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A mitochondrial prolyl aminopeptidase PAP2 releases N-terminal proline and regulates proline homeostasis during stress response

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Running Title

A mitochondrial prolyl aminopeptidase involved in proline cleavage.

Keywords

Amino acid, *Arabidopsis thaliana*, drought, mitochondria, pollen, peptide processing pathway, proline, prolyl aminopeptidase, salinity, senescence.

Summary

Most mitochondrial proteins are synthesised in the cytosol and targeted into the organelle via N-terminal targeting peptides that are cleaved upon import. The free targeting peptide is subsequently processed in a stepwise manner, with single amino acids released as final products. Here, we have characterised a proline-cleaving aminopeptidase in *Arabidopsis thaliana*, prolyl aminopeptidase-2 (PAP2, At3g61540). Activity assays show that PAP2 has a preferred activity to hydrolyse N-terminal proline. Protein localisation studies revealed that PAP2 is exclusively targeted to mitochondria. Characterisation of *pap2* mutants show defective pollen, enhanced dark-induced senescence, and increased susceptibility to abiotic stresses, which are likely attributed to a reduced level of accumulated free proline. Taken together, these results demonstrate the role of PAP2 in proline cleavage from mitochondrial peptides and proline homeostasis, which is required for the development of male gametophyte, tolerance to abiotic stresses, and leaf senescence.

Introduction

Mitochondria are energy-generating endosymbiotic organelles that host numerous complex biochemical and biomolecular pathways (Millar *et al.*, 2011). Due to progressive lateral gene transfer over billions of years, the majority of mitochondrial proteins are encoded in the nucleus, translated in the cytosol, and targeted into the correct compartment by specialised translocator machinery (Schleiff and Becker, 2011; Duncan *et al.*, 2013; Murcha *et al.*, 2014; Ghifari *et al.*, 2018). Around 70% of mitochondrial precursor proteins are translated with a cleavable N-terminal targeting peptide called a presequence that acts as a signal for correct sorting (Kmiec *et al.*, 2014). Upon successful protein import, the presequence is cleaved off by the Mitochondrial Processing Peptidase (MPP), generating free peptides. However, the accumulation of free peptides can be detrimental to organellar integrity and activity as an enrichment of peptides disrupts mitochondrial protein import and membrane integrity (Zardeneta and Horowitz, 1992; Hugosson *et al.*, 1994; Nicolay *et al.*, 1994; van't Hof and de Kruijff, 1995; Bruce, 2000). To minimise the adverse effects of free presequences, mitochondria have evolved peptidolytic mechanisms to degrade peptides into single amino acids (Kmiec *et al.*, 2014, 2018a; Teixeira *et al.*, 2017). Released amino acids may either be recycled for *in organello* protein synthesis or exported out of the organelle to be incorporated within various cellular metabolic pathways (Hildebrandt *et al.*, 2015; Teixeira *et al.*, 2017).

Over the past few decades, the targeting peptide processing pathway has been well described in mitochondria and chloroplasts of *Arabidopsis thaliana* (Arabidopsis). After the initial cleavage by MPP or the stromal processing peptidase (SPP), free targeting peptides are trimmed into smaller fragments by the Presequence Protease (PreP) (Bhushan *et al.*, 2003; Moberg *et al.*, 2003; Ståhl *et al.*, 2005) and Organellar Oligopeptidase (OOP) (Kmiec *et al.*, 2013). Peptide degradation by PreP/OOP yields peptide fragments ranging between 3–7 aa that become substrates for further amino acid cleavage by organellar aminopeptidases (Kmiec *et al.*, 2013; Teixeira *et al.*, 2017). Several aminopeptidases that have been characterised in Arabidopsis belong to the metallopeptidase families, including meiotic prophase aminopeptidase-1 (MPA1, also designated as alanyl aminopeptidase AAP) and aminopeptidase-M1 (APM1) that belong

to M1 family (Murphy *et al.*, 2002; Sánchez-Morán *et al.*, 2004; Hosein *et al.*, 2010), leucyl aminopeptidase-1 (LAP1), LAP2, and LAP3 that belong to M17 (Polge *et al.*, 2009; Waditee-Sirisattha *et al.*, 2011), and aspartyl aminopeptidase-1 (DAP1) and DAP2 that belong to M18 (Walling, 2006; Park *et al.*, 2017). Among these enzymes, aminopeptidases MPA1 (M1), LAP2 (M17-20), LAP3 (M17-10), and DAP2 (M18) have been found to be dually localised in both mitochondria and chloroplasts and involved in hydrolysing amino acids during the peptide processing pathway (Teixeira *et al.*, 2017; Kmiec *et al.*, 2018b). These enzymes show distinct substrate specificities and collectively are capable of cleaving almost all proteinogenic N-terminal amino acids. LAP2 exhibits a preferred activity towards hydrophobic and aromatic amino acids (LMFW), while DAP2 shows strong activity towards acidic residues (DE) (Waditee-Sirisattha *et al.*, 2011; Park *et al.*, 2017; Teixeira *et al.*, 2017). On the other hand, MPA1 exhibits a wide range of hydrolytic activity towards polar, non-polar, and aromatic residues (Murphy *et al.*, 2002; Teixeira *et al.*, 2017). Despite their activity to a wide range of amino acids, these metallopeptidases showed no activity towards proline, as determined by *in vitro* and *in organello* activity assays (Teixeira *et al.*, 2017). In addition, peptide degradation studies using isolated mitochondrial fractions revealed that N-terminal proline is released from short peptides (Kmiec *et al.*, 2018b), therefore suggesting the presence of a proline-cleaving aminopeptidase.

Proline contributes to around 5–7% of the total targeting peptide composition (Zybailov *et al.*, 2008; Huang *et al.*, 2009; Ghifari *et al.*, 2019). Despite the low contribution of proline to the overall amino acid composition of targeting peptides, proline residues within the presequence have been shown to be important for efficient translocation, such as import to the chloroplast envelope membrane (Lee *et al.*, 2018). Proline differs from other proteinogenic amino acids whereby its side-chain binds both amino and carboxylic groups, creating a cyclic imino acid group that renders its conformational rigidity (Cunningham and O'Connor, 1997; Szabados and Savouré, 2010). In a peptidolytic event, proline located within precursors or near the amino termini of biologically active peptides serves as structural elements that often hinder general proteolytic activity (Cunningham and O'Connor, 1997).

Prolyl aminopeptidases (PAPs) are the only known group of N-terminal proline-cleaving aminopeptidases. First characterised in *Escherichia coli* (Sarid *et al.*, 1959),

PAPs are conserved across life's kingdoms, including bacteria (Yoshimoto *et al.*, 1983; Gilbert *et al.*, 1994; Kitazono *et al.*, 1994b, 1996), protozoa (da Silva *et al.*, 2016), fungi (Basten *et al.*, 2005; Mahon *et al.*, 2009; Li *et al.*, 2010), plants (Waters and Dalling, 1983; Szawłowska *et al.*, 2006; Marinova *et al.*, 2008), and animals (Khilji *et al.*, 1979). Studies in bacteria have shown that a conserved serine residue is essential for PAP catalytic activity (Kitazono *et al.*, 1994a), hence categorised as a S33 serine peptidase in MEROPS. PAP catalytic activity and structure has been most comprehensively described in bacteria, showing specificity against N-terminal proline and hydroxyproline (Ito *et al.*, 2013). Bacterial PAP primarily targets smaller peptides of up to 12 aa long, whereby its activity decreases with longer peptides (Ito *et al.*, 2013). Overexpression of an Arabidopsis PAP1 isoform (At2g14260.2) resulted in an increase of accumulated free proline in leaf tissues and a higher tolerance to drought and high salinity stresses (Sun *et al.*, 2013). Similarly, overexpression of wheat (*Triticum aestivum*) PAP in Arabidopsis increased the level of accumulated proline in leaf that enhances survival rate and biomass of plants following zinc chloride (ZnCl₂) exposure (Wang *et al.*, 2015a).

In this study, we functionally characterise a putative Arabidopsis PAP, encoded by At3g61540 and named prolyl aminopeptidase-2 (PAP2). We have determined the sub-cellular localisation of PAP2, identified its enzymatic activity, and investigated its physiological roles during plant development. Sub-cellular localisation studies demonstrate that PAP2 is targeted to mitochondria and amino acid cleavage assays show that PAP2 prefers to hydrolyse N-terminal proline. Loss of function mutants of PAP2 exhibit altered plant male gametophyte development and exhibit enhanced susceptibility to various abiotic stresses which can be attributed to altered proline levels. Taken together, our results demonstrate that PAP2 is involved in the release of N-terminal proline in mitochondria and contribute to further understanding the role of prolyl aminopeptidase in regulating proline homeostasis during plant development and stress response.

Results

Proline aminopeptidases have conserved serine peptidase motifs

Arabidopsis contains two PAP homologues PAP1 (At2g14260) and PAP2 (At3g61540), where one homologue PAP1 has been previously characterised (Sun *et al.*, 2013). Based on sequence homology, all queried plant species contain two PAP homologues that are homologous to Arabidopsis PAP1 and PAP2, as well as moss *Physcomitrella patens* and the green algae *Chlamydomonas reinhardtii* (**Figure 1A**). However, only the PAP1 homologue was found in red algae *Cyanidioschyzon merolae*, suggesting a divergent evolutionary path between the two homologues. Phylogenetic analysis of prolyl aminopeptidase homologues from various organisms show that PAP1 and PAP2 from all plant species investigated branch separately from each other (**Figure 1A**). This is despite having a conserved Ser-Asp-His catalytic triad found in PAP homologues of land plants, algae, and bacteria (**Figure 1B**). Both PAP1 and PAP2 contain not only the conserved Ser-His-Asp triad, but also conserved features specific to S33 serine peptidase family according to MEROPS classification (Rawlings *et al.*, 2018), including α/β hydrolase folds and three distinct S33 fingerprint motifs (**Figure 1C**). One of these motifs contains the conserved catalytic serine motif GX**S**ZG, where X and Z represent any amino acid and bulky hydrophobic amino acid, respectively (Ito *et al.*, 2013). In most PAP1 homologues, X and Z are respectively substituted by glycine and tryptophan, whereas in most PAP2 homologues, X and Z are represented by glutamine and phenylalanine/tyrosine (**Figure 1B**).

PAP2 is targeted to mitochondria

TargetP (Emanuelsson *et al.*, 2007) predicts that PAP2 contains a targeting peptide of 49 aa long and is likely to be targeted to the chloroplast (TargetP score: 0.421) with a smaller probability of being targeted to the mitochondria (score: 0.179), (**Supplementary Figure S1**). PAP2 has also been detected in chloroplasts by mass spectrometry deposited in AT_CHLORO database (Ferro *et al.*, 2010). However, an independent mass spectrometry analysis also identified PAP2 in mitochondria (Senkler *et al.*, 2017). Therefore to confirm the sub-cellular localisation of PAP2, we carried out fluorescence tagging of the first 100 residues of PAP2 using both *in vivo* transient and *in planta* stable transformations (**Figure 2**). *In vivo* biolistic transformations of Arabidopsis cell cultures show no overlap of PAP2-GFP signals with the chloroplastic RFP marker (SSU-RFP)

(**Figure 2A**, top panel), whereas co-localisation of PAP2-GFP with the mitochondrial RFP marker (AOX-RFP) was clearly observed (**Figure 2A**, bottom panel) suggesting an exclusive mitochondrial localisation. We confirmed this result using Arabidopsis plants stably transformed with PAP2-GFP. In root hair, PAP2-GFP signals appear to be small, rod and round-shaped distinctive of mitochondria. Similarly, in leaf mesophyll cells, PAP2-GFP signals are observed as small rods with no overlap to chlorophyll auto-fluorescence (**Figure 2B**), confirming the mitochondrial targeting of PAP2.

PAP2 can release N-terminal proline

In bacteria, prolyl aminopeptidases have been shown to release N-terminal proline or hydroxyproline from short peptides (Kitazono *et al.*, 1994b, 1996). To investigate the molecular activity of PAP2, we expressed and purified the recombinant protein alongside previously characterised organellar aminopeptidases LAP2 (M17) and DAP2 (M18) (Waditee-Sirisattha *et al.*, 2011; Park *et al.*, 2017; Teixeira *et al.*, 2017; Kmiec *et al.*, 2018b). Synthetic probes consisting of an N-terminal amino acid bound to a chromophore (Leu-MCA, Lys-MCA, Asp-MCA, and Pro-ACC) were used as substrates. As the peptide bond between amino acid and chromophore is cleaved, fluorescent signals are released and detected as hydrolytic activity of the enzymes. This assay demonstrated that LAP2 has a preference against N-terminal leucine, whilst DAP2 prefers N-terminal aspartate as a substrate (**Figure 3A**), which are consistent with previously published results (Waditee-Sirisattha *et al.*, 2011; Park *et al.*, 2017; Teixeira *et al.*, 2017). On the other hand, PAP2 showed a preferred activity in cleaving N-terminal proline, with a substantially less activity to other amino acids tested (**Figure 3A**). Proline-cleaving activity by PAP2 is substantially higher than any other peptidases tested, demonstrating PAP2 has preferred activity towards N-terminal proline. Taken together, this enzymatic activity assay confirmed that PAP2 has a preferred cleaving activity to N-terminal proline, compared to N-terminal leucine, lysin, or aspartate that represent non-polar, polar cationic, and polar anionic residues, respectively. Therefore, we propose that PAP2 is a proline-cleaving aminopeptidase that releases N-terminal proline in mitochondria (**Figure 3B**).

Deletion of PAP2 affects pollen viability

To study the potential function of PAP2 in plant growth and development, we obtained two T-DNA insertion lines *pap2-1* (SAIL_738H05) and *pap2-2* (GABI_615G01) (**Figure 4A**). The lines were PCR-screened for homozygosity and the locations of the T-DNA inserts were confirmed by Sanger sequencing (**Supplementary Figure S2, Figure 4A**). Quantitative RT-PCR confirms loss of function with a significant depletion of transcript compared to the Col-0 that was restored almost to Col-0 levels upon complementation (35S::*PAP2*) (**Figure 4B**, indicated by *; $p < 0.01$, $n = 6$). Stage-growth progression analysis (Boyes *et al.*, 2001) during seed germination, seedling development, and full-cycle growth was carried out for all lines. However, no significant differences were observed in *pap2* mutants under normal growth conditions (**Supplementary Figure S2**).

Proline has been demonstrated to affect the flowering time and development of the reproduction system, including the formation and maturation of the plant male gametophyte or pollen (Mattioli *et al.*, 2008, 2009a,b; Funck *et al.*, 2012). As PAP2 has proline-releasing activity and is highly expressed in all Arabidopsis organs including floral tissues (**Supplementary Figure S1**), we investigated the pollen phenotype of *pap2-1* and *pap2-2* mutants to assess the role of PAP2 in pollen development. Healthy pollen contains protoplasm, which is composed of the cytoplasm, nucleus, and colourless organelles such as mitochondria that retain the magenta fuchsin acid dye. In contrast, aberrant or non-viable pollen only contain cell walls that accumulate the malachite green pigment (Alexander, 1969). As a result, non-viable pollens appear pale green and shrivelled (indicated by arrows), whereas viable pollen stain purple and shaped globular (**Figure 4C**). All lines tested showed a similar total pollen count (**Figure 4D**), whilst *pap2-1* and *pap2-2* displayed a significantly lower percentage of viable pollen compared to Col-0 with 70% and 60% viable seeds for *pap2-1* and *pap2-2* ($p < 0.001$, $n = 10$), respectively. This phenotype, however, was restored in the complementation line (**Figure 4D**). To determine if the defective pollen phenotype was a result of altered proline levels, free proline abundance was measured in floral samples using the proline-ninhydrin colorimetric assay (Troll and Lindsley, 1955; Bates *et al.*, 1973). *pap2-1* and *pap2-2* showed a significant decrease in floral free proline by up to three-fold compared to Col-0 ($p < 0.001$, $n = 6$), which was restored in the complemented line 35S::*PAP2* (**Figure 4E**). In contrast, no significant change in proline content was detected in leaf samples of *pap2-1*

and *pap2-2* compared to Col-0 (**Figure 4E**). These findings indicate that the loss of function of PAP2 has decreased the level of accumulated free proline in pollen and floral tissues, which may directly affect the pollen viability.

Deletion of PAP2 enhances plant susceptibility to abiotic stresses

Proline accumulation has been associated with tolerance to a range of abiotic stresses (Yoshida *et al.*, 1995; Choudhary *et al.*, 2005). Additionally, PAP homologues in plants were previously identified to have a role in conferring tolerance to various abiotic stresses (Sun *et al.*, 2013; Wang *et al.*, 2015a). To investigate the potential role of PAP2 in the plant stress response, we examined *pap2-1* and *pap2-2* under salt and drought stress during vegetative growth (**Figure 5A**). When plants were exposed to NaCl and ZnCl₂, *pap2-1* and *pap2-2* were observed to have wilted leaves and an apparent delay in growth compared to Col-0. During drought treatment, *pap2-1* and *pap2-2* also exhibited a delay in growth with chlorosis evident in the leaves (**Figure 5A**). Complementation of PAP2 restored the growth delay and stress phenotype (**Figure 5A**). Measurements of total leaf proline content in stress-treated plants was carried out and proline content was significantly lower in *pap2-1* and *pap2-2* compared to Col-0 and 35S::PAP2 (**Figure 5B**) ($p < 0.05$, $n = 6$). These findings reiterate that proline accumulates in leaves during stress and suggests the PAP2 may play a role in stress tolerance by modulating proline homeostasis.

We further investigated the role of PAP2 in stress tolerance but this time during seed germination, as mitochondria are the main energy source during this important developmental stage. Under normal conditions, no significant differences could be observed across genotypes (**Figure 5C**). However, during salt (NaCl and ZnCl₂) treatment, the percentage of germinated *pap2-1* and *pap2-2* seeds were significantly lower compared to Col-0 and 35S::PAP2 after 36 h and 60 h following exposure to light under NaCl and ZnCl₂ media, respectively (**Figure 5C**) (indicated by * $p < 0.05$, $n = 3$).

Deletion of PAP2 results in enhanced dark-induced senescence

During leaf senescence, chloroplasts are progressively degraded and the energy status is solely regulated by mitochondria (Keech *et al.*, 2007). To compensate for the loss of primary energy from chloroplasts, mitochondria provides energy from alternative sources,

such as the catabolism of amino acids, including proline (Keech *et al.*, 2007; Chrobok *et al.*, 2016). To investigate the potential role of PAP2 during this process, dark-induced senescence assays were carried out on *pap2-1*, *pap2-2* and *atg5* (*autophagy-related protein 5*), a well known mutant that displays early leaf senescence (Sakuraba *et al.*, 2014). Leaf senescence during vegetative growth was induced by dark treatment on detached leaves or by wrapping attached leaves with aluminium foil during normal plant growth (Pružinská *et al.*, 2005). Prior to treatment, detached leaves of 5-week-old plants showed no significant differences in chlorophyll content ($p > 0.05$; $n = 3$) (**Figure 6A**). Following 3 d dark treatment, *pap2-1*, *pap2-2* and *atg5* displayed earlier leaf yellowing (**Figure 6A**) and significantly lower chlorophyll content compared to Col-0 and 35S::PAP2 (*pap2-1*, $p < 0.01$; *pap2-2*, $p < 0.05$; *At5g* $p < 0.005$, $n = 3$) (**Figure 6A**). Following 3-d treatment, the chlorophyll content of Col-0 and 35S::PAP2 was observed to decrease to 45%, whilst both *pap2* mutants exhibited a decrease of around 65% and *atg5* decreased to around 77% (**Figure 6A**). Dark-induced senescence of attached leaves was also carried out with chlorophyll content measured four times at 2 d intervals (**Figure 6B**). As previously reported (Sakuraba *et al.*, 2014), the *atg5* mutant displayed an early senescence phenotype with significant decreases in chlorophyll content at all four time points (**Figure 6B**) (indicated by * $p < 0.01$; $n = 3$). A significant decrease in chlorophyll content in *pap2-1* and *pap2-2* was apparent only from 4-d following treatment ($p < 0.05$, $n = 3$) and this accelerated leaf senescence was restored in the 35S::PAP2 complemented line (**Figure 6B**).

Discussion

In this study, we have demonstrated that PAP2 is a mitochondrial-targeted active aminopeptidase that has preferred activity towards N-terminal proline relative to the other amino acids tested: leucine (non-polar), lysine (polar cationic), and aspartate (polar anionic). Phenotypic studies using loss of function mutants demonstrate that PAP2 is involved in proline homeostasis during plant development and in response to abiotic stresses. Deletion of PAP2 significantly reduces proline content in floral tissues that negatively affects pollen viability. The *pap2* mutants also displayed an enhanced susceptibility to abiotic stresses such as high salinity and drought during vegetative growth and germination. Furthermore, early senescence could be induced by dark

treatment of *pap2* mutants demonstrating the role of PAP2 in proline homeostasis during stress exposure and development.

Sequence analysis reveals that PAP2 shares a conserved Ser-His-Asp catalytic triad and S33 protein family fingerprint motifs to PAP1 (**Figure 1C**), a previously characterised in Arabidopsis PAP homologue (Sun *et al.*, 2013), that is evolutionarily distant to PAP2 (**Figure 1A**). Sun *et al.*, had showed that the shorter isoform of PAP1 (At2g14260.2, 329 aa) is localised to the cytosol as determined by biolistic transformation of onion epidermal cells (Sun *et al.*, 2013). However, the longer isoform of PAP1 (At2g14260.1, 380 aa) (**Figure 1C**) is predicted to contain a putative chloroplast targeting peptide (**Supplementary Figure S1**). Deletion of PAP1 in Arabidopsis decreased overall proline content and enhanced the plants susceptibility to osmotic stresses such as high salinity and drought (Sun *et al.*, 2013), suggesting a role in maintaining proline homeostasis during stress. Similarly, overexpression of wheat PAP1 in Arabidopsis also renders an increased tolerance to zinc stress, which is also likely to be attributed to the increased proline level (Wang *et al.*, 2015a). However, despite being identified to have a role in proline homeostasis, the molecular activity of PAP1 and its correct localisation remains to be confirmed.

Biochemical analyses of recombinant PAP2 shows that it is able to release N-terminal proline, whereby other aminopeptidases tested exhibited substantially less activity towards proline. PAP2 also exhibited substantially lower activity against other amino acids tested (**Figure 3**), which demonstrates that a substrate with an N-terminal proline is a PAP2 preferred substrate. The activity of Arabidopsis PAP2 is similar to the proline-cleaving activity of bacterial PAP homologues (Yoshimoto *et al.*, 1983; Kitazono *et al.*, 1996; Murai *et al.*, 2004; Yamamoto *et al.*, 2010), which is unsurprising given the high sequence conservation and conserved catalytic motifs (**Figure 1**). Using both *in vivo* transient biolistic transformation and *in planta* stable GFP-tagged lines of PAP2, we determined that PAP2 is exclusively targeted to mitochondria (**Figure 2**), supported by an independent proteomic study that identified PAP2 within mitochondrial matrix fractions (Senkler *et al.*, 2017). The activity and sub-cellular location of PAP2 shown here supports previous studies which proposed the presence of yet unidentified prolyl aminopeptidases within the mitochondrial matrix and thought to be involved in amino acid recovery (Teixeira *et al.*, 2017; Kmiec *et al.*, 2018b).

Amino acid recovery is the last stage of a multi-step peptide processing pathway, which is catalysed by M1, M17, and M18 metallo-aminopeptidases (Teixeira *et al.*, 2017; Kmiec *et al.*, 2018b; Ghifari *et al.*, 2019). These metallo-aminopeptidases have been shown to release almost all proteinogenic amino acids, except proline (Kmiec *et al.*, 2018b). In addition, loss of function mutants of MPA1 (M1), LAP2 (M17) and DAP2 (M18) were observed to exhibit altered amino acid recovery in both chloroplasts and mitochondria, with no effect to proline cleavage (Teixeira *et al.*, 2017; Kmiec *et al.*, 2018b). Therefore, the localisation and molecular activity of PAP2 affirm the role of PAP2 in releasing N-terminal proline from proteins or peptides generated by the mitochondrial peptide processing pathway (**Figure 3B**).

Using T-DNA insertion lines we explored the role of PAP2 in proline homeostasis during normal plant growth, development, and abiotic stress response. Under normal growth conditions, *pap2* mutants showed no significant growth and developmental differences compared to Col-0 (**Supplementary Figure 2**). This result demonstrates that the lack of N-terminal proline hydrolysis has no direct deleterious impact to plant vegetative growth and development. It is also possible that disruption of organellar amino acid recovery from peptides has no obvious impact to the growth rate of whole plants as also observed in the deletions of MPA1 (M1) and LAP3 (M17-20) (Teixeira *et al.*, 2017). The disruption of amino acid recovery is likely compensated by the various metabolic processes that can maintain amino acid homeostasis, such as biosynthesis and turnover (Hildebrandt *et al.*, 2015; Batista-Silva *et al.*, 2019).

However, when we further investigated the *pap2* mutants, we found that deletion of PAP2 severely affects pollen viability with a corresponding decrease to floral proline content (**Figure 4**). Previous studies demonstrated that failure of proline accumulation in flower, anther, and pollen disrupts pollen development and maturation as the development of pollen cell wall and microspore is compromised (Mattioli *et al.*, 2008, 2009b, 2012, 2018). The level of pollen proline content appears to positively correlate with pollen viability and fertility (Mattioli *et al.*, 2018). Disruption of the enzymes directly involved in proline biosynthesis, such as Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) and reductase (P5CR) resulted in severely aberrant pollen and retarded reproductive development (Funck *et al.*, 2012; Mattioli *et al.*, 2012). Our results further demonstrate the importance

of proline accumulation in pollen development and the role of PAP2 in maintaining the level of free proline in generative tissues.

Functional loss of PAP2 resulted in an increased sensitivity to high salinity and drought stress with decreased proline content in *pap2* leaves compared to Col-0 (**Figure 5**). Proline has been widely characterised as an important osmolyte that accumulates during plant response to osmotic stresses. Proline contributes in substituting primary energy metabolites as an alternative substrate, scavenging reactive oxygen species (ROS), stabilising cell wall and sub-cellular structure, and acting as a signalling molecule to maintain homeostasis of other metabolites (Lehmann *et al.*, 2010; Kishor *et al.*, 2015; Wang *et al.*, 2015b; Batista-Silva *et al.*, 2019; Launay *et al.*, 2019). The role of prolyl aminopeptidase homologues in plant abiotic stress tolerance has also been previously reported. Overexpression of Arabidopsis and wheat PAP1 in Arabidopsis enhanced resistance to drought and high salinity stresses including NaCl and ZnCl₂, likely due to an increased accumulation of free proline (Sun *et al.*, 2013; Wang *et al.*, 2015a). The enhanced sensitivity of *pap2* mutants to salt stresses and the corresponding lower accumulated proline presented here further supports the role of PAP2 in maintaining proline homeostasis in plants during stress.

Senescence is the last stage of leaf development whereby cellular materials are gradually degraded and converted into transportable nutrients (Lim *et al.*, 2007). To supply the cellular energy demands following chloroplast degradation, mitochondria undergo the catabolism of amino acids such glutamic acid, aspartic acid, alanine, serine, glycine, and proline to provide energy needed for nutrient transport (Chrobok *et al.*, 2016). Moreover, mitochondrial proline catabolism is required to fuel leaf senescence by restoring the energy loss from decreases in the TCA cycle and mitochondrial respiration (Launay *et al.*, 2019). Proline levels have been shown gradually decrease during leaf senescence in Arabidopsis and tobacco (Masclaux *et al.*, 2000; Chrobok *et al.*, 2016) with mitochondrial proline likely to be oxidised and converted into glutamate to provide additional energy to assist in nitrogen remobilisation, essential process in the leaf senescence (Chrobok *et al.*, 2016; Launay *et al.*, 2019).

PAP2 is highly expressed in all rosette leaf developmental stages, including senescing leaves (**Supplementary Figure S2**). Our findings show that *pap2* knockouts exhibit an accelerated dark-induced senescence when tested in both detached and attached leaves

(Figure 6). During leaf senescence, proline can be obtained from the degradation of organelle and cell wall proteins, which have high proline and hydroxyproline content (Ihsan *et al.*, 2017). Inactivation of PAP2 combined with the down-regulation of mitochondrial amino acid biosynthesis during senescence (Breeze *et al.*, 2011), may further decrease proline abundance and consequently accelerate senescence progression. The involvement of organellar aminopeptidase activity during leaf senescence has also been previously observed with LAP2 whereby *Arabidopsis lap2* mutants also exhibited an early leaf senescence phenotype (Waditee-Sirisattha *et al.*, 2011). LAP2, which has been shown to release non-polar N-terminal amino acids such as leucine, phenylalanine, and tryptophan from targeting peptides (Teixeira *et al.*, 2017; Kmiec *et al.*, 2018b), was also shown to regulate intracellular amino acid turnover (Waditee-Sirisattha *et al.*, 2011). The senescence phenotype of *lap2* mutants was attributed to diminished GABA (γ -aminobutyric acid), a metabolite responsible for stress signalling in plants, which is derived from glutamate metabolism (Waditee-Sirisattha *et al.*, 2011) an important precursor for nitrogen remobilisation (Lin and Wu, 2004; Chrobok *et al.*, 2016). As glutamate can be synthesised from proline catabolism (Funck *et al.*, 2008), it can be inferred that proline catabolism is also important for leaf senescence and decreases in proline abundance accelerates leaf senescence progression.

Conclusion

In summary, this study demonstrated the presence of a *Arabidopsis* prolyl aminopeptidase named PAP2 that contains the conserved catalytic residues and fingerprint motifs of S33 aminopeptidase family. PAP2 is localised to the mitochondria and has the ability to release N-terminal proline, demonstrating its role in amino acid recovery in mitochondrial peptide processing. *pap2* mutants exhibit pollen defects and enhanced sensitivity to abiotic stresses which is attributed to decreased proline content therefore indicating that PAP2 is required for maintaining proline homeostasis during pollen development, stress response, and leaf senescence.

Methods

Bioinformatics and phylogenetic analysis

Prolyl aminopeptidase homologues in plants were identified and retrieved from Phytozome v12.1 (<https://phytozome.jgi.doe.gov/>) (Goodstein *et al.*, 2012). Homologues from bacteria, fungi, and animals were identified by Protein BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and sequences retrieved from Uniprot (<http://www.uniprot.org/>). Protein sequence alignments were carried out using MEGA7 with MUSCLE parameters (Edgar, 2004). The phylogenetic tree was constructed in MEGA7 using the Jones-Taylor-Thornton (JTT) maximum-likelihood method (Jones *et al.*, 1992) with 1,000 bootstrap replications. Protein domains were identified using InterPro (<http://www.ebi.ac.uk/interpro/search/sequence-search>) (Finn *et al.*, 2017). Targeting peptides cleavage sites were predicted using TargetP (Emanuelsson *et al.*, 2007). Sequence logo was generated using WebLogo (Crooks *et al.*, 2004).

Cloning and plasmid constructs

Gateway® site-specific recombination technology (Invitrogen) (Katzen, 2007) was used to clone all constructs in this study from Arabidopsis Columbia-0 (Col-0) cDNA using gene-specific primers (**Supplementary Table S1**). Sequences encoding full-length or the first N-terminal 100 amino acids of PAP2 were cloned into pDONR201 and subsequently into pDEST-cGFP (C-terminal green fluorescent protein) (Carrie *et al.*, 2009) for *in vivo* cell culture biolistic transformation, pGWB5 for *in planta* GFP expression (Nakagawa *et al.*, 2007), and pB2GW7 for gene complementation (Karimi *et al.*, 2002). For protein expression, PAP2 cDNA was cloned into pET15b as described previously (Kmiec *et al.*, 2013, 2018; Teixeira *et al.*, 2017).

In vivo biolistic transformation, *in planta* stable expression, and fluorescence microscopy

For *in vivo* localisation assay, C-terminal red fluorescent protein (RFP) attached with soybean (*Glycine max*) alternative oxidase (AOX) (Carrie *et al.*, 2008) and pea (*Pisum sativum*) small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Carrie *et al.*, 2009) were used as mitochondrial and chloroplastic targeting markers, respectively. Biolistic transformation was performed using the Particle Delivery System

PDS-1000/He (Bio-Rad). Initially, 5 µg of GFP and RFP-tagged plasmids were co-precipitated onto gold nanoparticles (Bio-Rad) before being bombarded with pressurised helium into 4-day old Arabidopsis PSB-D Col-0 cell culture (Menges and Murray, 2002). Transformants were incubated at 22°C for 18-24 h in the dark before being visualised using a fluorescent BX61 microscope (Olympus). For *in planta* localisation analysis, Arabidopsis Col-0 plants were stably transformed with PGWB5–PAP2 using the *Agrobacterium tumefaciens* floral dip method. Fluorescence was visualised using the excitation/emission at 460-480/495-540 nm (GFP), 535-555/570-625 nm (RFP), 540-550/565-575 nm (TRITC). Images were captured using Cell^R Xcellence RT (Olympus).

Protein expression, purification and activity assays

Recombinant PAP2, LAP2, and DAP2 were expressed with N-terminal 6xHis tags in *E.coli* Rosetta-2 competent cells (Novagen). Proteins were purified with Ni-NTA agarose columns as previously described (Kmieć *et al.*, 2013, 2018b; Teixeira *et al.*, 2017). To measure aminopeptidase enzymatic activity, 5 µg of purified protein was incubated with 0.5 µg of fluorogenic substrates (Leu-MCA, Lys-MCA, Asp-ACC, Pro-ACC; MCA = 4-methyl-coumaryl-7-amide, ACC = 7-amino-4-carbamoyl-methyl-coumarin) as previously described (Teixeira *et al.*, 2017). All measurements were carried out in triplicate and results obtained as the relative fluorescence unit per second (RFU/s).

T-DNA insertional and complementation lines

Arabidopsis transfer DNA (T-DNA) insertional lines for PAP2, *pap2-1* (SAIL_738H05) and *pap2-2* (GABI_615G01) were obtained from the Arabidopsis Biological Resource Centre (ABRC) or Nottingham Arabidopsis Stock Centre (NASC). Insertional lines were screened for homozygosity using the recommended exon-specific primers designed in SALK T-DNA Primer Design (<http://signal.salk.edu/tdnaprimers.2.html>) (**Supplementary Table S1**). A PAP2 complemented line (35S::PAP2) was generated by stably transforming *pap2-2* with pB2GW7-PAP2 using the *Agrobacterium tumefaciens* floral dip method. Quantitative real-time PCR (qRT-PCR) was carried out to measure relative transcript abundance. Total RNA was isolated from 14-day-old seedlings using RNeasy Plant Kit (Qiagen) according to manufacturer's instructions. Total cDNA was then synthesised from RNA using random primers and the reverse transcriptase system

(Invitrogen). Standards for quantitative RT-PCR were synthesised from Col-0 cDNA using exon-specific primers (**Supplementary Table S1**) and purified using QIAQuick PCR Purification Kit (Qiagen). QRT-PCR was performed in LightCycler 480 (Roche) using 2X iQ SYBR Green Supermix (Bio-Rad). All measurements were performed in duplicates from three biological replicates. For statistical analysis, transcript abundance was normalised and compared with Col-0 to determine relative abundance. Student's t-test ($p < 0.05$) was used to determine any significant differences between Col-0 and mutants.

Plant materials, growth, and phenotyping

For biolistic transformation, Arabidopsis PSB-D cell cultures (Menges and Murray, 2002) were grown in a medium containing 4.3 g/L Linsmaier-Skoog salt, 30 g/L sucrose, 0.5 mg/L 1-naphthaleneacetic acid (NAA), and 50 μ g/L kinetin (pH adjusted to 5.7 with KOH). Plate-based and soil-based plant phenotyping was performed according to previously described methods (Boyes *et al.*, 2001). For soil-based phenotyping, plants were grown on a soil-vermiculite-perlite mix (3:1:1) under long-day conditions (22°C, 16-h day and 8-h night, 100 μ mol/m²s light intensity) following 3-d cold and dark stratification. For plate-mediated phenotyping seeds were chlorine gas-sterilised and sown on half-strength Murashige-Skoog (MS) media (composed of 2.15 g/L MS basal salt medium powder, 0.1% v/v Gamborg B5 vitamins, 0.5 g/L morpholine-ethanesulfonic acid (MES), 7.5 g/L agar, 1.5% (w/v) sucrose, pH adjusted to 5.7 with KOH) following 3-d cold and dark stratification.

Stress treatment and senescence

For salt stress, plants grown on soil were watered with deionised water supplemented with NaCl and ZnCl₂. Salt stress was applied with increasing concentrations (NaCl: 100, 150, 200, and 250 mM; ZnCl₂ 10, 15, 20, 25 mM) with a 4-d interval. Each tray containing 20 pots were watered with 800 mL salt solution. For drought stress, watering was withheld for 14 days as described previously (Waditee-Sirisattha *et al.*, 2011). For salt stress phenotyping during germination, sterilised seeds were sown on half-strength MS media supplemented with various salt concentrations (NaCl 100 mM, ZnCl₂ 20 mM). Germination rates were determined from a minimum of 100 seeds in three independent replicates following exposure to light over 0-60 h as described previously (Wang *et al.*,

2014). Senescence phenotyping was conducted on detached leaves incubated in the dark and on attached leaves during normal growth conditions (Pružinská *et al.*, 2005). In both detached and attached leaf senescence assays, rosette leaf number 5, 6, and 7 were used from 5-week-old plants. For detached leaf assays, leaves were placed on a Whatman filter paper, slightly dampened with water and incubated in the dark for 3 days. For attached leaf assays, rosette leaves were dark-treated by wrapping with aluminium foil for 8 d. The chlorophyll content of detached and attached leaves was measured using the SPAD-502 chlorophyll meter (Minolta) and averaged from measurements taken in three distinct areas. A minimum of three individual plants were used for all chlorophyll measurement.

Pollen staining

Pollen staining was carried out on freshly opened flowers at the first stage of flower production (stage 6.10) using the Alexander's staining method (Alexander, 1969). For viability assays, pollen grains were collected by dipping a freshly opened flower to a droplet of staining solution on a glass slide. The slide was then covered with a coverslip, heated, and dried at 60°C before imaging under a light microscope. For pollen counts, a single flower was incubated in 100 µL staining solution, vortexed, and a 1 µL aliquot was taken to count healthy and aberrant pollen under a light microscope. All measurements were carried out in biological triplicate from at least five flowers from five individual plants of each genotype ($n = 5$). Student's t-test was used to determine significantly different datasets ($p < 0.05$).

Proline content assay

Proline content was measured using ninhydrin-based colorimetric assay (Bates *et al.*, 1973). Floral and rosette leaf samples were collected at stage 6.10 (initial flower production stage) (Boyes *et al.*, 2001). Free proline was extracted by heating ground tissue with 70% (v/v) ethanol at 60°C. Standards and samples were heated to 95°C for 20 min with ninhydrin reaction mixture (1% w/v ninhydrin in 60% v/v acetic acid and 20% v/v ethanol). After cooling to room temperature in the dark, the mixture was centrifuged at 10,000 g for 1 min and the absorbance (520 nm) measured on SPECTROStar microplate

reader (BMG Labtech). Every measurement was carried out in biological triplicate and averaged from at least three biological replicates for each genotype.

Data Availability Statement

All relevant data can be found within the manuscript and its supporting materials

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Author Contribution

PFT, EG, and MWM designed the experiments; ASG, PFT, BK, and AP performed the experimental procedures; ASG, PFT, and MWM analysed and interpreted the data; ASG and MWM wrote the manuscript with inputs from all co-authors.

Conflict of Interest

The authors declare no conflict of interest.

Supporting Materials Legends

Supplementary Table S1. Sequence of primers used in the study.

Supplementary Figure S1. Bioinformatics analyses of prolyl aminopeptidase sequences.

Supplementary Figure S2. Genotyping and phenotypic analyses of T-DNA insertional lines under normal conditions.

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Figure Legends

Figure 1. Sequence and phylogenetic analyses of proline aminopeptidases

A. Phylogenetic tree of proline aminopeptidase homologues from various species of plants, algae, bacteria, fungi, and mammals. The tree was built based on protein sequences aligned with MUSCLE and was constructed using maximum-likelihood method and Jones-Thornton-Taylor (JTT) approach with 1,000 bootstrap replications. Branch length denotes evolutionary distance and numbers on each branch represent the percentage of bootstrap support.

B. Multiple sequence alignment of two proline aminopeptidase homologues in *Arabidopsis*: prolyl aminopeptidase-1 (PAP1, At2g12460) and prolyl aminopeptidase-2 (PAP2, At3g61540) with orthologues from various species. Highly conserved and identical amino acids are highlighted in grey and blue (red asterisks), respectively. Conserved catalytic triad Ser-Asp-His (S-D-H) are highlighted in green.

C. Schematic diagram of *Arabidopsis* proline aminopeptidases protein domains. Both proteins contain α/β hydrolase fold (turquoise) and three S33 fingerprint motifs (red) illustrated in WebLogo diagrams based on previous alignment with the catalytic serine motif (GXSBG) is highlighted (grey box). Putative targeting sequence (dark blue) is based on TargetP prediction.

Abbreviations: At, *Arabidopsis thaliana*; As, *Aeromonas sobria*; Bc, *Bacillus coagulans*; Br, *Brassica rapa*; Cm, *Cyanidioschyzon merolae*; Cr, *Chlamydomonas reinhardtii*; Hs, *Homo sapiens*; Ld, *Lactobacillus delbrueckii*; Mm, *Mus musculus*; Nm, *Neisseria meningitidis*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Sc, *Saccharomyces cerevisiae*; Sl, *Solanum lycopersicum*; Sm, *Serratia marcescens*; Zm, *Zea mays*.

Figure 2. PAP2 is localised to the mitochondria

A. Fluorescence visualisation of *in vivo* transient transformation of Arabidopsis Col-0 cell culture. PAP2 tagged to GFP was co-transformed with either a chloroplastic marker (SSU-RFP) or mitochondrial marker (AOX-RFP). Chloroplasts (C) and mitochondria (M) are indicated with arrows (scale bar: 20 μ m).

B. Visualisation of *in planta* stably transformed Col-0 with PAP2 tagged to GFP and expressed under cauliflower mosaic virus (CaMV) 35S promoter. Fluorescence was captured in leaf mesophyll cells and root hairs for PAP2-GFP and chlorophyll auto-fluorescence, while bright field was captured to determine the cell layout. Chloroplasts (C) and mitochondria (M) are indicated by arrows (scale bar: 20 μ m).

Figure 3. PAP2 shows specificity for proline

A. Enzyme hydrolytic activity of recombinant purified Arabidopsis aminopeptidases and oligopeptidase. Aminopeptidase activity was measured using fluorogenic probes: Leu-MCA, Lys-MCA, Asp-ACC, and Pro-ACC. Data is presented as average $\pm$ SD of three independent replications. Significantly different datasets are shown in asterisks (Student's t-test, $p < 0.05$, $n = 3$).

B. Schematic illustration highlighting the possible role of PAP2 in the mitochondrial peptide processing pathway. After import by the mitochondrial translocase of the inner membrane (TIM) the mitochondrial presequence is cleaved by the mitochondrial processing peptidase (MPP). The cleaved presequences and peptides generated from general mitochondrial proteolysis undergoes sequential degradation by presequence peptidase (PreP) and organellar oligopeptidase (OOP) to generate intermediate short peptide fragments. Short peptides are subsequently degraded by the aminopeptidases MPA1, LAP2, DAP2, and PAP2 to release single amino acids.

Figure 4. Deletion of PAP2 results in defective pollen viability and altered proline content

A. Schematic diagram of the *PAP2* gene structure in Arabidopsis (At3g61540) with exons (blue boxes) and introns (grey lines). The T-DNA insert locations for *pap2-1* and *pap2-2* the amplicons used for quantitative real-time polymerase chain reaction (QRT-PCR) are indicated.

B. Reverse-transcription polymerase chain reaction (RT-PCR) and QRT-PCR of *PAP2* transcript from Col-0, *pap2-1*, *pap2-2* and the complemented line *35S::PAP2*. *ACTIN2* was used as a positive control. Significantly different datasets are shown in asterisks (Student's t-test, *** $p < 0.001$, $n = 3$).

C. Alexander-stained stamen and pollen of Col-0, *pap2-1*, *pap2-2* and *35S::PAP2*. Aberrant (non-viable) pollens appear green and shrivelled as indicated by the arrows. Healthy viable pollen are spherical and stain purple. Scale bar = 100 μm .

D. Total pollen counts per flower measured from at least six freshly opened flowers from different plants of Col-0, *pap2-1*, *pap2-2* and *35S::PAP2* (mean $\pm$ SD; $n = 6$) and percentage of viable pollens of each genotype (mean $\pm$ SD; $n = 10$). Significantly different datasets are shown in asterisks (Student's t-test, $p < 0.05$).

E. Total free proline content per fresh weight (FW) of floral and leaf samples of each genotype Col-0, *pap2-1*, *pap2-2* and *35S::PAP2*. Samples were collected at the initial flowering stage (Stage 6.10, Boyes et al., 2003). Proline concentration was measured using proline-ninhydrin colorimetric assay (mean $\pm$ SD; $n = 3$). Significantly different datasets are shown in asterisks (Student's t-test, $p < 0.05$).

Figure 5. Deletion of PAP2 results in enhanced sensitivity to salt and drought treatment and altered proline content

A. Representative images of four week old Col-0, *pap2-1*, *pap2-2* and 35S::PAP2 exposed to various abiotic stresses over two weeks. Phenotypes were analysed from at least 40 plants of each genotype in three independent replications.

B. Proline content measurements of leaves excised from plants following stress treatment. Proline was measured using proline-ninhydrin colorimetric assay (mean $\pm$ SD; $n=3$). Significantly different datasets are shown in asterisks (Student's t-test, $p<0.05$).

C. Germination rates of Col-0, *pap2-1*, *pap2-2* and 35S::PAP2 at normal growth conditions (solid lines) and under salt (NaCl and ZnCl₂) stress (dotted lines). Germination rates were observed from 0-60 h post exposure to light from three independent replications. Germination rates is expressed as percentage of seeds displaying radicle emergence at a given time point (minimum $n=100$) (mean $\pm$ SD; $n=3$). Significantly different datasets are shown in asterisks (Student's t-test, $p<0.05$).

Figure 6. Deletion of PAP2 results in enhanced dark-induced senescence

A. Individual leaves (rosette leaves 5, 6, and 7) detached from 5-week old plants and incubated in the dark for 3 days to induce leaf senescence. The left-hand panel shows representative images and the right-hand panel shows chlorophyll content (measured using SPAD-502 chlorophyll meter) before (0-d) and following dark treatment (3-d) (mean $\pm$ SD; $n=3$). Significantly different datasets are shown in asterisks (Student's t-test, $p<0.05$).

B. Representative images of 5-week-old plants under normal conditions (16 h light/8 h dark photoperiod) and following 8-d of dark-induced senescence (24 h dark photoperiod). Rosette leaves 5, 6, and 7 used for chlorophyll measurements are indicated by circles. The right-hand panel shows chlorophyll concentration per leaf area (in nmol cm^{-2}) of each genotype under normal conditions (dotted lines) and during dark treatment (solid lines). Chlorophyll concentration was converted from SPAD-502 readings using a formula described in Ling *et al.*, 2011 (Ling *et al.*, 2011) (mean $\pm$ SD $n=3$). Significantly different datasets are shown in asterisks (Student's t-test, $p<0.05$).

A

B

C

AChloroplastic marker
Mitochondrial marker **B**

Leaf mesophyll cell

Root hair

A **B**