Lack of Vacuolar H\(^+\)-Pyrophosphatase and Cytosolic Pyrophosphatases Causes Fatal Developmental Defects in Arabidopsis thaliana

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The cytosolic level of inorganic pyrophosphate (PPi) is finely regulated, with PPi hydrolyzed primarily by the vacuolar H\(^+\)-pyrophosphatase (H\(^+\)-PPase, VHP1/FUGU5/AVP1) and secondarily by five cytosolic soluble pyrophosphatases (sPPases; PPa1–PPa5) in Arabidopsis thaliana. Loss-of-function mutants of H\(^+\)-PPase (fugu5s) have been reported to show atrophic phenotypes in their rosette leaves when nitrate is the sole nitrogen source in the culture medium. For this phenotype, two questions remain unanswered: why does atrophy depend on physical contact between shoots and the medium, and how does ammonium prevent such atrophy. To understand the mechanism driving this phenotype, we analyzed the growth and phenotypes of mutants on ammonium-free medium in detail. fugu5-1 showed cuticle defects, cell swelling, reduced β-glucan levels, and vein malformation in the leaves, suggesting cell wall weakening and cell lethality. Based on the observation in the double mutants fugu5-1 ppa1 and fugu5-1 ppa4 of more severe atrophy compared to fugu5-1, the nitrogen-dependent phenotype might be linked to PPi metabolism. To elucidate the role of ammonium in this process, we examined the fluctuations of sPPase mRNA levels and the possibility of alternative PPi-removing factors, such as other types of pyrophosphatase. First, we found that both the protein and mRNA levels of sPPases were unaffected by the nitrogen source. Second, to assess the influence of other PPi-removing factors, we examined the phenotypes of triple knockout mutants of H\(^+\)-PPase and two sPPases on ammonium-containing medium. Both fugu5 ppa1 ppa2 and fugu5 ppa1 ppa4 had nearly lethal embryonic phenotypes, with the survivors showing striking dwarfism and abnormal morphology. Moreover, fugu5 ppa1\(^{+/-}\) ppa4 showed severe atrophy at the leaf margins. The other triple mutants, fugu5 ppa1 ppa5 and fugu5 ppa2 ppa4, exhibited death of root hairs and were nearly sterile due to deformed pistils, respectively, even when grown on standard medium. Together,
INTRODUCTION

Vacuolar H\textsuperscript{+}-translocating inorganic pyrophosphatase (H\textsuperscript{+}-PPase; gene, VHP1) has two physiological roles: hydrolysis of PPI in the cytosol and active translocation of protons into plant vacuoles. PPI is generated as a byproduct of the synthesis of macromolecules, such as DNA, RNA, proteins, and polysaccharides (Stitt, 1998; Maeshima, 2000; Heinonen, 2001). Excessive accumulation of PPI in the cytosol suppresses macromolecule biosynthesis based on the law of mass action. On the other hand, several bi-directional enzymes involved in glycolysis, including pyrophosphate-dependent phosphofructokinase (PFK), UGPase, and pyruvate phosphate dikinase (PPDK) can both utilize and produce PPI (Hajirezaei et al., 1993; Heinonen, 2001; Park et al., 2010; Chastain et al., 2011). Thus, sufficient PPI is also essential for metabolic activities in plant cells. In addition to scavenging PPI, H\textsuperscript{+}-PPase acts as a proton pump along with vacuolar H\textsuperscript{+}-ATPase to maintain acidic pH within the vacuolar lumen, which occupies the largest volume within plant cells (Maeshima and Yoshida, 1989; Nakanishi et al., 2003). In most young tissues of plants, H\textsuperscript{+}-PPase accounts for 10% of vacuolar membrane proteins by weight (Maeshima, 2001). Therefore, loss of H\textsuperscript{+}-PPase activity is expected to markedly suppress plant growth. Surprisingly, the loss of H\textsuperscript{+}-PPase function had a relatively mild phenotypic effects. A T-DNA insertion H\textsuperscript{+}-PPase knockout mutant vhp1-1 of Arabidopsis thaliana (A. thaliana) and amino acid exchange and deletion mutants of H\textsuperscript{+}-PPase (fugu5) showed abnormal cotyledon shape, with fewer and larger cells, when seedlings were grown in the absence of sucrose (Ferjani et al., 2011), as well as a mild suppression of plant growth (Asaoka et al., 2016), and delayed stomatal closure (Asaoka et al., 2019). Very recently, excess PPI has been reported to limit cotyledon pavement cell morphogenesis and to alter cotyledon flatness (Gunji et al., 2020). On the contrary, V-ATPase knockout mutant vha-a2 vha-a3 showed severe growth defect and higher vacuolar pH than fugu5, suggesting that V-ATPase is the primary vacuolar proton pump (Krebs et al., 2010; Kriegel et al., 2015). Although another H\textsuperscript{+}-PPase knockout mutant allele avp1-1 (Li et al., 2005) displayed severe auxin-related growth defects, Kriegel et al. (2015) unambiguously demonstrated that avp1-1 growth defects are due to a secondary T-DNA insertion in ARF-GEF GNOM gene, which is essential for PIN cycling. Few other studies suggested that H\textsuperscript{+}-Pase can act as PPI synthase, providing PPI to sucrose oxidation pathway to energize sucrose loading into phloem (Pizzio et al., 2015; Khadilkar et al., 2016; Scholz-Starke et al., 2019).

The oblong shape of fugu5 cotyledons recovered upon the addition of sucrose to the growth medium, as this phenotype was triggered by lowered sucrose production from seed storage lipids (Takahashi et al., 2017). Previous research into metabolite changes in fugu5 seedlings using capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS) and mathematical analysis revealed that UGPase is the major target of PPI’s inhibitory effect on gluconeogenesis, which ultimately leads to reduced sucrose production (Ferjani et al., 2018). In addition, double knockout mutants of H\textsuperscript{+}-PPase and cytosolic soluble PPase (sPPase) exhibited marked changes in morphology and metabolites, including defect of cell wall components and excessive accumulation of starch, while sPPase quadruple mutants displayed a normal phenotype (Segami et al., 2018). Thus, H\textsuperscript{+}-PPase has a greater impact on PPI homeostasis than that of sPPases.

Some non-plant species such as the purple photosynthetic bacterium Rhodospirillum rubrum, parasitic protozoa, Streptomyces, hyperthermophilic bacteria, and Agrobacterium possess H\textsuperscript{+}-PPases (Baltscheffsky et al., 1999; Maeshima, 2000; Pérez-Castiñeira et al., 2001; Seufferheld et al., 2003; Hirono et al., 2007). In organisms without H\textsuperscript{+}-PPase, such as Escherichia coli and Saccharomyces cerevisiae, loss of sPPase causes growth arrest and cell death due to hyperaccumulation of PPI (Chen et al., 1990; Serrano-Bueno et al., 2013) which were rescued by genetic complementation with H\textsuperscript{+}-PPase (Pérez-Castiñeira et al., 2002). However, no lethal phenotype has been reported in plants to date. Other PPases such as PPI-dependent phosphofructokinase (Stitt, 1998; Amthor et al., 2019) may contribute to PPI hydrolysis in plants. In this study, we prepared multiple knockout mutants of H\textsuperscript{+}-PPase and sPPase(s) to identify lethal conditions in plants and to evaluate the physiological contributions of four cytosolic soluble PPases (PPa1, PPa2, PPa4, and PPa5).

Recently, growth of fugu5 and vhp1 was found to be severely suppressed and cell death was observed at the basal region of the true leaves when grown on ammonium-free medium (Fukuda et al., 2016), which is commonly used for hydroponics. The phenotype was rescued either by addition of ammonium to the growth medium at more than 1 mM or genetic insertion of the yeast sPase IPP1, indicating that excessive accumulation of PPI causes the observed phenotypic effects (Fukuda et al., 2016). Based on these observations, we explored the changes in the tissues of mutant lines grown under these specific conditions. In this study, we found that deletion of both H\textsuperscript{+}-PPase and
sPPase resulted in marked changes in the morphology and construction of cells and tissues, cell surface components, cell death rate, and development of plants, even in those grown on standard growth medium. These results reveal the importance of PPI homeostasis for nitrogen metabolism and amino acid biosynthesis as well as macromolecule and sucrose biosynthesis in plants. Here, we discuss the biochemical and physiological effects of excessive PPI on cell morphology and cell fate, with consideration of macromolecule biosynthesis and differences in nitrogen assimilation between roots and shoots.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

*A. thaliana* (accession Columbia-0; hereafter referred to as wild type, WT) seeds, which were provided by the RIKEN BioResource Center (Tsukuba, Japan), were surface-sterilized, placed in the dark at 4°C for 2 days and then sown on plates of 0.5× Murashige-Skoog (MS) medium containing 2.5 mM MES-KOH (pH 5.7), 1% (w/v) sucrose, and 0.6% gellan gum (0.5 × MS plates) at 22°C under long-day conditions (light/dark regime of 16 h/8 h, cool-white lamps, 90 μmol/m² s). In addition to WT, two loss-of-function mutant alleles of H⁺-PPase (Ferjani et al., 2007, 2011), also in the Columbia-0 background, were characterized under the same conditions. The PPa5-GFP which expresses cfSGFP2-tagged PPa5 under the control of its own promoter, the loss-of-function mutants of cytosolic soluble PPase (*ppa1, ppa2, ppa4*, and *ppa5*) and double mutants (*ppa1 ppa5, fugu5 ppa1, fugu5 ppa2, fugu5 ppa4*, and *fugu5 ppa5*) were prepared as described previously (Segami et al., 2018). Triple mutants (*fugu5 ppa1 ppa2, fugu5 ppa1 ppa4, fugu5 ppa1 ppa5, and fugu5 ppa2 ppa4*) were prepared through crossing of the corresponding mutant lines.

Molecular Genetics Research Laboratory (MGRL) culture medium and a modified form of it supplemented with NH₄⁺ (MGRLAm) were prepared for examination of the effects of the NO₃⁻ and NH₄⁺ ions on plant growth. Basal MGRL medium for gel plates contained 1.5 mM NaN₃, 0.26 mM Na₂HPO₄, 1.5 mM MgSO₄, 2.0 mM Ca(NO₃)₂, 3.0 mM KNO₃, 12 μM Fe(III)-EDTA, 10 μM MnSO₄, 30 μM H₃BO₃, 1.0 μM ZnSO₄, 1.0 μM CuSO₄, 24 mM (NH₄)₆Mo₇O₂₄, 130 mM CoCl₂, 2% sucrose, and 0.4% gellan gum (Fujiwara et al., 1992; Naito et al., 1994). MGRLAm medium contained 3.0 mM NH₄Cl and 3.0 mM KCl instead of 3.0 mM KNO₃, and thus contained 4 mM NO₃⁻ and 3 mM NH₄⁺ as the sole nitrogen sources. Medium pH of MGRL and MGRLAm were adjusted to 5.8.

Morphological Observations

Whole plants were observed and photographed using an EOS D60 (Canon) or EOS Kiss X7 digital camera (Canon) and a stereo microscopic microscope (SZ61; Olympus) equipped with a CCD camera (DP50, Olympus or DXS500, Olympus). Embryos were cleared using Hoyzer’s solution (Feng and Ma, 2017) and observed with a BX51 upright microscope (Olympus) equipped with a CCD camera (DP72, Olympus).

For observation of leaf veins, 10-, 15-, and 20-day-old leaves were fixed in a solution (ethanol:acetic acid = 3:1) at room temperature. The fixed specimens were washed in an ethanol series (70%, 50%, 30%, and 15%) and rinsed with ethanol (10% xylitol, 15% sodium deoxycholate, and 25% urea) (Kurihara et al., 2015).

To observe the cross sections of rosette leaves, leaf samples including the leaf margin were cut to a size of 2 × 2 mm with a razor. Both ends of the leaves were sliced to allow the fixative solution to permeate well into the sample, and then the samples were immersed in a fixative solution (3% glutaraldehyde, and 50 mM Na-Pi, pH 7.0) and degassed thoroughly. The samples were embedded in 5% agar and sliced to 40 μm thickness with a microtome (VT1200 s, Leica).

Scanning Electron Microscopy (SEM)

Leaves were dissected from 10- and 20-DAG plants. They were mounted on a stub with adhesive carbon tape, and then transferred directly to a specimen chamber of the low-vacuum SEM (TM3030, Hitachi). Leaf epidermal cells were observed at 0–4°C, under low-vacuum conditions (30~50 Pa).

Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) observations were conducted with an upright FV1000-D confocal laser scanning microscope (Olympus). For fluorescein diacetate (FDA) and propidium iodide (PI) staining, samples were soaked in dye solution containing 5 μg/ml FDA, 10 μg/ml PI, and 100 mM sorbitol. After 1 min, the samples were observed via CLSM. For calcofluor white staining, samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 60 min under a vacuum at room temperature. The fixed tissues were washed twice for 1 min in PBS and cleared with ClearSee (Kurihara et al., 2015). The cleared samples were stained with 0.1% calcofluor white in ClearSee solution for 60 min and then washed with ClearSee solution for 30 min. The stained samples were observed via CLSM. UPLSAPO10X or UPLSAPO60XW (Olympus) was used as the objective lens. The excitation wavelength and transmission range for emission were 473 nm and 485 to 560 nm for FDA and green fluorescent protein (GFP), 559 nm and 617 to 717 nm for PI, and 405 nm and 425 to 475 nm for calcofluor white.

Immunoblotting

Preparation of the soluble fraction from *A. thaliana* plants and immunoblotting were conducted as described previously (Segami et al., 2018). To detect PPA isoymes, a peptide-specific antibody for *A. thaliana* PPa1–PPa5 (C+MPIMIDQGEKDDKII) was used.

Toluidine Blue Staining

Whole plants grown on plate medium were stained with 0.1% toluidine blue for 2 min. After washing three times with distilled water, leaves were observed via a stereo microscope (SZ61, Olympus).

Image Analysis

Image analysis of leaf surface, leaf vein and area quantification was performed using an ImageJ Fiji (Schindelin et al., 2012).
For quantification of the leaf areole density, in brief, the vessel patterns were traced manually, and subjected to “Analyze Particles” function to extract total areole area. Areole total area was divided by total leaf area. The obtained quotient was shown as an indicator of the leaf vein continuity.

For calculation of undulation index (UI), the cell perimeter and cell area of pavement cells were measured on SEM images (3~7 leaves per one sample, more than 15 cells from one leaf). The complexity of the pavement cells was quantified by calculating the UI (Thomas et al., 2003) using the following equation (Kürschner, 1997):

\[ UI = \frac{p}{2\pi \sqrt{A/\pi}} \]

where \( UI \) (dimensionless) is the undulation index, \( p \) (µm) is the cell perimeter, and \( A \) (µm²) is the cell area.

Quantification of leaf and seed area was performed using an ImageJ macro, as shown in Supplementary Figure 8. In brief, to select the desired area, Color Thresholder 2.0.0-rc-69/1.52p software was used. Then, the auto-generated macro code constructed by Color Thresholder was pasted into the macro at the indicated line.

RESULTS

Morphological Phenotypes of Leaf of fugu5

In fugu5, dead cells were observed in a highly proliferative region, namely the petiole-blade junction, of leaves grown on MGRL (Fukuda et al., 2016). To investigate further morphological changes in the other regions, we carefully observed the leaf veins, tissue construction of palisade mesophyll, and cell arrangement of the epidermis of true leaves. The first true leaves of 10-day-old plants grown on MGRL plates or plates with modified MGRL medium supplemented with 3 mM NH₄Cl (MGRL⁰⁰) were fixed with a solution of ethanol and acetate, and then treated with ClearSee to visualize leaf veins. Normal networks of leaf veins were observed in WT grown on either MGRL or MGRL⁰⁰ plates (Figures 1A,B). In contrast, fugu5-1 and fugu5-3 leaves, particularly in the distal leaf region, did not form the normal network of veins when grown on MGRL plates (Figures 1C,E, arrows). This defect was clearly rescued when fugu5-1 and fugu5-3 was cultivated on MGRL⁰⁰ (Figures 1D,F). Statistical analysis of the images using the quotient of the areole area and total leaf area values confirm that fugu5s grown on MGRL had abnormal networks of leaf veins, in other word, networks with low leaf vein continuity (Figure 1G).

Next, we observed cross sections of true leaves to investigate tissue construction. Generally, leaf mesophyll tissue is composed of four to five layers of cells of similar size. In 20-day-old plants, leaves from WT grown on both MGRL and MGRL⁰⁰ and fugu5-1 grown on MGRL⁰⁰ showed the normal arrangement of cells of regular size (Figures 2A,D,E). In fugu5-1 leaves, the alignment of cells (cell layers) was irregular and cell size was variable (Figures 2B,C). Furthermore, several large cells were present in the pavement cells of fugu5-1 grown on MGRL (Figure 2B, arrowhead). fugu5-1 ppa1 and fugu5-1 ppa4 plants grown on MGRL⁰⁰ showed severe phenotypic effects, including cell swelling and abnormal cell alignment (Supplementary Figure 1), suggesting that PPI accumulation caused cell swelling in leaves. Notably, fugu5-1 ppa1 grown on MGRL showed cell death in the adaxial side of the leaf, while fugu5-1 ppa4 showed cell death in the leaf margins (Supplementary Figure 1).

To detect morphological differences in pavement cells of 20-day-old leaves, SEM analysis was performed. Pavement cells usually exhibit puzzle-cell formation, but very recently Gunji et al. (2020) reported that the complexity of cotyledon pavement cells was reduced by PPI accumulation via inhibiting microtubule dynamics. On both MGRL and MGRL⁰⁰, WT leaves showed normal pavement cell structure (Figures 2F,G). However, fugu5-1 grown on MGRL showed obviously simplified cells (Figure 2H), while fugu5-1 grown on MGRL⁰⁰ showed puzzle-cell formation (Figure 2I), suggesting ammonium deficient conditions likely increased PPI level in pavement cells. Statistical analysis of UI, which indicates the degree of cell structure complexity, confirmed the above observations (Figure 2J).

To check whether nitrogen content was affected by fugu5 mutation, ammonium and nitrate contents were analyzed in plant shoots. For ammonium content, there was no significant difference between the WT and fugu5-1, although shoots grown on MGRL⁰⁰ accumulated twice more ammonium than MGRL in 10-DAG plants (Supplementary Figure 2A). On the other hands, in 10-DAG plants, there was no large difference in nitrate content except a little decrease in the WT grown on MGRL⁰⁰ (Supplementary Figure 2C). In 20-DAG plants grown on MGRL⁰⁰, both the WT and fugu5-1 consumed ammonium and nitrate (Supplementary Figures 2B,D). Together, these results imply that there were no large differences between WT and fugu5-1 in nitrogen usage.

Defects in the Cell Wall and Cuticle Layer of Mutant Leaf Epidermis

In roots, fugu5-1 ppa1 showed cell swelling, which was likely caused by decreased cellulose and root tip burst due to hypotonic treatment (Segami et al., 2018). Previously, we reported that contact of leaves with the culture medium was closely related to the occurrence of leaf atrophy (Fukuda et al., 2016). Therefore, we estimated that cell death in fugu5 leaves on MGRL is caused by extracellular abiotic stresses.

To check for deficiencies of cell wall components, we stained 10-day-old leaves with calcofluor white, a fluorescent dye that binds to β-glucan (Anderson et al., 2010), and observed the epidermal cells at leaf margins with CLSM (Figure 3). fugu5-1 grown on MGRL showed a strong decrease in the fluorescence signal and fugu5-1 grown on MGRL⁰⁰ showed a mild decrease in signal intensity compared with WT grown on both MGRL and MGRL⁰⁰. The double mutants fugu5-1 ppa1 and fugu5-1 ppa4, particularly the former, which exhibited a severe leaf phenotype (Supplementary Figure 1), also showed markedly low signals. These results suggest a relationship between the β-glucan content and mechanical strength of the cell wall.
Generally, the surface of plant shoot tissues is covered with a thin hydrophobic layer called the cuticle (Fich et al., 2016). The cuticle prevents water loss from the leaf surface and entry of water and solutes. The cuticle is a multilayered structure composed of waxes and related hydrocarbons deposited on the leaf epidermis. To examine the integrity of the cuticles of WT and fugu5 leaves, fresh leaves were stained with toluidine blue, which is a water-soluble dye with high affinity for acidic components. If the leaf surface lacks a cuticle layer, the dye would stain the cells (Tanaka et al., 2004). WT leaves grown on both plate media did not stain (Figures 4A,B). In contrast, fugu5-1 leaves grown on MGRL stained moderately (10-day-old seedling; Figure 4A) or strongly at the rim of basal region (20-day-old; Figure 4B), which coincides with the atrophic region. These results indicate that the epidermal cells of fugu5-1 are unable to synthesize the components to generate adequate cuticles.

Double Mutants Grown in Ammonium-Free Medium Exhibit Severe Growth Defects

Leaf atrophy in fugu5-1 depends on other factors, such as contact of leaves with the medium surface and agar concentration (i.e., medium hardiness) (Fukuda et al., 2016). Therefore, the observed phenotypes varied among independent experiments. Additionally, we found striking leaf atrophy in fugu5-1 ppa1 and fugu5-1 ppa4 plants grown on MGRL. These mutants are known to show a weak leaf atrophy phenotype when grown in half-strength MS, which contains 10 mM ammonium (Segami et al., 2018). All individuals of the double mutants failed to fully expand leaves when grown on MGRL (Figures 5A,B). This phenotype was stable, and the variance of leaf area for seedlings grown on MGRL was significantly lower than that on
Fukuda et al. PPases Knockout Causes Fatal Defect

FIGURE 2 | Abnormal epidermal and mesophyll cells in leaves of fugu5 grown on MGRL culture medium. WT and fugu5-1 were grown on an MGRL or MGRL\textsuperscript{Am} plates for 20 days. (A–E) Rosette leaves were fixed and sectioned for observation. Images of leaf cross sections from WT (A) and fugu5-1 (B, C) grown on an MGRL plates, and from WT (D) and fugu5-1 (E) grown on MGRL\textsuperscript{Am} plates were taken using a upright microscope. Arrowheads indicate the swollen cells in epidermis. (F–I) SEM images of leaf adaxial side. Leaves of (F) WT and (H) fugu5-1 grown on an MGRL plates and leaves of (G) WT and (I) fugu5-1 grown on an MGRL\textsuperscript{Am} plates observed. (J) Ul of pavement cells. First leaves of 10-DAG plants and third or fourth leaves of 20-DAG plants were used for the observations above, except for severely atrophied fugu5-1 grown on MGRL, in which the leaf stage was hardly distinguished. Different letters above each bar indicate statistically significant differences (P < 0.05, Tukey's HSD test), and asterisks indicate statistically significant differences at *P < 0.05, **P < 0.01, ***P < 0.001 (Welch two sample t-test, n > 50). Error bars indicate SD (n > 50).
MGRL Am for each line (F test; Figure 5C). Moreover, in 6-DAG seedlings grown with plastic sheets, which prevent direct contact of cotyledons with the growth medium, fugu5-1 ppa1 and fugu5-1 ppa4 grown on MGRL showed chlorosis in the emerging true leaves (Figure 5D, arrows). These results suggest that the phenotype exhibited by fugu5-1 ppa1 and fugu5-1 ppa4 grown on MGRL was independent of plant-medium contact and appeared at younger stage than that of fugu5-1.
A primary question of this study is how ammonium ions prevent atrophy of fugu5 mutants. It has been shown that atrophy is triggered by excess PPI (Fukuda et al., 2016). Therefore, we postulated three possible effects of ammonium: (1) induction of sPPase expression; (2) induction of the expression of other pyrophosphatases; and/or (3) suppression of PPi generation in the mutants.

To test the first possibility, we prepared soluble fractions from WT and mutants grown on MGRL and MGRLAm, and then performed immunoblotting using anti-sPPase, which collectively detects five sPPases, PPa1 to PPa5, to examine their protein level (Segami et al., 2018). There was no marked change in the level of sPPases between samples grown on the two types of media (Figure 6). In addition, we measured the mRNA levels of four sPPases and four PFPs (At1g12000, At1g20950, At1g76550, and At4g04040) and found no marked differences between MGRL and MGRLAm conditions (Supplementary Figure 3 and Supplementary Table 1). These results suggest that ammonium does not induce the expression of sPPases or PFPs.

**Seed Viability Defects of fugu5 ppa1 ppa2 and fugu5 ppa1 ppa4**

To further validate the existence of other PPI-hydrolysis enzymes or PPI-removing factors, we prepared double and triple knockout mutants for various combinations of H⁺-PPase and sPPases. If these mutants are lethal, the existence of other functional PPI removal mechanisms is unlikely. All double mutants of H⁺-PPase and sPPase, including the most severe mutant fugu5 ppa1, showed normal fertility (data not shown). However, numerous shrunken seeds were observed in the triple heterozygous mutants fugu5-1 ppa1 ppa2 and fugu5-1 ppa1 ppa4 (Supplementary Figure 4A). It is worth to notice that non-shrunken seeds from fugu5 ppa1−/+ ppa2 and fugu5 ppa1−/+ ppa4 parental lines showed massive increase in size (Supplementary Figure 4B). The other line with shrunken seeds, fugu5-1−/+ ppa2, showed less size increase, suggesting that nutrient surplus due to inhibited development of 25% of seeds did not cause the size increase. Interestingly, fugu5-1 and sPPase quadruple mutant ppa1,2,4,5 formed larger seeds. It appears again that increased PPI somehow affected seed storage oil metabolism, in agreement with the previous report by Meyer et al. (2012), in which seed specific overexpression of PPa1 and RNAi of PPa1 or PPa4 have been analyzed.

To test the fertility of the triple mutants, we tested the genotypes of heterozygous mutant progeny. Both fugu5-1 ppa1 ppa2 and fugu5-1 ppa1 ppa4 germinated from freshly prepared seeds, but not from old seeds that had been stored for at least 1 year (Tables 1, 2). The germination rates of fugu5 ppa1 ppa2 and fugu5 ppa1 ppa4 were considerably reduced. These results suggest that both fugu5 ppa1 ppa2 and fugu5 ppa1 ppa4 are nearly lethal and not tolerant of long-term storage.

To determine when these mutations caused seed defects, we observed ovules and embryos. At 14 DAP, when the valves turned yellow, about 75% of seeds of both fugu5-1−/+ ppa1 ppa2 and fugu5-1−/+ ppa4 showed normal brown coloration, while the remaining 25% of seeds appeared abnormal, either green or shrunken, indicating that the ratio of these defective seeds followed Mendelian segregation (Figures 7A,B). In fugu5-1 ppa1−/+ ppa4, green seeds contained green embryos with their cotyledons and hypocotyls fused together (Figure 7A). The genotypes of most green embryos were identified as fugu5-1 ppa1 ppa4 homozygous (Supplementary Figure 5).

**Undeveloped Embryos in fugu5 ppa1 ppa2 and fugu5 ppa1 ppa4**

At 7–8 DAP, WT and all double mutants had green ovules, which is the normal color at this developmental stage (Figure 7C). The color of ovules varies during embryonic development, and WT at 5 DAP had a torpedo-stage embryo (Figure 7D) with yellow ovule coloration (Figure 7C). At 8 DAP, triple mutants...
**Figure 5** | Effect of NH$_4^+$ in culture medium on growth. (A) Growth of WT, fugu5-1, fugu5-3, ppa1,2,4,5, fugu5-1 ppa1, and fugu5-1 ppa4 seedlings for 17 days on MGRL (without NH$_4^+$) or MGRL$^\text{Am}$ (3 mM NH$_4^+$) plates. (B) Magnified images of fugu5-1 ppa1 and fugu5-1 ppa4 grown on MGRL or MGRL$^\text{Am}$. (C) Leaf area was calculated using photographs and represented with boxplots and dot plots. Asterisks above the boxplots indicate statistically significant differences of mean values compared with the WT of each medium condition (**$P$ < 0.01, ***$P$ < 0.001, Steel test), and blue asterisks between pair indicate statistically significant differences of variances (**$P$ < 0.001, F test). (D) Seedlings were grown on MGRL or MGRL$^\text{Am}$ plates for 6 days using plastic sheets to prevent contact between cotyledons and medium. Arrows indicate areas of chlorosis (small true leaves).

*fugu5$^+/-$ ppa1 ppa2* and *fugu5 ppa1$^+/-$ ppa4* heterozygous, contained about 25% yellow ovules (Figure 7C). For further analysis of the embryos in these ovules, we observed transparent ovules cleared with Hoyer’s solution (Feng and Ma, 2017). Yellow ovules of fugu5$^+/-$ ppa1 ppa2 and fugu5 ppa1$^+/-$ ppa4 heterozygous had malformed spherical embryos, whereas the embryos of green ovules exhibited bent cotyledons. The size of yellow ovule embryos was larger than that of the heart stage of the WT at 4 DAP and smaller than that of the WT torpedo stage at 5 DAP (Figures 7D,E, yellow ovule). In particular, fugu5 ppa1$^+/-$ ppa4 had larger embryos than did fugu5$^+/-$ ppa1 ppa2 heterozygous. These observations indicate that
### TABLE 1 | Segregation ratios of triple mutants by genotype.

| Genotype of parent | Storage | Genotype of seedlings | Total | ~/- | +/- | +/+ | Ungerminated |
|--------------------|---------|-----------------------|-------|-----|-----|-----|-------------|
| fugu5-1+/− ppa1 ppa2 | 0 days  | 84                    | 4     | 45  | 20  | 15  |             |
|                    | 4 years | 41                    | 0     | 19  | 7   | 15* |             |
| fugu5-1 ppa1+/− ppa2 | 1 year  | 74                    | 0     | 39  | 32  | 3*  |             |
| fugu5-1 ppa1 ppa2p/− | 1 year  | 82                    | 0     | 45  | 23  | 14* |             |
| fugu5-1 ppa1+/− ppa4 | 0 days  | 90                    | 8     | 44  | 29  | 9   |             |
| fugu5-3+/− ppa1 ppa4 | 2 weeks | 44                    | 2     | 27  | 15  | 13  |             |

*Storage refers to the period from seed harvest to sowing. *Some shrunken seeds were removed during the harvest process. In other cases, seeds were directly harvested from individual fruits and sowed immediately.

### TABLE 2 | Segregation ratios of triple mutants by phenotype.

| Genotype of parent | Storage | Total | Dwarf | Curled | Normal | Ungerminated |
|--------------------|---------|-------|-------|--------|--------|-------------|
| fugu5-1 ppa1+/− ppa4 | 1 year  | 89    | 0     | 57     | 24     | 8*          |
| fugu5-3+/− ppa1 ppa4 | 1 year  | 88    | 0     | 0      | 75     | 13*         |

Plants were classified by phenotype: Normal means no obvious phenotypic effects or marginal atrophy (ppa1 ppa4 or fugu5-1 ppa4-like), Curled indicates strong leaf edge atrophy (fugu5 ppa1+/− ppa4-like) and Dwarf represents severe growth defect (fugu5 ppa1 ppa4-like). See Figure 8B for morphological examples. *Some shrunken seeds were removed during the harvest process.

### FIGURE 6 | Immunoblot analysis of PPases in the soluble fractions from WT and mutants. (A) WT, a mutant expressing yeast cytosolic PPase (IPP1), fugu5-1, and fugu5-3 were grown on MGRL or MGRL<sup>Am</sup> plates for 10 (upper panel) or 18 days (lower panel). Soluble fractions were prepared and subjected to immunoblotting using anti-PPase. sPPases were detected at 27 and 25 kDa. (B) Relative intensities of immunostained bands at 27 kDa are indicated with orange (MGRL) and blue boxes (MGRL<sup>Am</sup>).
embryos were not simply delayed in development but also grew abnormally.

**Indispensability of H⁺-PPase and sPPases for Shoot Development**

Both triple mutants *fugu5-1 ppa1 ppa2* and *fugu5-1 ppa1 ppa4* showed extremely reduced shoot growth (*Figures 8A–C*). On the other hand, the heterozygous mutant *fugu5-1 +/- ppa1 ppa2* showed normal morphology and no significant differences from *ppa1 ppa2* (*Figures 8A,B*), suggesting that a single copy of the VHP1 gene is sufficient to compensate for lack of PPa1 and PPa2. Another heterozygous mutant, *fugu5-1 ppa1 +/- ppa4*, showed decreased leaf area compared with *fugu5-1 ppa4* (*Figure 8C*) and a strong leaf edge curling phenotype, even when grown
FIGURE 8 | Defect in development of the triple mutants fugu5-1 ppa1 ppa2 and fugu5 ppa1 ppa4. (A) 18-DAG plants germinated from fugu5-1+/− ppa1 ppa2 seeds. Red square indicates fugu5-1 ppa1 ppa2, yellow triangles indicate fugu5-1+/− ppa1 ppa2 and blue circle indicates ppa1 ppa2. (B) 18-DAG plants germinated from fugu5-1 ppa1+/− ppa4 seeds. Red square indicates fugu5-1 ppa1 ppa4, yellow triangles indicate fugu5-1+/− ppa1 ppa4 and blue circle indicates ppa1 ppa4. (C) Comparison of leaf area of 12-DAG plants. Different letters above each plot indicate statistically significant differences (P < 0.05, Tukey’s HSD test). (D) Phenotype of plant from fugu5-1 ppa1 ppa2. (E) Phenotype of fugu5-1 ppa1 ppa4. Plants were grown on half-strength MS plates with sucrose.
on MS plates, on which fugu5 showed no atrophic symptoms (Figure 8B, yellow triangle). At 8-DAG, both fugu5-1 ppa1 ppa2 and fugu5-1 ppa1 ppa4 showed extremely abnormal shapes including apparent lack of cotyledons, short roots, and distorted leaves (Figures 8D,E). At 26-DAG, several individuals of fugu5-1 ppa1 ppa2 and fugu5-1 ppa1 ppa4 successfully developed leaves, but they remained very small compared with heterozygous or double mutant plants (Figures 8D,E, right and lower panels), and were especially damaged in fugu5-1 ppa1 ppa4 (Figure 8E, right panel). In severe cases, individuals of fugu5-1 ppa1 ppa2 and fugu5-1 ppa1 ppa4 had no expanded leaves (Figures 8D,E, left panels). These results indicate that PPa1, PPa2, and PPa4 are active in the true leaves and contribute to PPi homeostasis in the fugu5 background.

In MGRL medium, the double mutants fugu5-1 ppa2 and fugu5-1 ppa5 exhibited reduced leaf area but not severe atrophy...
(Figures 5A,C). In addition, fugu5-1 ppa1+/− ppa2 showed no leaf atrophy on MS medium (Supplementary Figure 6A), despite significantly reduced leaf area and root length (Supplementary Figures 6B–E), suggesting that PPa2 does not contribute to preventing leaf atrophy.

Another triple mutant fugu5-3 ppa2 ppa4 had full viability but retarded cotyledon development compared with its heterozygous siblings fugu5-3 ppa2+/− ppa4 and fugu5-3 ppa2 ppa4+/− (Supplementary Figures 7A,B). Their cotyledon shape was distorted, and more than half of the plants formed single cotyledons. Six out of 26 seedlings of this triple mutant failed to form true leaves and their growth was prematurely arrested (Supplementary Figures 7A,C). These observations indicate that VHP1, PPa2, and PPa4 are essential for normal development of the cotyledons and shoot apical meristem at an early developmental stage, and that PPa1 alone can maintain PPase activity at a sufficient level for viability.

In addition, fugu5-3 ppa2 ppa4 showed severe floral phenotypic changes, including enlarged and distorted petals (Supplementary Figure 7D). Although few seeds were obtained from the mutant plant, hand pollination using WT pollen was not successful. In contrast, fugu5-3 ppa2 ppa4 pollen succeeded in pollinating WT pistils (data not shown), suggesting that the pistil of fugu5-3 ppa2 ppa4 is impaired. The petals and sepals of the mutant also showed morphological abnormalities (Supplementary Figure 7D).

### DISCUSSION

**Physiological Mechanism of Leaf Atrophy**

Leaf atrophy was observed in fugu5 mutants when grown on ammonium-free medium. This phenotype has been shown to be triggered by excessive PPi, and thus could be rescued through heterologous expression of the yeast sPPase IPP1 in fugu5 mutants (Fukuda et al., 2016). In this study, we focused on two questions: why the atrophic phenotype depends on direct contact of shoots with medium, and the mechanism through which ammonium ions prevent leaf atrophy. To address the first question, we analyzed mutant leaves and observed several new phenotypes: irregular and disconnected leaf veins...
(Figure 1), abnormal mesophyll tissue organization, cell swelling and severely simplified pavement cells (Figure 2), decrement of β-glucans (Figure 3), and partial lack of cuticle layer in the rosette leaves (Figure 4). These observations led us to consider dysfunctional biosynthesis of cell wall components and cutin. During biosynthesis of these macromolecules, PPi is generated (Heinonen, 2001). The increased level of PPi may suppress biosynthesis of these macromolecules and thus cause defects in cell wall construction and strength in growing leaves.

In the case of interrupted veins (Figure 1), a similar phenotype was reported in a triple mutant of glycosyltransferases in the cellulose synthase-like D (CSDL) family, csld2 csld3 csld5, which transfer mannose from GDP-mannose onto an endogenous acceptor (Yin et al., 2011). GDP-mannose is a nucleotide-sugar that releases PPi from its synthesis reaction, and PPi accumulation likely inhibits this reaction, as recently reported for UDP-glucose (Ferjani et al., 2018). Indeed, the etiolated hypocotyl of fugu5-1 ppa1 contained a reduced level of mannose (Segami et al., 2018). Along with the decreased level of β-glucans in the leaf surface (Figure 3), cell wall synthesis was markedly inhibited in fugu5-1 leaves grown on ammonium-free medium.

The cuticle, which acts as plant skin, is essential for the protection of leaves and structural support of tissues. Therefore, the lack of cuticle layer in fugu5 grown on MGRL medium may result in serious damage to its tissues. In the early pathway of cuticle synthesis, ligation of CoA with long chain fatty acid by acyl-CoA synthetase (EC6.2.1.3) releases PPi into the cytosol (Pulsifer et al., 2012; Fich et al., 2016). This finding strongly suggests that the lack of VHP1 increases the PPi level in leaf epidermal cells and suppresses the formation of cuticle. Cells with immature cell walls (Figure 3) lacking the cuticle layer (Figure 4) might be physically weak and sensitive to osmotic fluctuations in the extracellular environment. Taken together, these findings indicate that leaf atrophy in fugu5 might be caused by cell death due to external stresses, such as infiltration of solutes from the growth medium.

The Importance of H+--PPase and sPPases in Leaf Development

To address the second question regarding the physiological mechanism through which ammonium ion prevents the atrophy in fugu5 and other mutants, we hypothesized three possible effects of ammonium in the mutants: induction of sPPases, induction of other pyrophosphatases, and suppression of PPi generation. The first hypothesis was clearly contradicted by the induction of other pyrophosphatases, and suppression of PPi effects of ammonium in the mutants: induction of sPPases, and PPi homeostasis.

We constructed four triple mutants, fugu5-1 ppa1 ppa2, fugu5-1 ppa1 ppa4, fugu5-1 ppa1 ppa5, and fugu5-3 ppa2 ppa4, and tested their growth on half-strength MS plates, which contained 10 mM NH₄⁺. fugu5-1 ppa1 ppa2 and fugu5-1 ppa1 ppa4 showed the severest phenotypic effects, which were nearly lethal (Tables 1, 2 and Figure 8). Among their heterozygous counterparts, fugu5-1 ppa1+/− ppa4 showed significant leaf edge atrophy (Figures 8A,B), whereas fugu5-1 ppa1+/− ppa2 showed no atrophic leaf symptoms (Supplementary Figure 6A). These results indicate that PPa4 is more important than PPa2 for preventing leaf atrophy (Supplementary Figures 6B,C).

The other triple mutant, fugu5-3 ppa2 ppa4, showed no additional effects on leaf phenotype compared to the former two mutants (Supplementary Figure 7). Comparison of fugu5-3 ppa2 ppa4 with fugu5-1 ppa1 ppa2 and fugu5-1 ppa1 ppa4 clearly indicates the importance of PPa1 to leaf development. In GFP localization analysis for our previous study (Segami et al., 2018), VHP1-GFP and PPa1-GFP were expressed in all leaf cell types, whereas PPa2-GFP and PPa4-GFP were preferentially expressed in mesophyll and epidermis cells, respectively. Therefore, the severe phenotype of fugu5-1 ppa1 ppa2 (Figure 8D) may be related to mesophyll dysfunction, whereas fugu5-1 ppa1 ppa4 (Figure 8E) suffers from defects in epidermal development. PPa5-GFP is also expressed in leaf epidermis, but is limited to mature leaves, whereas PPa4-GFP is highly expressed during the early stages of leaf development. Combined with the result that fugu5-1 ppa1 ppa5 and fugu5 ppa5 did not exhibit strong leaf atrophy (Figures 5A, 9C), this shows that the atrophic phenotype is associated with young leaves, in agreement with the notion that PPi is abundantly released in proliferating young tissues. Therefore, the combination of VHP1, PPa1, PPa2, and PPa4 might be essential for proper leaf development. The present observations of nearly lethal phenotypic effects in multiple mutants of VHP1 and PPases strongly suggest that other PPi utilizing enzymes or PPi hydrolyzing enzymes, if any exist, are not involved in PPi homeostasis.

Phenotypes of Embryos and Root Hairs in Triple Mutants

The triple mutants fugu5-1 ppa1 ppa2 and fugu5-1 ppa1 ppa4 both showed nearly lethal phenotypes. Their embryos exhibited retarded growth and developed without showing the typical heart-stage and torpedo-stage shapes, becoming round in shape at 8 DAP. Finally, fugu5-1 ppa1 ppa4 made green embryos lacking differentiation into cotyledons and hypocotyls in seeds at 14 DAP (Figure 7A and Supplementary Figure 5). In this study, we could not obtain triple homozygous mutants from seeds stored that were over 1 year (Tables 1, 2). In combination with the observation that fugu5-1 ppa1 ppa4 embryos remained green in color until 14 DAP (Figure 7A and Supplementary Figure 5), this result suggests that seed maturation in the mutant is insufficient for maintaining seed longevity during long-term storage. The high ratio of shrunken seeds to normal-sized ones (Figure 7B) and low germination rate suggest that PPa2 is more important than PPa4 during embryogenesis.
fugu5-1 ppa1 ppa5 showed phenotypes of short roots, short root cells, fewer root hairs, and necrosis of the root epidermis (Figures 9A,B,E,F). Root epidermis is divided into two types: root hair cells and non-root hair cells (Galway et al., 1994; Berger et al., 1998). Notably, root epidermis is normally viable at an early developmental stage, before root-hair formation (Figure 9E). PPa5-GFP was expressed primarily in root hairs in the root elongation zone (Figure 9D) and PPa3-GFP was also expressed in root hair cells, whereas PPa4-GFP was mainly detected in non-root hair cells and PPa2-GFP was expressed only during the early developmental stage (Segami et al., 2018). These observations coincide with the defective root hair phenotype of fugu5-1 ppa1 ppa5, in which root hair line cells died (Figure 9E). In this triple mutant, most root hairs died, but several elongated hairs were observed (Figures 9A,E), suggesting that the formation and elongation of root hairs require precise regulation of P Pi at a critical level and the remaining PPa3 activity was insufficient to maintain viability.

Possible Mechanism for Rescue of Mutant Phenotype by Ammonium

To explore the relationship between P Pi production and absence of ammonium supply, here we considered organ-specific nitrogen metabolism. Most plants incorporate and utilize ammonium preferentially over nitrate (Howitt and Udvardi, 2000; Konishi et al., 2017). Thus, A. thaliana incorporates NH4+ preferentially from MGRLAm medium and converts NH4+ to amino acids in roots to reduce the toxic NH4+ levels (Hachiya and Sakakibara, 2017). The amino acids thus generated are transported from the roots to shoots (Konishi et al., 2017). In contrast, NO3− is incorporated by roots, and then transported directly from roots to shoots because NO3− has no toxicity to plant cells. In shoots, NO3− is reduced to NH4+ via NAD(P)H reducing equivalents that are mainly supplied by photosynthesis and is subsequently incorporated into glutamine. The assimilation of nitrate is assumed to act as a strong consumer of reducing power, and therefore ammonium utilization greatly decreases the energy consumption required to synthesize organic N compounds (Williams et al., 1987; Hachiya and Sakakibara, 2017; Gakière et al., 2018).

Considering the differences in nitrogen metabolism between roots and shoots, metabolism of NO3− into amino acids occurs preferentially in shoots grown on MGRL medium. Although NO3− reduction itself includes no P Pi-generating reactions, for example, the biosynthesis of NAD, which is involved in NO3− reduction, generates P Pi (Heinonen, 2001; Gakière et al., 2018). We predict that the elevated level of P Pi formed from NO3− reduction related pathway in shoots causes cellular dysfunction and atrophy in fugu5 plants grown on MGRL medium. The addition of ammonium to the medium might reduce the generation of P Pi in shoots by promoting amino acid synthesis in roots. This organ-specific nitrogen metabolism for synthesis of amino acids rescues the phenotype of fugu5. To further test this hypothesis, we will analyze transcriptomic and metabolomic data and then identify the pathways generating high P Pi levels under ammonium-free conditions.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

MF co-coordinated the project, contributed to phenotyping, analyzed the data, and drafted the manuscript. MMi conducted microscopy and calcofluor white staining. MMA conceived of and initiated the project, obtained funding, and contributed to the manuscript. SS conducted association analysis, constructed multiple mutants, wrote and finalized the manuscript. AF provided the fugu5 mutants, AVP1pro::IPP1 transgenic lines and contributed to the manuscript. SK conducted image analysis. RS conducted RT-qPCR analysis. TT contributed to seed phenotype analysis. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00655/full#supplementary-material

REFERENCES

Amthor, J. S., Bar-Even, A., Hanson, A. D., Millar, A. H., Stitt, M., Sweetlove, L. J., et al. (2019). Engineering strategies to boost crop productivity by cutting respiratory carbon loss. Plant Cell 31, 297–314. doi: 10.1105/tpc.18.00743

Anderson, C. T., Carroll, A., Akhmetova, L., and Somerville, C. (2010). Real-time imaging of cellulose reorientation during cell wall expansion
Hirono, M., Nakanishi, Y., and Maeshima, M. (2007). Identification of amino acids in developing seeds is influenced by the expression of pyrophosphate:fructose-6-phosphate phosphotransferase show no visible phenotype and only minor changes in metabolic fluxes in their tubers. *Planta* 193, 16–30. doi: 10.1007/BF00198868

Heinonen, J. K. (2001). *Biological Role of Inorganic Pyrophosphate*. Boston, MA: Springer. doi: 10.1007/978-1-4615-1433-1436

Hirono, M., Nakashita, Y., and Maeshima, M. (2007). Identification of amino acid residues participating in the energy coupling and proton transport of Streptomyces coelicolor A3(2) H\(^+\)-pyrophosphatase. *Biochim. Biophys. Acta Bioenerg.* 1767, 1401–1411. doi: 10.1016/j.bbabio.2007.09.007

Howitt, S. M., and Udvardi, M. K. (2000). Structure, function and regulation of ammonium transporters in plants. *Biochim. Biophys. Acta* 1465, 152–170. doi: 10.1016/S0005-2738(00)00136-X

Khadirlar, A. S., Yadav, U. P., Salazar, C., Shulaev, V., Paez-Valencia, J., Pizzi, G. A., et al. (2016). Constitutive and companion cell-specific overexpression of AVP1, encoding a proton-pumping pyrophosphatase, enhances biomass accumulation, phloem loading, and long-distance transport. *Plant Physiol.* 170, 401–414. doi: 10.1093/tpc/tnw045

Koniishi, N., Ishiyama, K., Beier, M. P., Inoue, E., Kanno, K., Yamaya, T., et al. (2017). Contributions of two cytosolic glutamine synthetase isoforms to ammonium assimilation in *Arabidopsis* roots. *J. Exp. Bot.* 68, 613–625. doi: 10.1093/jxb/erw454

Krebs, M., Beyhl, D., Görlich, E., Al-Rasheid, K. A. S., Marten, I., Stierhof, Y. D., et al. (2010). Arabidopsis V-ATPase activity at the tonoplast is required for efficient nutrient storage but not for sugar accumulation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3251–3256. doi: 10.1073/pnas.0913051107

Krieger, A., Andrés, Z., Medzhirdzaszy, A., Krüger, F., Scholl, D., Delang, S., et al. (2015). Job sharing in the endomebrane system: vacuolar acidification requires the combined activity of V-ATPase and V-PPase. *Plant Cell* 27, 3383–3396. doi: 10.1105/tpc.15.00733

Kurilara, D., Mizuta, Y., Sato, Y., and Higashiyama, T. (2015). ClearSee: a rapid optical clearing reagent for whole-plant fluorescence imaging. *Development* 142, 4168–4179. doi: 10.1242/dev.127611

Kürschner, W. M. (1997). The anatomical diversity of recent and fossil leaves of the durmast oak (*Quercus petraea Liebl*! Q pseudoacastanica Goeppert)—implications for their use as biosensors of palaeoatmospheric CO2 levels. *Rev. Palaeobot. Palynol.* 96, 1–30. doi: 10.1016/S0034-6667(96)00051-6

Li, J., Yang, H., Peer, W. A., Richter, G., Blakeslee, J., Bandypadhyay, A., et al. (2005). Plant Science: *Arabidopsis* H\(^+\)-PPase AVP1 regulates auxin-oxidated organ development. *Science* 310, 121–125. doi: 10.1126/science.1115711

Maeshima, M. (2000). Vacular H\(^+\)-pyrophosphatase. *Biochim. Biophys. Acta* 1465, 37–51.

Maeshima, M. (2001). Tonoplast transporters: organization and function. *Annu. Rev. Plant Biol.* 52, 469–497. doi: 10.1146/annurev.arplant.52.1.469

Maeshima, M., and Yoshida, S. (1989). Purification and properties of vacuolar membrane proton-translocating inorganic pyrophosphatase from mung bean. *J. Biol. Chem.* 264, 20068–20073.

Meyer, K., Stecca, K. L., Ewell-Hicks, K., Allen, S. M., and Everard, J. D. (2012). Oil and protein accumulation in developing seeds is influenced by the expression of a cytosolic pyrophosphatase in *Arabidopsis*. *Plant Physiol.* 159, 1221–1234.

Naito, S., Hirai, M. Y., Chino, M., and Komeda, Y. (1994). Expression of a soybean seed storage protein gene in transgenic tobacco plants. *Planta* 193, 988–999. doi: 10.1007/BF00939325

Nakanishi, Y., Yabbe, I., and Maeshima, M. (2003). Patch clamp analysis of a vacuolar pH\(^+\)-pyrophosphatase: A tightly membrane-bound family. *Plant Physiol.* 132, 524–530. doi: 10.1104/pp.103.032569

Nakanishi, Y., Yabbe, I., and Maeshima, M. (2003). Patch clamp analysis of a vacuolar pH\(^+\)-pyrophosphatase: A tightly membrane-bound family. *Plant Physiol.* 132, 524–530. doi: 10.1104/pp.103.032569

Park, J., I., Ishimizu, T., Suwabe, K., Sudo, K., Hakozaki, H., et al. (2010). UDP-glucose pyrophosphorylase is rate limiting in vegetative and reproductive phases in *Arabidopsis thaliana*. *Plant Cell Physiol.* 51, 981–996. doi: 10.1093/pcp/pqp057

Pérez-Castillejo, J. R., López-Marqués, R. L., Losada, M., and Serrano, A. (2001). A thermostable K\(^+\)-stimulated vacuolar-type pyrophosphatase from the hyperthermophilic bacterium *Thermotoga maritima*. *FEBS Lett.* 496, 6–11. doi: 10.1016/S0014-5793(01)02390-2

Pérez-Castillejo, J. R., López-Marqués, R. L., Villalba, J. M., Losada, M., and Serrano, A. (2002). Functional complementation of yeast cytosolic pyrophosphatase by bacterial and plant H\(^+\)-translocating pyrophosphatases. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15914–15919. doi: 10.1073/pnas.24262539

Pérez, G. A., Paez-Valencia, J., Khadirlar, A. S., Regmi, K., Patron-Soberero, A., Zhang, S., et al. (2015). Arabidopsis type I proton-pumping pyrophosphatase expresss strongly in phloe, where it is required for pyrophosphate metabolism and photosynthetic partitioning. *Plant Physiol.* 167, 1541–1553. doi: 10.1104/pp.114.254342

Frontiers in Plant Science | www.frontiersin.org 17 May 2020 | Volume 11 | Article 655
Pulsifer, I. P., Kluge, S., and Rowland, O. (2012). *Arabidopsis* LONG-CHAIN ACYL-COA SYNTHETASE 1 (LACS1), LACS2, and LACS3 facilitate fatty acid uptake in yeast. *Plant Physiol. Biochem.* 51, 31–39. doi: 10.1016/j.plaphy.2011.10.003

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Scholz-Starke, J., Primo, C., Yang, J., Kandel, R., Gaxiola, R. A., and Hirschi, K. D. (2019). The flip side of the *Arabidopsis* type I proton-pumping pyrophosphatase (AVP1): using a transmembrane H\(^+\) gradient to synthesize pyrophosphate. *J. Biol. Chem.* 294, 1290–1299. doi: 10.1074/jbc.RA118.006315

Segami, S., Tomoyama, T., Sakamoto, S., Gunji, S., Fukuda, M., Kinoshita, S., et al. (2018). Vacuolar H\(^+\)-pyrophosphatase and cytosolic soluble pyrophosphatases cooperatively regulate pyrophosphate levels in *Arabidopsis thaliana*. *Plant Cell* 30, 1040–1061. doi: 10.1105/tpc.17.00911

Seufferheld, M., Vieira, M. C. F., Ruiz, F. A., Rodrigues, C. O., Moreno, S. N. J., and Docampo, R. (2003). Identification of organelles in bacteria similar to acidocalcisomes of unicellular eukaryotes. *J. Biol. Chem.* 278, 29971–29978. doi: 10.1074/jbc.M304548200

Stitt, M. (1998). Pyrophosphate as an energy donor in the cytosol of plant cells: an enigmatic alternative to ATP. *Bot. Acta* 111, 167–175. doi: 10.1111/j.1438-8677.1998.tb0092x

Takahashi, K., Morimoto, R., Tabet, H., Asaoka, M., Ishida, M., Maeshima, M., et al. (2017). Compensated cell enlargement in fugu5 is specifically triggered by lowered sucrose production from seed storage lipids. *Plant Cell Physiol.* 58, 668–678. doi: 10.1093/pcp/pcx021

Tanaka, T., Tanaka, H., Machida, C., Watanabe, M., and Machida, Y. (2004). A new method for rapid visualization of defects in leaf cuticle reveals five intrinsic patterns of surface defects in *Arabidopsis*. *Plant J.* 37, 139–146. doi: 10.1046/j.1365-313X.2003.01946.x

Thomas, P. W., Woodward, F. L., and Quick, W. P. (2003). Systematic irradiance signalling in tobacco. *New Phytol.* 161, 193–198. doi: 10.1046/j.1469-8137.2003.00954.x

Williams, K., Percival, F., Merino, J., and Mooney, H. A. (1987). Estimation of tissue construction cost from heat of combustion and organic nitrogen content. *Plant, Cell Environ.* 10, 725–734. doi: 10.1111/1365-3040.ep11604754

Yin, L., Verhertbruggen, Y., Okawa, A., Manisseri, C., Knierim, B., Prak, L., et al. (2011). The cooperative activities of CSLD2, CSLD3, and CSLD5 are required for normal *Arabidopsis* development. *Mol. Plant* 4, 1024–1037. doi: 10.1093/mp/ssr026

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.