SHORT REPORT

The plant \(i\text{-AAA}\) protease controls the turnover of an essential mitochondrial protein import component

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ABSTRACT

Mitochondria are multifunctional organelles that play a central role in energy metabolism. Owing to the life-essential functions of these organelles, mitochondrial content, quality and dynamics are tightly controlled. Across the species, highly conserved ATP-dependent proteases prevent malfunction of mitochondria through versatile activities. This study focuses on a molecular function of the plant mitochondrial inner membrane-embedded AAA protease (denoted \(i\text{-AAA}\)) FTSH4, providing its first bona fide substrate. Here, we report that the abundance of the Tim17-2 protein, an essential component of the TIM17:23 translocase (Tim17-2 together with Tim50 and Tim23), is directly controlled by the proteolytic activity of FTSH4. Plants that are lacking functional FTSH4 protease are characterized by significantly enhanced capacity of preprotein import through the TIM17:23-dependent pathway. Taken together, with the observation that FTSH4 prevents accumulation of Tim17-2, our data point towards the role of this \(i\text{-AAA}\) protease in the regulation of mitochondrial biogenesis in plants.

KEY WORDS: AAA protease, ATP-dependent proteolysis, Mitochondrial protein import, TIM17:23 translocase

INTRODUCTION

Mitochondria are essential, double membrane-bound organelles that are the main sites of ATP production in eukaryotic cells. Continuous degradation and biogenesis processes enable the maintenance of healthy mitochondrial populations and allow the mitochondrial proteome to adapt in response to the fluctuating cellular requirements (Nunnari and Suomalainen, 2012; Harbauer et al., 2014; Palikaras and Tavernarakis, 2014). The vast majority of mitochondrial proteins are nuclear encoded and synthesized as preproteins in the cytosol that are transported into destined sub-compartments of the organelle, mitochondrial content, quality and dynamics are tightly controlled. Across the species, highly conserved ATP-dependent proteases prevent malfunction of mitochondria through versatile activities. This study focuses on a molecular function of the plant mitochondrial inner membrane-embedded AAA protease (denoted \(i\text{-AAA}\)) FTSH4, providing its first bona fide substrate. Here, we report that the abundance of the Tim17-2 protein, an essential component of the TIM17:23 translocase (Tim17-2 together with Tim50 and Tim23), is directly controlled by the proteolytic activity of FTSH4. Plants that are lacking functional FTSH4 protease are characterized by significantly enhanced capacity of preprotein import through the TIM17:23-dependent pathway. Taken together, with the observation that FTSH4 prevents accumulation of Tim17-2, our data point towards the role of this \(i\text{-AAA}\) protease in the regulation of mitochondrial biogenesis in plants.

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RESULTS AND DISCUSSION

Mitochondria devoid of FTSH4 accumulate Tim17-2

To understand the role of FTSH4 protease in the maintenance of mitochondrial function, we searched for primary changes caused by...
the loss of this i-AAA protease. Consequently, to diminish the risk of secondary effects, we sought to identify mitochondrial proteins that accumulate in ftsh4 mutants cultivated under optimal growth conditions, where no significant alterations in mitochondrial activity were observed (22°C with a long-day photoperiod, cycles of 8 h dark and 16 h light) (Smakowska et al., 2016). Interestingly, we found a substantial increase in the steady-state levels of Tim17-2 in mitochondria isolated from both ftsh4-1 and ftsh4-2 lines (Fig. 1; Fig. S1). Tim17-2 is a highly conserved, essential component of the inner mitochondrial membrane protein translocase – the TIM17:23 complex – that transports the majority of mitochondrial proteome (Wasilewski et al., 2016; Schulz et al., 2015). As evident from studies performed in yeast, Tim17 plays multiple roles within the TIM17:23 translocase. It regulates pore structure and voltage gating of the Tim23 channel, and is required for the promotion of the inner-membrane insertion of preproteins and direct interaction of the TIM17:23 core with the presequence translocase-associated motor (Martinez-Caballero et al., 2007; Chacińska et al., 2005). Consequently, the precise level and stoichiometry of Tim17 may be important for the optimal functioning of TIM17:23. *A. thaliana* contains three isoforms of Tim17, of which only Tim17-2 is essential and constitutively expressed (Murcha et al., 2014). We found that steady-state levels of other TIM17:23 subunits as well as levels of components of protein translocases from the outer mitochondrial membrane, Sam50 and Tom40, and of the intermembrane space-localized small Tim chaperone, Tim9, were unaffected upon FTSH4 loss (Fig. 1). These results show that the increase in Tim17-2 levels in ftsh4 mutants is specific and not caused by a general upregulation of mitochondrial preprotein import machineries. To further address correlation between the FTSH4

Fig. 1. Tim17-2 protein levels are elevated in ftsh4 mutant. Representative immunoblots of mitochondrial fractions prepared from wild type and ftsh4-1 cells (upper panel). Quantification of steady-state levels of mitochondrial proteins in ftsh4 mutant relatively to wild type (set to 1). Bars represent mean±s.d. from n≥3 (lower panel). IM, inner membrane; OM, outer membrane; IMS, intermembrane space.

Fig. 2. A substantial portion of Tim17-2 is present as low molecular mass sub-complexes in mitochondria lacking FTSH4 protease. (A) Western blot analysis of mitochondrial fractions from wild type and ftsh4 mutants subjected to alkaline carbonate extraction. Equal amounts from the whole mitochondria (Total), supernatant fractions (soluble and peripheral membrane proteins) and pellet fractions (membrane-integrated proteins) were resolved with SDS-PAGE, immobilized on PVDF membrane and analyzed with specific antibodies. (B) Mitochondria isolated from wild-type and ftsh4-1 plants were lysed with digitonin and subjected to BN-PAGE followed by SDS-PAGE in the second dimension. TIM17:23 complexes were probed with anti-Tim17-2 and -Tim50 antibodies.
protease and Tim17-2 levels we generated a *ftsh4-1* complementation line (*ftsh4-1* FTSH4), in which FTSH4 was expressed under the constitutive CaMV 35S promoter (Fig. S2). Overproduction of FTSH4 rescued the morphological phenotype of *ftsh4-1* line (Fig. S3) and led to a decrease in Tim17-2 steady-state levels (Fig. S2). Next, we questioned whether Tim17-2 present in excessive amounts in *ftsh4* mutants is correctly integrated into the mitochondrial membrane. To address this, we isolated mitochondria from wild-type and mutant plants and performed alkaline carbonate extraction that allows for distinguishing between integral membrane proteins (pellet) and soluble or peripheral membrane proteins (supernatant). Both in wild-type and *ftsh4* mitochondria, Tim17-2 was exclusively found in pellet fractions after sodium carbonate treatment, indicating integration into the inner membrane (Fig. 2A). To confirm specificity of the assay, mtHsp70 (also known as mtHsc70-1, a soluble protein of mitochondrial matrix) and Slp1 (mitochondrial inner membrane protein that is partially extracted with sodium carbonate; Gehl et al., 2014) were used as controls.

We also addressed Tim17-2 distribution within mitochondrial membrane protein complexes by Blue native (BN)-PAGE followed by a second-dimension electrophoresis under denaturing conditions (2D-BN/SDS-PAGE) (Fig. 2B). In wild-type mitochondria, Tim17-2 mainly co-migrated with Tim50 as does the TIM17:23 translocase (Wang et al., 2012). A small portion of Tim17-2 in a low molecular mass sub-complex was detected. In contrast, a substantial fraction of Tim17-2 was present as a low molecular mass sub-complex in *ftsh4* mutant. Furthermore, the ratio of TIM17:23 complexes in *ftsh4* mutants was noticeably altered. Taken together, we conclude that FTSH4 controls the abundance of Tim17-2 in the mitochondrial membrane and that lack of this protease exerts an impact on the architecture of TIM17:23 translocase.

**Proteolytic activity of FTSH4 is required to prevent accumulation of Tim17-2**

Specific accumulation of Tim17-2 in *ftsh4* mutant mitochondria strongly suggests that FTSH4 proteolytic activity is required for the turnover of this essential protein import component. In order to address whether Tim17-2 represents a proteolytic substrate of FTSH4, we generated a mutant line expressing a proteolytically inactive variant of FTSH4 [FTSH4(H486Y)] in the *ftsh4-1* background. The overproduction of FTSH4(H486Y) was confirmed by immunoblot analysis (Fig. 3A). Expression of the proteolytically inactive variant of FTSH4 neither restored the morphological phenotype of *ftsh4-1* line (Fig. S3) nor downregulated levels of Tim17-2 (Fig. 3A), demonstrating the importance of the proteolytic function of this i-AAA protease in vivo. To obtain further evidence for the role of FTSH4 proteolytic activity, we performed a series of experiments using different approaches.

**Fig. 3. Proteolytic activity of FTSH4 is required to prevent Tim17-2 accumulation.** (A) Representative immunoblot of mitochondrial fractions prepared from wild-type, *ftsh4-1* and *ftsh4-1* FTSH4(H486Y) plants (left panel). The density of the Tim17-2 band is shown underneath the blot (Quanf.). Quantification of steady-state levels of Tim17-2 and SHMT in *ftsh4-1* FTSH4 (H486Y) mutants relative to wild type (set to 1). Bars represent means±s.d. from n=3 (right panel). (B) Immunoblot of purified mitochondria obtained from *ftsh4-1* FTSH4(H486Y) and wild-type plants. Mitochondria were incubated for 0 to 4 h at 35°C in a buffer supplemented with ATP (upper panel). Quantification of Tim17-2 and Slp1 stability in *ftsh4-1* FTSH4(H486Y) mutant and wild-type mitochondria. Results represent means±s.d. from n=3 (lower panel). (C) Immunoblot showing co-immunoprecipitation of Tim17-2 with FLAG-tagged proteolytically inactive FTSH4. Mitochondria from *ftsh4-1* FTSH4(H486Y) and *ftsh4-1* control were lysed with digitonin and immunoprecipitated with anti-FLAG affinity matrix. The precipitated proteins were then immunoblotted with antibodies against the indicated proteins. IN, input (5%); FT, flow-through (5%); W, wash; E, eluate. (D) Immunoblot showing co-immunoprecipitation of Tim17-2 with FLAG-tagged proteolytically inactive FTSH4. Mitochondria from *ftsh4-1* FTSH4(H486Y) were lysed with digitonin and incubated with or without antibodies raised against Tim17-2 and Protein-A-Sepharose. The precipitated proteins were then immunoblotted with antibodies against the indicated proteins. W, wash; E, eluate.
activity in the regulation of Tim17-2 levels, we analyzed turnover of Tim17-2 in isolated mitochondria. We observed a time-dependent decrease in Tim17-2 amounts in wild-type mitochondria, while the levels of this protein remained stable in mitochondria isolated from plants expressing proteolytically inactive FTSH4 (Fig. 3B). Moreover, we found that Tim17-2 specifically co-precipitates with FTSH4(H486Y) (Fig. 3C,D). We conclude from these studies that Tim17-2 represents a bona fide substrate of the FTSH4 protease.

Lack of FTSH4 protease is associated with enhanced capacity for the import of model substrates for the TIM17:23 translocase

An imbalance in levels of different complex subunits might lead to changes in the activity of the whole protein complex (König et al., 2016). Since our results suggest that FTSH4 protease controls Tim17-2 levels and by this modulates the architecture of TIM17:23 translocase, we questioned how the loss of FTSH4 influences mitochondrial protein import. To establish the role of FTSH4 in this process, we analyzed the capacity for in vitro uptake of the alternative oxidase (AOX, Genbank X68702), a precursor known to be imported via TIM17:23 translocase (Murcha et al., 2005), into fish4-1 mutant mitochondria (Fig. 4A). We found that mitochondria devoid of FTSH4 protease display a substantially increased rate of import for the radiolabeled AOX precursor. In contrast, in vitro uptake of the adenine nucleotide transporter (ANT) (Murcha et al., 2005), a precursor known to be imported via the TIM22 translocase complex, showed no difference in the rate of import in mitochondria isolated from fish4-1 compared to in wild type (Fig. 4B). Furthermore, we found that overexpression of FTSH4 protease in a fish4-1 background led not only to a reduction in Tim17-2 levels (Fig. S2), but also to a decrease in the rate of AOX import (Fig. S4). This is in line with previous findings, where the mammalian homolog of FTSH4, YME1L, was shown to downregulate the activity of TIM17:23 translocase by mediating the proteolysis of Tim17A isoform (Rainbolt et al., 2013). Increased import capacity through TIM17:23 translocase in fish4-1 mutant suggests that a low molecular mass Tim17-2 complex, which is present in elevated amounts in mutant mitochondria, could represent a functional translocase involved in import of preproteins. On the other hand, the TIM17:23 translocase is a very dynamic complex in which its subunits alter their conformations and undergo association and dissociation to facilitate preprotein import (Schulz et al., 2015). Therefore, we cannot rule out the possibility that the presence of excessive amounts of Tim17-2 induces changes in the architecture and dynamics of TIM17:23 translocase that have a stimulatory impact on import of at least a specific set of preproteins. Noteworthy, Tim17-2 contains a C-terminal extension that protrudes into the outer mitochondrial membrane providing a physical link between the inner and outer mitochondrial membranes (Murcha et al., 2005). Thus, accumulation of Tim17-2 in the fish4 mutant could enhance the tethering between mitochondrial membranes facilitating preprotein import through the TIM17:23 complex (Donzeau et al., 2000). However, the molecular mechanism detailing how the Tim17-2 levels control the rate of preprotein import into the plant mitochondria remains to be elucidated.

Conclusions

In conclusion, our report suggests that the FTSH4 protease has a novel role in the regulation of preprotein influx into the mitochondria by regulating the abundance of Tim17-2 levels. These findings provide new insights into the significance of ATP-dependent proteolysis in the maintenance of mitochondrial proteostasis in plants.

MATERIALS AND METHODS

Reagents

A detailed list of reagents used in this study is provided in Table S1.

Plant material and growth conditions

Arabidopsis thaliana lines used were: ecotype Col-0 wild type, T-DNA insertion lines fish4-1 (SALK_035107/TAIR) and fish4-2 (GABI_103H09/
TAIR) (Gibala et al., 2009), the *ftsh4-1* FTSH4 complementation line and *ftsh4-1* FTSH4(H486Y) mutant line, and were grown on 0.5× MS medium supplemented with 3% (w/v) sucrose in chambers in a 16 h light and 8 h dark (long-day photoperiod) at 22°C for 2 weeks with a light intensity of 150 µmol m⁻² s⁻¹. To test the morphological phenotype of *A. thaliana* lines, plants were grown in soil in a climate-controlled chamber in a 8 h light and 16 h dark (short-day photoperiod) at 22°C for 10 weeks.

**Generation of *ftsh4-1* FTSH4 complementation line**

FTSH4 cDNA sequence was amplified using primers FTSH4.FLAGfor and FTSH4.FLAGrev (Table S2) and cloned into pENTR/D-TOPO (Invitrogen). The resulting plasmid, FTSH4.FLAG pENTR, was used in a gateway LR recombination reaction with destination vector pGWB514, and a final construct, FTSH4.FLAG pGWB514, was obtained. The *ftsh4-1* FTSH4 complementation line was made by the *Agrobacterium tumefaciens*-mediated transformation (Bernhardt et al., 2012).

**Generation of the *ftsh4-1* FTSH4(H486Y) mutant line**

In order to abolish the proteolytic activity of FTSH4 protease, a highly conserved histidine at the position 486 (a Zn²⁺-binding site, in the HEexH motif) was mutated into a tyrosine residue (Westphal et al., 2012). The mutation was introduced on FTSH4.FLAG pENTR plasmid using a QuickChange Lightening site-directed mutagenesis kit (Agilent Technologies) and primers Mut_prot_H_FP and Mut_prot_H_RP (Table S2) resulting in a FTSH4(HIS).FLAG pENTR plasmid. Next, the Gateway LR recombination reaction with destination vector pGWB514, and a final construct, FTSH4.FLAG pGWB514, was obtained. The *ftsh4-1* FTSH4 complementation line was made by the *Agrobacterium tumefaciens*-mediated transformation (Bernhardt et al., 2012).

**Immunoblot analysis**

Selected proteins were probed with the specific antibodies indicated in Table S3. Proteins were visualized with enhanced chemiluminescence (Mruk and Cheng, 2011) using a G-BOX ChemiXR5 (Syngene, UK) and the results were quantified with ImageJ software.

**Isolation of mitochondria from *Arabidopsis thaliana***

Isolation of mitochondria from 14-day-old seedlings was performed accordingly to a well-established procedure (Murcha and Whelan, 2015).

**2D-BN/SDS-PAGE analysis**

Mitochondria were solubilized in digitonin-containing buffer and 2D-BN/SDS-PAGE analysis was performed as described previously (Wittig et al., 2006; Wang et al., 2012).

**In vitro uptake of mitochondrial ³²S-labeled precursor proteins**

Radiolabeling of model mitochondrial precursors (AOX; Genbank X68702) and ANT (Genbank X57556) with [³²S] methionine and their import into mitochondria was performed as described previously (Duncan et al., 2015). Radiolabeled proteins were visualized by digital autoradiography (PharosFX Plus Systems, Bio-Rad) and analyzed with Quantity One software (Bio-Rad).

**Carbonate extraction assay**

To discriminate between membrane-integrated and soluble mitochondrial proteins, alkaline carbonate extraction assay was performed as described previously (Fujiki et al., 1982).

**Analysis of the kinetics of protein degradation in isolated mitochondria**

The kinetics of protein degradation in isolated mitochondria was analyzed by incubation of mitochondria in assay buffer (300 mM sucrose, 10 mM MOPS-KOH, 80 mM KCl, 5 mM MgCl₂, 8 mM ATP, 12.5 µM ZnSO₄, pH 7.2) at 35°C, followed by SDS-PAGE and immunoblotting.

**Co-immunoprecipitation**

In order to perform immunoprecipitation of FLAG-tagged FTSH4(H486Y) protein, mitochondria were resuspended in digitonin solubilization buffer (1% digitonin, 20 mM Tris-HCl, 0.1 mM EDTA, 100 mM NaCl, 10% glycerol, pH 7.7) at 1 mg/ml. PMSF and EDTA-free protease inhibitor cocktail were added and samples were incubated for 30 min at 4°C with mixing. After a clarifying centrifugation (18,000 g for 15 min), solubilized material was loaded on anti-FLAG affinity matrix and incubated under constant rotation for 1.5 h at 4°C. After excessive washing steps proteins were eluted and subjected to SDS-PAGE and immunoblot analysis. Immunoprecipitation assays using antibodies raised against Tim17-2 and Protein-A–Sepharose resin were performed as described previously (Wang et al., 2012).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

M.O. and H.J. designed the study. M.O. and K.P. performed the experiments. M.W.M. provided the materials. All authors analyzed the data. M.O. wrote the manuscript with contribution from other authors.

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**Supplementary information**

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**References**


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