 1). Phylogenetic analysis of Tim50 across eukaryotic systems suggests that this protein was acquired early in eukaryotes and has evolved independently in each of the major lineages [91]. Interestingly, AtTim50 is the only subunit of the TIM17:23 complex encoded by one gene in plants. Whilst Tim50 is essential in yeast, a recent report by Kumar et al., 2012 characterized a At*tim50* knock-out line (SALK_059376) as being viable with only minimal phenotypic abnormalities such as reduced hypocotyl length and enlarged mitochondria [112]. In contrast, another study has shown AtTim50 to be an essential gene for early embryonic development [18]. The apparent minimal phenotypic aberrations in the AtTim50 knockout characterized by Kumar et al., 2012 is surprising, given the essential nature of Tim50 in other systems such as yeast, Drosophila and *Caenorhabditis elegans* [113–115]. Thus, further characterisation of this mutant would clarify if AtTim50 is in fact an essential protein in Arabidopsis.

In order to clarify this, we examined all available AtTim50 insertional knock-out lines in Arabidopsis. Genotyping identified a homozygous line (SALK_000523) with the insertion located at position +2211 bp from the translation start site (Fig. 4A). Immunodetection with an antibody raised against Arabidopsis Tim50 [13], with mitochondria isolated from 2 week old homozygous seedlings detected a band, with an apparent molecular weight of 42 kDa, compared to a band with an apparent molecular weight of 43 kDa from wild-type mitochondria (Fig. 4A). Considering the position of the T-DNA within the At*Tim50* gene, western blot analysis suggests that the T-DNA insert results in a truncated protein (Fig. 4A). Phenotypic examination showed this plant to have a retarded growth phenotype when compared to Col-0 (Fig. 4A). The previously published At*tim50* knock-out line (SALK_059376) [112], was then also screened for homozygosity and immunodetected was carried out using isolated mitochondria (Fig. 4B). Immunodetection of AtTim50 showed the presence of a 43 kDa band (Fig. 4B). The intensity of this AtTim50 detection suggests that the T-DNA insert within SALK_059376 results in a At*Tim50* knock-down (Fig. 4B), with equal protein loading confirmed using antibodies against porin. To ensure correct genotyping was carried out, additional screening primers were used as outlined previously [112] and sequencing of the T-DNA insert confirms its location within the 4th intron. Overall, the observed retarded development seen here, when only a truncated AtTim50 protein is present (Fig. 4A), supports the previous report of AtTim50 as an essential Arabidopsis protein, necessary for seed viability in Arabidopsis [18].

4.8. Tim44

In addition to Tim50, Tim44 also associates with the TIM17:23 complex, likely as part of the plant equivalent of the TIM17:23 PAM complex (Fig. 1). In an effort to gain insight into AtTim44 function, T-DNA insertional knock-out lines of At*tim44* were also examined (Table 1). In plants, AtTim44 is encoded by two genes and it was shown that knocking out each of these individually does not affect plant development (Table 1). However, crossing these, i.e. the At*tim44-1* (−/−) homozygous knock-out line and At*tim44-2* (−/−) homozygous knock-out line failed to produce any double knock-out homozygous lines (Table 1). Analysis of the At*tim44-1* (−/−)::At*tim44-2* (+/−) plant, revealed early embryonic abortion within the silique at a ratio of 34 ± 8% consistent with an embryo lethal phenotype (Fig. 2). Thus, this suggests that both isoforms of AtTim44 can functionally replace each other and that AtTim44 is an essential protein for seed viability (Fig. 2).

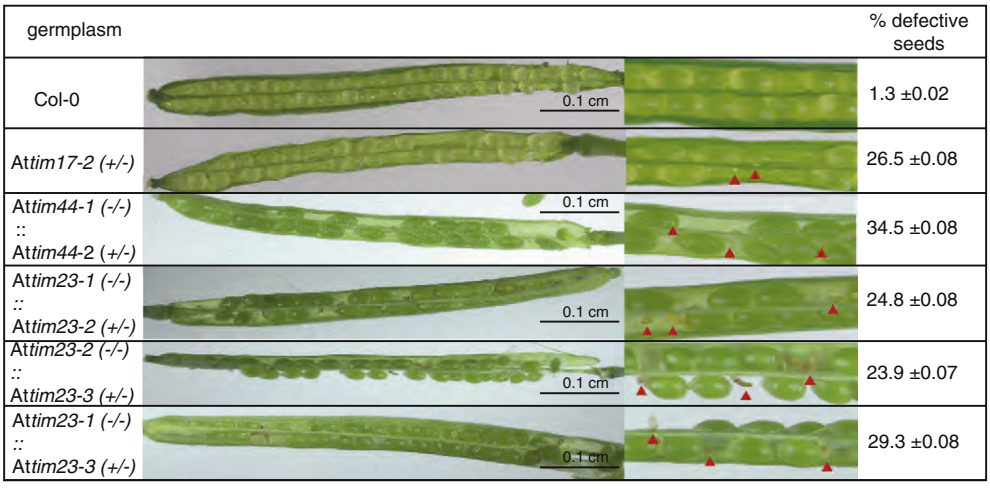

Fig. 2. Germplasms resulting in defective embryo development. Seed sets for At*tim17-2* (+/−) heterozygous knock-out and double knock-out lines, At*tim44-1* (−/−)::At*tim44-1* (+/−), At*tim23-1* (−/−)::At*tim23-2* (+/−), At*tim23-1* (−/−)::At*tim23-3* (+/−) and At*tim23-2* (−/−)::At*tim23-3* (+/−) exhibit seed lethal phenotypes. The germplasms were screened to confirm genotype and siliques dissected to confirm the lethality of gene inactivation. Values indicate the average percentage of aborted progeny from three biological replicates ± S.D (n = 60). Scale bar, 0.1 cm. Arrow indicates defective embryo. Abbreviations: (+/−) = heterozygous knock-out, (−/−) = homozygous knock-out, Col-0 = wild-type.

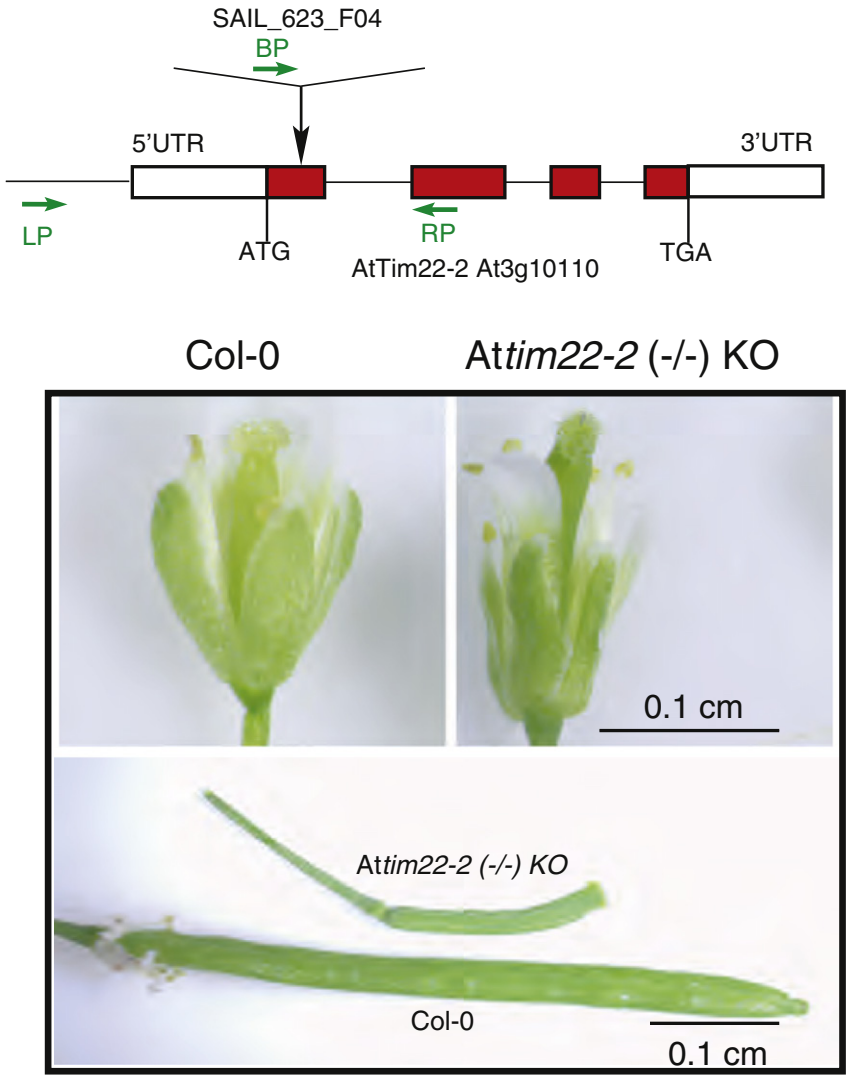

Fig. 3. Deletion of At*Tim22-2* results in plant sterility. The T-DNA insertional knock-out line for AtTim22-2 (At3g10110) (SAIL_623_F03) was screened for homozygosity. At*tim22-2* homozygous plants exhibit smaller flowers and poorly developed siliques with little to no seed. Scale bar, 0.1 cm, (−/−) = homozygous knock-out.

5. Matrix

5.1. MPP and PrepP

As mitochondrial proteins are imported from the inner membrane into the matrix, processing steps are necessary before the imported protein is fully functional. To do this, the mitochondrial processing peptidase (MPP) and the Presequence Peptidase (PrepP) are utilised (Fig. 1) [116]. In Arabidopsis MPP is encoded by two genes, denoted α-MPP (At3g16480) and β-MPP (At3g02090). MPP functions to cleave the targeting signal prior to assembly and interestingly, although there is orthology (31 and 41% respectively) between these and their yeast counterparts, it has been shown that there are significant differences in plants. Specifically, the plant MPP has been shown to be an integral component of the respiratory cytochrome bc1 complex and thus is bi-functional, involved in both processing and electron transfer [117–119]. This unique feature is plant specific, not seen in yeast and mammals, and provides an example of proteins shared between the protein import and respiratory chain complexes [117].

In Arabidopsis PrepP is encoded by 2 genes, AtPrepP1 and AtPrepP2 exhibiting 87% identity. These encode peptidases involved in the degradation of presequences following MPP cleavage [120,121]. Initially identified in plants, AtPrepP has the ability to compliment its yeast non-essential ortholog (Mop112/Cym1) suggesting functional conservation [122]. However it may yet have undergone neo-functionalisation as AtPrepP1 and AtPrep2 are both co-localised in both mitochondria and chloroplast [123]. Deletion of both isoforms of AtPrepP results in minor growth aberrations suggesting additional proteases may function to degrade presequences [124].

6. Linking mitochondrial biogenesis to mitochondrial activity

In yeast, the TIM17:23 complex has been identified to form dynamic supercomplexes with both the TOM40 channel and Complex III and Complex IV of the mitochondrial respiratory chain via Tim21 [125], proposed to promote efficient translocation at contact sites by utilising the membrane potential of the inner membrane [3,125]. Examination of the AtTim23-2 over-expressing line revealed that Complex I was reduced in abundance by ~90% [13]. Further analysis of independent Complex I knock-out lines showed that AtTim23-2 was conversely increased in abundance, thus supporting the link between the TIM17:23 complex and respiratory chain Complex I in plants [13,126]. Further analysis of the connection between TIM17:23 and Complex I showed that another PRAT protein B14.7, previously shown by two independent groups to be a subunit of Complex I [94,95], was also a subunit of TIM17:23 [13]. Furthermore, AtTim23-2 and AtB14.7 imported and assembled into

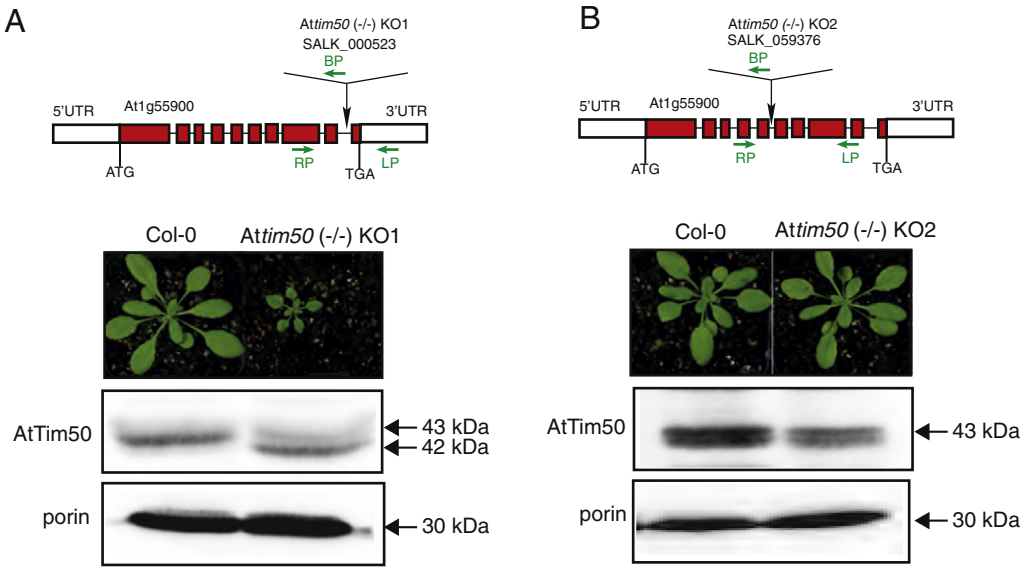

Fig. 4. Identification of A*ttim50* mutants. The T-DNA insertional knock-out lines for AtTim50 (At1g55900) were screened for homozygosity and immunodetected with AtTim50 antibody to confirm loss of function. (A) The line, SALK_000523 (A*ttim50* (−/−) KO1) with the T-DNA insert at position +2211 in the last intron, displays an aberrant growth phenotype compared to wild-type (Col-0). Immunodetection of mitochondria isolated from 2 week old seedlings indicates that the insertion results in a truncated AtTim50 of apparent molecular weight of 42 kDa. (B) The A*ttim50* (−/−) KO2 line, SALK_059376, previously identified to be a A*ttim50* knock-out [59] was rescreened for homozygosity. Homozygous lines display no apparent growth aberrations and immunodetection against AtTim50 suggests that the T-DNA insertion results in a knock-down, not a knock-out. The mitochondrial control (Porin) shows equal protein loading. Abbreviations: RP = Right border screening primer, LP = Left border screening primer, BP = T-DNA border screening primer, kDa = kilodaltons (−/−) = homozygous knock-out.

Complex I and TIM17:23 confirming that these two complexes share both of these subunits. The overexpression of AtTim23-2 also resulted in a 2–3-fold increase in transcript abundance of mitochondrial encoded genes, organelle translation, and protein import. Transcript analysis of nuclear genes encoding mitochondrial proteins also displayed an increase in abundance, in particular for genes encoding proteins involved in mitochondrial biogenesis [13,126]. These findings uncovered a link between TIM17:23 and Complex I, and importantly revealed that by changing the abundance of AtTim23-2, mitochondrial biogenesis could be altered. Thus, an important regulatory mechanism linking mitochondrial biogenesis and activity has been uncovered and highlights the dynamic and complex regulatory mechanisms of protein import in higher eukaryotes. A recent study in yeast has identified a novel subunit of the TIM17:23 complex, Mgr2 involved in coupling TIM17:23 to the TOM complex and the respiratory chain, is required for efficient import by stabilizing the TOM-TIM supercomplex [127]. In Arabidopsis there appears to be one ortholog encoded by At3g07910 that exhibits a low percentage identity (32%). However, it does contain a ROMO1 (reactive mitochondrial oxygen species modulator 1) conserved domain also found in yeast Mgr2. More interestingly At3g07910 similarly contains regions of homology to several PRAT family members and thus is a likely candidate for further characterization in plant mitochondria.

6.1. AtB14.7 (At2g42210) is essential for seed viability

Screening of the T-DNA insertional knock-out line for A*tB14.7* (At2g42210) (SAIL_434_E06) (Fig. 5) failed to identify any homozygous genotypes and subsequent self fertilisation and screening of heterozygous progeny failed to identify any homozygous lines. Inspection of silique development in the heterozygous germplasms (AtB14.7 (+/−)) found defective seeds at a ratio comparable to the defective seed ratios exhibited in wild-type (Col-0). Pollen viability and total pollen counts were investigated by Alexander staining and found to be significantly lower in heterozygous plants compared to wild-type (Col-0) (Fig. 5). Thus, AtB14.7 is essential for seed viability in Arabidopsis.

7. The essential role of mitochondrial import during germination

In this review, we have shown that several mitochondrial inner membrane import components are essential for seed viability in Arabidopsis (Fig. 1–5; Table 1), consistent with their relatively high levels of transcript abundance during germination. It has been reported that genes peaking in expression during germination are enriched in mitochondrial proteins, particularly those encoding RNA processing functions such as the mitochondrial pentatripeptide repeat (PPR) containing proteins [87]. Thus, the high expression of several inner membrane import components during seed germination [87] supports the findings that a loss-of-function of these results in an embryo lethal phenotype (Table 1). Close examination of mitochondrial biogenesis during germination revealed that at transcript and protein abundance of mitochondrial import component did not correlate during germination [17]. Specifically, it was seen that the proteins encoding import components are present very early during germination, peaking in abundance before the respective transcripts in Arabidopsis [17]. This is also supported by the findings in rice, where it was shown that within 30 min of imbibition (water up-take), mitochondrial protein import begins [15]. Taken together, the recent findings linking AtTim23-2 protein abundance with altered mitochondrial biogenesis [13], as well as the observed embryo lethal phenotypes presented here after the loss-of-function of several inner membrane import components (Table 1), indicate that mitochondrial protein import plays a crucial role in early germination in plants, facilitating a move from a dormant metabolic inert state to an active metabolic state, to drive germination.

8. Concluding remarks

- The conservation of inner membrane translocases

In yeast, all components of the TIM17:23-sort complex are essential for viability, with the exception of Tim21 [109]. As shown above, in Arabidopsis; AtTim17, AtTim23, AtTim44 and AtTim50 are also shown to be essential subunits for seed viability (Table 1), whilst AtTim21

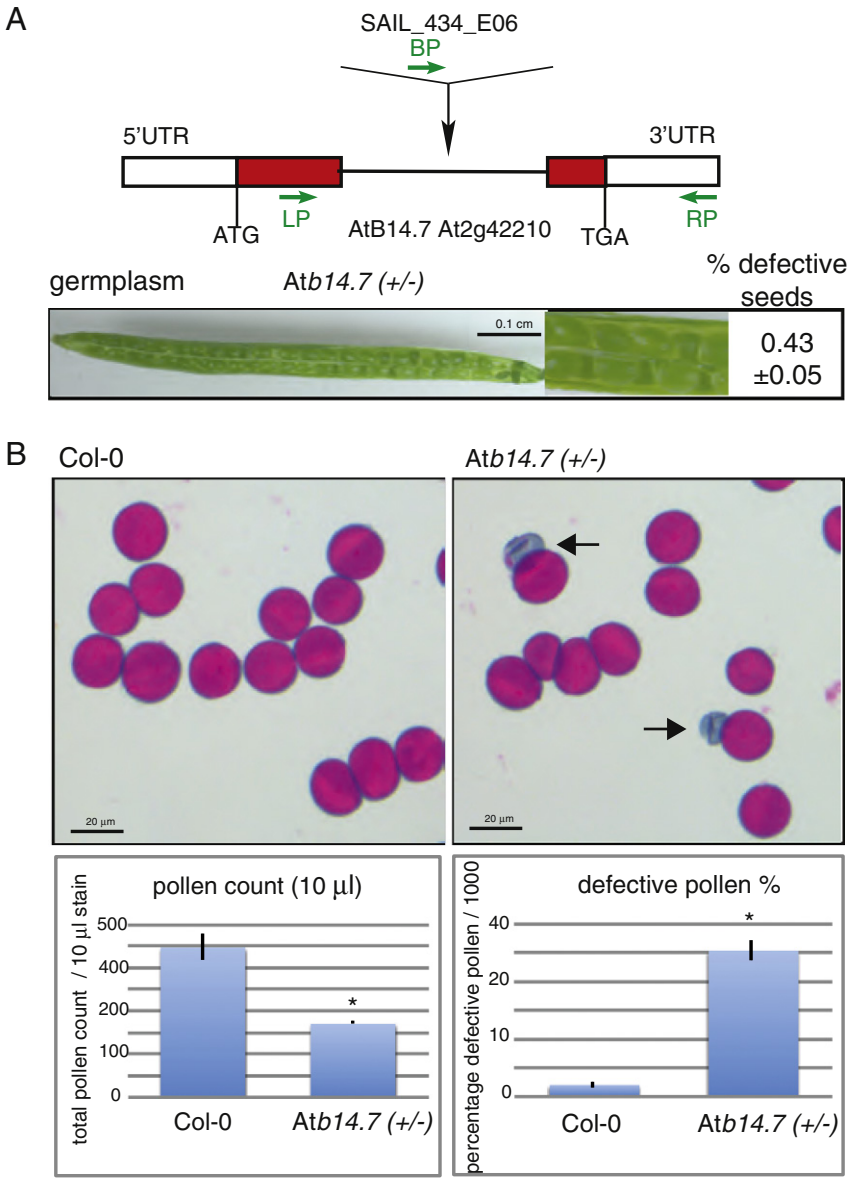

Fig. 5. Identification of a A*tb14.7* heterozygous mutant. (A) Screening of the T-DNA insertional knock-out line for At*B14.7* (SAIL_434_E06) failed to identify any homozygous lines. Dissection of A*tb14.7* (+/−) heterozygous plants show all seed sets to be viable and subsequent genotyping only identified Col-0 or heterozygous germplasm. Values indicate the average percentage of aborted progeny per silique ± S.D (n = 60) from three biological replicates. Scale bar, 0.1 cm. (B) The number of pollen grains and percentage of defective pollen from A*tb14.7* (+/−) heterozygous plants as determined by Alexander staining. Arrows indicate defective pollen grains. * indicates statistically significant difference compared with wild-type (Col-0) ($p < 0.01$). Scale bars, 20 µm. Error bars indicate S.E, (+/−) = heterozygous knock-out.

has acquired an essential function during vegetative growth [111] (Table 1). This shows a high level of conservation for the essential subunits of plant mitochondrial import apparatus.

- The acquisition and divergence of function

In plants, the presence of multi-gene families has resulted in neo-specialisation and neo-functionalisation of the mitochondrial import components. At*Tim17* and At*Tim23* are examples, where expansion of these gene families in plants resulted in the different isoforms acquiring new functions or showing different tissue specific expression. Of the three isoforms of AtTim17; AtTim17-2 maintains the essential function, whilst the loss-of-function of AtTim17-1 and AtTim17-3 did not result in embryo lethal phenotypes (Table 1). Interestingly, expression profiling revealed higher expression of At*Tim17-1* during seed development, and further examination using At*tim17-1* knock-outs revealed a germination specific phenotype (Wang and Murcha — unpublished data), suggesting neo-specialisation, with this isoform having a unique role in seed development and germination. The C-terminal extension of AtTim17-2 appears to contain conserved motifs predicted to bind nucleic acids, and as plant mitochondria have the ability to import tRNA, this merits an investigation of the role of AtTim17-2 in the import of tRNA [5].

- New levels of dynamics with the respiratory chain and supercomplex structures

Whilst only one gene encodes TIM23 in yeast, three genes encode for AtTim23. Interestingly, this has allowed some redundancy, where it was seen that whilst deletion of one gene has no effect on seed viability, the deletion of two AtTim23 encoding genes does result in an embryo lethal phenotype (Fig. 2). Whilst there appears to be few predicted structural differences between the three AtTim23 isoforms, expression analysis indicates differences in abundance with At*Tim23-2* showing the highest

transcript abundance [22]. Studies in Arabidopsis have shown that AtTim23-2 interacts with respiratory Complex I and III [13], which raises the possibility that different isoforms may have preference for protein–protein interactions. These differences could then facilitate the formation of dynamic and variant forms of complexes and supercomplexes within the inner mitochondrial membrane. In addition, it has been shown that AtB14.7 is also an essential inner membrane PRAT protein (Table 1). It has previously been shown that AtB14.7 has a dual-location, both within the TIM17:23 complex and the respiratory Complex I in Arabidopsis [13]. However, the specific role of AtB14.7 has yet to be elucidated. B14.7 has been characterized as a Complex I subunit in *N. crassa*, bovine and now Arabidopsis [13], suggesting an important function in higher eukaryotes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.09.026.

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