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Regulation of pyrophosphate levels by H$^+$-PPase is central for proper resumption of early plant development

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The synthesis of DNA, RNA, and de novo proteins is fundamental for early development of the seedling after germination, but such processes release pyrophosphate (PPi) as a byproduct of ATP hydrolysis. The over-accumulation of the inhibitory metabolite PPi in the cytosol hinders these biosynthetic reactions. All living organisms possess ubiquitous enzymes collectively called inorganic pyrophosphatases (PPases), which catalyze the hydrolysis of PPi into two orthophosphate (Pi) molecules. Defects in PPase activity cause severe developmental defects and/or growth arrest in several organisms. In higher plants, a proton-translocating vacuolar PPase (H$^+$-PPase) uses the energy of PPi hydrolysis to acidify the vacuole. However, the biological implications of PPi hydrolysis are vague due to the widespread belief that the major role of H$^+$-PPase in plants is vacuolar acidification. We have shown that the Arabidopsis *fugu5* mutant phenotype, caused by a defect in H$^+$-PPase activity, is rescued by complementation with the yeast cytosolic PPase IPP1. In addition, our analyses have revealed that increased cytosolic PPi levels impair postgerminative development in *fugu5* by inhibiting gluconeogenesis. This led us to the conclusion that the role of H$^+$-PPase as a proton-pump is negligible. Here, we present further evidence of the growth-boosting effects of removing PPi in later stages of plant vegetative development, and briefly discuss the biological role of PPases and their potential applications in different disciplines and in various organisms.

Biosyntheses of macromolecules in living cells are characteristically accompanied by liberation of pyrophosphate (PPi), a byproduct of ATP hydrolysis.[1-3] PPi is formed in a variety of biosynthetic reactions and is immediately hydrolyzed to orthophosphate by pyrophosphatase (PPase); thus, the cytosolic concentration of PPi is thought to be tightly regulated.[4] Nevertheless, the physiological role of PPases remains unclear due to the drastic phenotypes that have been observed in the several PPase loss-of-function mutants of various organisms including *Arabidopsis thaliana* (Arabidopsis, hereafter).[5-8]

Role of H$^+$-PPase in Plant Seedling Development

PPases fall into two major classes, soluble PPases and membrane-bound H$^+$-translocating PPases (H$^+$-PPase), and show no sequence similarity with each other.[9] Our previous analysis of the Arabidopsis *fugu5* mutant revealed that it is defective in the vacuolar type H$^+$-PPase AVP1.[10] *fugu5* has an altered morphology associated with decreased cell number and increased cell size,[11] a phenotype that we have previously called "compensation,"[12-15] which is restricted to the cotyledons and the first pair of rosette leaves.[10] Interestingly, sucrose alone is sufficient to rescue both the visible and cellular *fugu5* mutant phenotypes.[10] Moreover, the *fugu5* mutant does not show detectable PPi hydrolysis activity, and the vacuolar H$^+$-ATPase protein level and its activity do not change, indicating that *fugu5* phenotypes are specifically caused by the loss of H$^+$-PPase activity. Biochemical analyses have also shown that the amount of sucrose decreases in germinating seedlings of *fugu5*, whereas PPi accumulates.[10] Together, these findings suggest that the elevated level of PPi (~2.5-fold increase) inhibits gluconeogenesis (~50% decrease in sucrose levels) in the *fugu5* mutant. Finally, we have demonstrated that *fugu5* mutant phenotypes are rescued by yeast cytosolic PPase IPP1 expression, which actively hydrolyzes cytosolic PPi but has no effect on vacuolar acidification. Based on these findings, we concluded that the major role of Arabidopsis vacuolar H$^+$-PPase during early seedling development is the removal of the inhibitory metabolite PPi rather than vacuole acidification.[10]

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The postgerminative growth phenotype was determined to further assess the effect of the *IPP1* gene on plant growth in later stages of vegetative development (**Fig. 1**). *fugu5* plants exhibited delayed growth, whereas this phenotype was totally recovered by introducing the $AVP1_{Pro}$:*IPP1* transgene (**Fig. 1**).[10] We measured leaf blade length, width, area, and petiole length in the first 10 rosette leaves (**Fig. 2**). Interestingly, our results revealed that the growth of transgenic plants totally recovered to wild-type levels (**Fig. 2**). These results suggest that *fugu5* mutant defects during the early stages of post-germinative development drastically hinder overall plant growth.

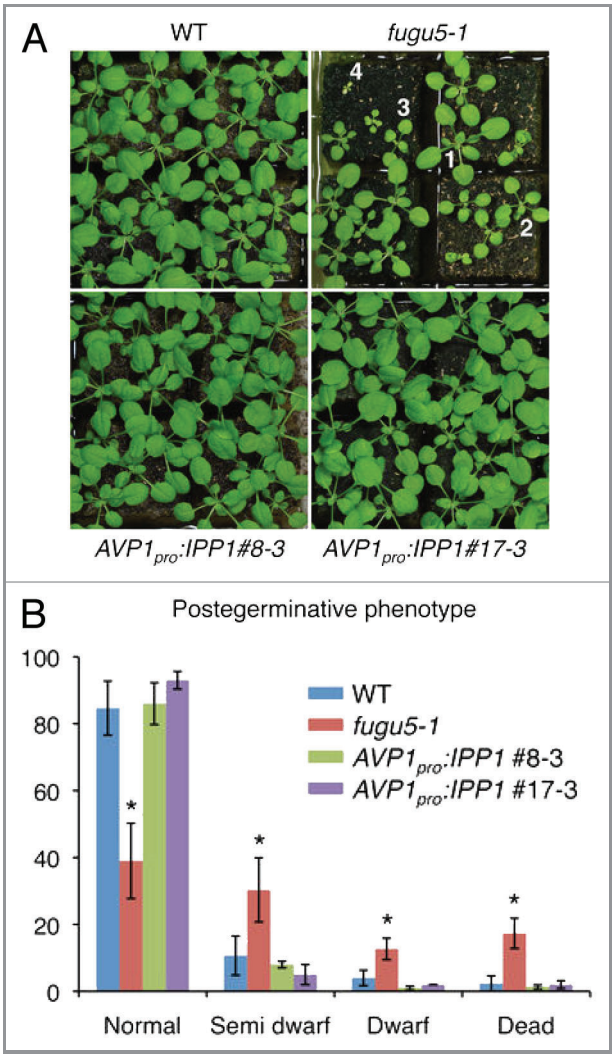

Figure 1. Heterologous *IPP1* expression rescues *fugu5* gross phenotypes. (A) Gross morphology of the wild type, *fugu5–1*, and two representative lines of $AVP1_{Pro}$:*IPP1* transgenic plants, $AVP1_{pro}$:*IPP1* #8–3 and $AVP1_{pro}$:*IPP1* #17–3, 28 d after sowing (DAS). Plants numbered 1–4 represent those used to determine postgerminative phenotypes. (1) Normal; (2) Semi dwarf; (3) Dwarf; (4) Dead. Normal, plants with the highest growth; semi-dwarf, plant size reduced by ~50% compared with normal plants; dwarf, plant size reduced by ~75% compared with normal plants. (B) Distribution of postgerminative phenotype determined at 21 DAS. Results are means ± SDs (n = 6). Asterisk, $p < 0.01$ (two-tailed Student t-test).

In a previous report, the loss-of-function mutant *avp1–1* has been reported to show severe phenotypes that could be interpreted as seedling lethal.[8] The reported phenotypes were not observed in our *fugu5* mutant alleles.[10] Two plausible explanations of such striking differences are as follows: First, the T-DNA insertion line *avp1–1* may have more than one T-DNA insertion site. Second, a large genomic region at the vicinity of *AVP1* gene was lost and/or rearranged during the T-DNA insertion process. As a straightforward way to address this issue, the above possibilities should be carefully checked using the *avp1–1* allele. Alternatively, whether *AVP1* gene can rescue the *avp1–1* mutant phenotypes or not, is fundamentally important to clarify this ambiguous situation.

On the other hand, the *AVP1* overexpression has been found to boost plant growth,[8] and to confer tolerance against salt and/or drought stress in several plant species including Arabidposis.[16-18] Still, it is difficult to discriminate between the contribution of vacuolar acidification and PPi hydrolysis and the observed phenotypes, provided that both activities should be upregulated in the *AVP1*OX lines. Interestingly, transgenic lines expressing the *IPP1* gene alone under the control of *AVP1* promoter in *fugu5* showed a slightly improved growth that sometimes exceeded that of the wild type.[10] Taken together, a direct comparative analysis of the effects of *IPP1* and *AVP1* overexpression on plant growth and/or stress tolerance should represent a powerful system to properly address the points above.

H^+-PPase as a Master Regulator of Cytosolic PPi Homeostasis

The Arabidopsis genome contains three genes that encode two types of H^+-PPase enzymes, a single gene for the type I enzyme *At*VHP1;1/AVP1,[8,10] which is exclusively localized on the tonoplast, and two genes for the type II enzyme, *At*VHP2;1 and *At*VHP2;2, which are exclusively localized on the Golgi apparatus with an extremely low expression level compared with that of *At*VHP1;1.[19] The physiological contribution of the type II H^+-PPases in vacuolar acidification and cytosolic PPi hydrolysis is fairly insignificant. This explains the absence of detectable PPi hydrolysis activity in the total membrane fraction *of fugu5*.[10] Therefore, it is evident that AVP1/FUGU5 is a master PPase regulator of cytosolic PPi levels, which is an important result for future studies related to PPi homeostasis in plants.

Molecular cloning and characterization of H^+-PPase has been also performed in several species of monocots such as barley (*Hordeum vulgare*) and rice (*Oryza sativa*).[20,21] For example, in the rice genome there are at least 6 genes encoding the type I H^+-PPase (*OsVHP1;1* to *OsVHP1;6*); with *OsVHP1;3* being markedly enhanced in deepwater rice by submersion.[21] In the starchy grains of cereals, for example, carbon is stored in the endosperm mainly in the form of starch, but the embryo, scutellum (single large cotyledon) and aleurone layer tissues that constitute a very small part of the total seed weight are enriched in triacylglycerol (TAG).[22,23] Therefore, it will be fundamentally important to determine whether H^+-PPase(s) plays a similarly critical role during the mobilization of starch and/or oil stores in nonoilseed plants.

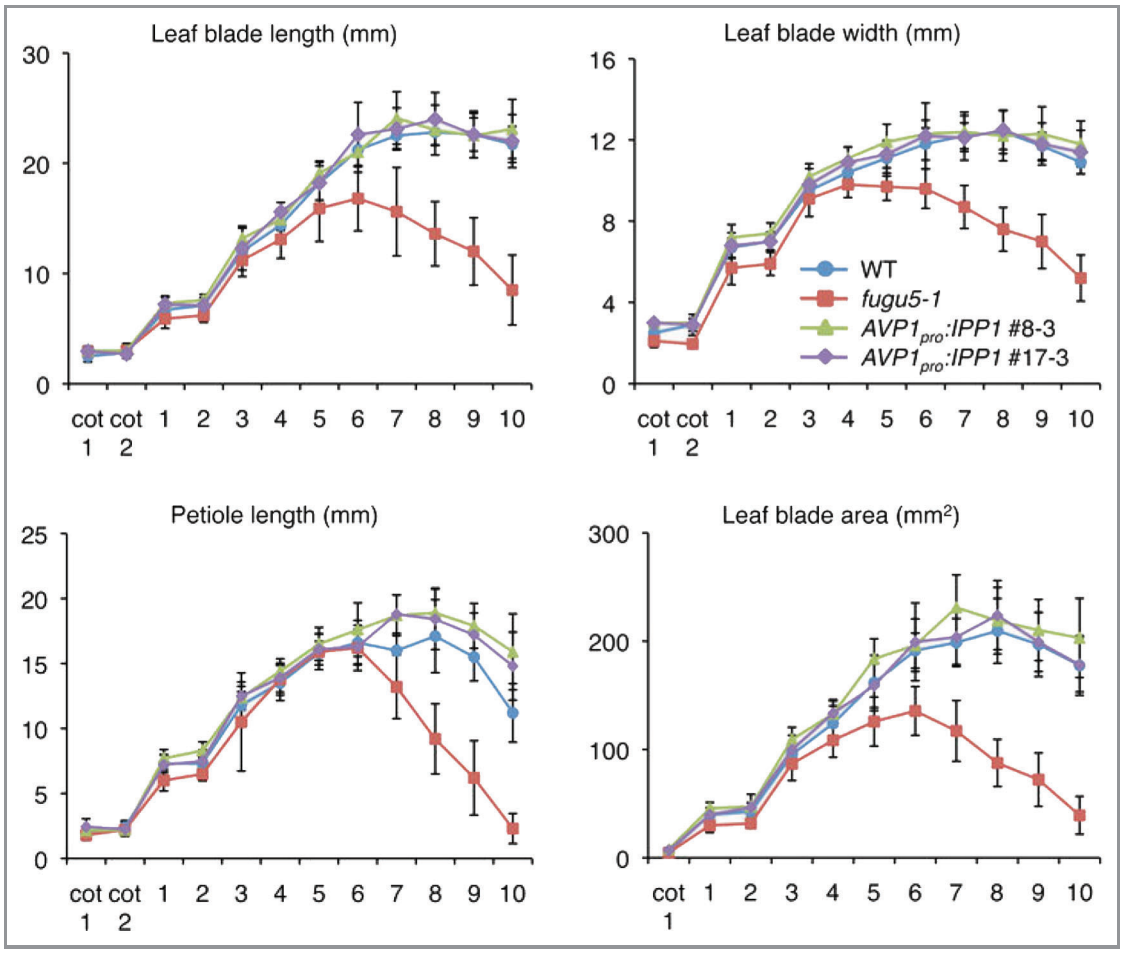

Figure 2. Heterologous *IPP1* gene expression restores the growth delay at later stages of vegetative development. Wild-type, *fugu5–1*, $AVP1_{pro}$:*IPP1* #8–3, and $AVP1_{pro}$:*IPP1* #17–3 were grown on rockwool, and the cotyledons and the first 10 rosette leaves were collected 31 d after sowing (DAS) to determine leaf blade length, width, area, and petiole length. The results are means ± SDs (n = 10). Note that growth of the *fugu5–1* was severely retarded.

Implication of H⁺-PPase in Postgerminative Oilseed Metabolism

Oilseed plants, including Arabidopsis and several food crop plants, such as *Helianthus annuus* (sunflower), *Brassica napus* (oilseed rape), *Arachis hypogaea* (peanut) and *Glycine max* (soybean) produce seeds that contain major oil reserves in the form of TAG.[24] After germination, these lipid stores are broken down providing carbon source for sucrose de novo synthesis in the cytosol (gluconeogenesis), and also intermediate metabolites used for energy production in the mitochondria (respiration).

Specialized metabolic pathways involving the β-oxidation, the glyoxylate cycle and gluconeogenesis play central role to ensure the efficient and rapid use of seed oil reserves to sustain seedling growth during its transition from heterotrophic to photoautotrophic growth. The enzymes involved in the above pathways have been extensively studied and elucidated. In spite of that, very recently, analyses of the *fugu5* mutants have provided the opportunity to discover the metabolic and physiological contribution of the H⁺-PPase during germination and postgerminative growth. Indeed, the removal of cytosolic PPi by the H⁺-PPase has a major contribution for sustaining gluconeogenesis.[10] The inhibitory effect of cytosolic PPi accumulation on several biosynthetic reactions has been generally recognized (**Fig. 3**). For the particular case of gluconeogenesis, the limiting step for sucrose synthesis under high PPi levels has to be determined.

Focus on Metabolic Significance of PPase Enzymes Toward Unraveling Their Role in Animal Systems

Our findings on plant PPase also provide clues for understanding the roles of animal PPases. In plants, H⁺-PPase activity is generally high in young tissues such as leaf primordia, which are characterized by high proliferative activity.[25] It is interesting to note that increased PPase expression in humans is associated with hyperthyroidism[26] and many types of cancer.[27-29] For example, cytosolic PPase is expressed 7.6-fold higher, and three isoforms of triosephosphate isomerase (a key component of the glycolytic pathway) are upregulated 2.1- to 2.7-fold higher, in human lung tumors than in normal lung tissues.[29] This suggests that the human cytosolic PPase may help clear toxic byproducts resulting from elevated metabolism in these tumors, such as PPi, to sustain

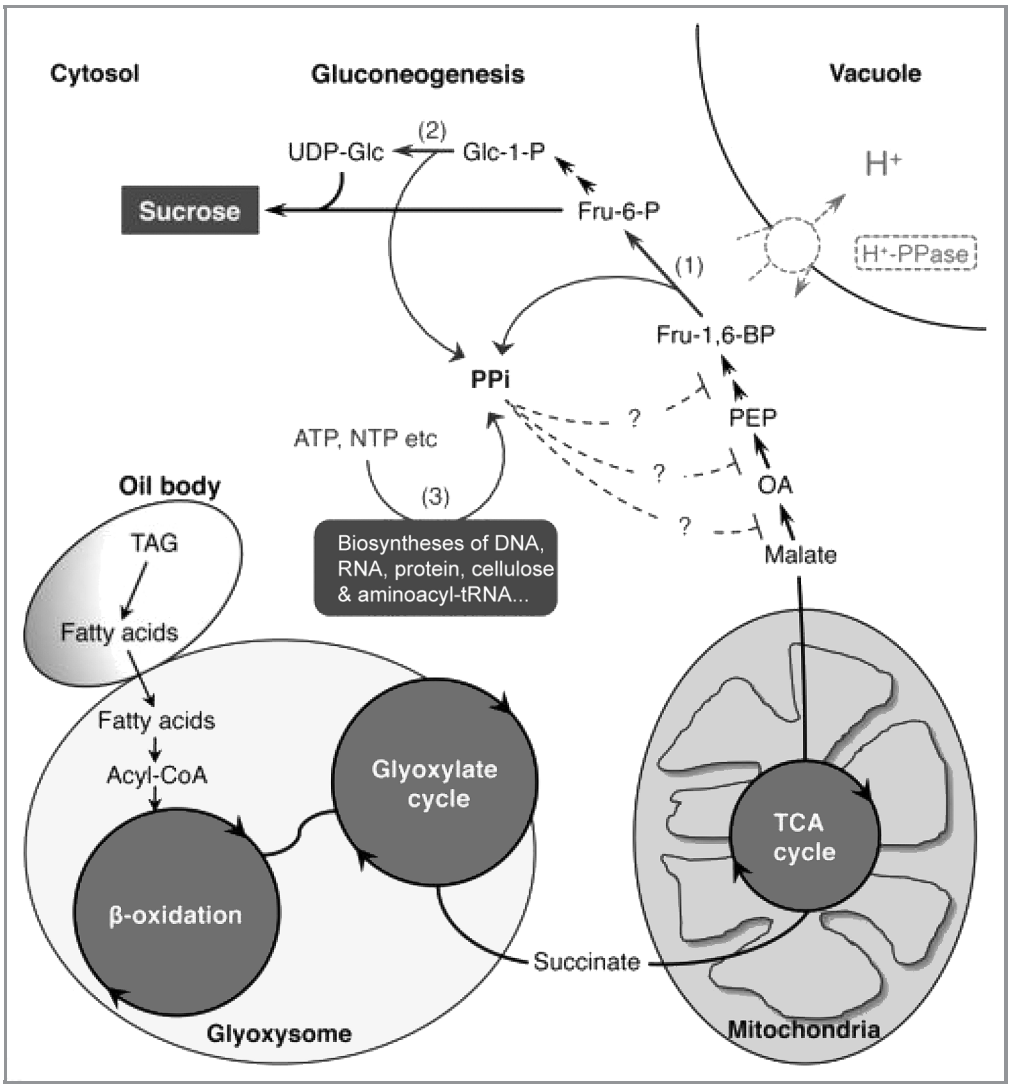

Figure 3. Schematic model of the impact of H^+-PPase loss-of-function on postgerminative metabolism in oilseeds. The metabolic pathways shown here were taken and modified from Ferjani et al. (2011). TAGs from oil bodies are broken down into fatty acids by the action of lipases, then transported into the glyoxysomes where they are converted to acetyl-CoA by β-oxidation and then to succinate by the glyoxylate cycle. Succinate passes from the glyoxysome to the mitochondria and enters the tricarboxylic acid (TCA) cycle, where it is converted to malate. Malate exported to the cytosol is oxidized to oxaloacetate (OA) and then sequentially converted to phosphoenolpyruvate (PEP), fructose 1,6-bisphosphate (Fru-1,6-BP); fructose 6-phosphate (Fru-6-P); Glucose 1-phosphate (Glc-1-P); and finally to UDP-glucose (UDP-Glc). During gluconeogenesis, PPi is generated by the reactions of PPi-dependent phosphofructokinase (1) and by the reaction of UDP-Glc pyrophosphorylase (2). Syntheses of macromolecules, such as cellulose and proteins also generate PPi as a by-product (3). PPi in the cytosol is usually consumed by the H^+-PPase. H^+-PPase loss-of-function causes an overaccumulation of PPi in the cytosol.[10] PPi inhibits cellular metabolism such as reactions (1) and (2) resulting in partial arrest of gluconeogenesis.[10] Reaction (3) is also susceptible to PPi inhibiton leading to either arrest or retardation of syntheses of macromolecules. Other metabolic reactions immediately upstream (dotted lines) or downstream of reaction (1) might be a potential target of PPi inhibition.

cell cycling and other cellular activities. In contrast, the null mutant *Caenorhabditis elegans pyp-1* shows developmental arrest at the L2 larval stage and exhibits gross defects in intestinal morphology and function.[7] This could explain the PYP-1::GFP expression pattern in the intestines and nervous system, as the intestines are highly metabolic organs involved not merely in digestion but also in storage and macromolecule synthesis.[7] Therefore, it appears that active synthesis of macromolecules, as a key process, may represent a common target of PPi inhibition leading to the observed phenotypes, given its central role in cellular and organismal growth. By focusing on the metabolic significance of PPases, which we believe are rather well conserved among all living things, we may gain valuable insights into PPase function in different organisms including vertebrates. Alternatively, PPases may represent a novel potential target of many cancers, which would allow the development of new promising specific inhibitors or drug-delivery systems to cure this lethal disease.

Final Remarks

We recently showed that removing PPi is essential to sustain cell proliferation during Arabidopsis germination, a stage in which vacuolar acidification seems to have no contribution.[10] However, the remainder of the compensation mechanism (i.e., excessive cell enlargement) remains unclear and will be addressed in future research. In our analyses, plants were always grown under standard culture conditions. Therefore, it would be of great interest to determine whether H^+-PPase plays a role as a proton pump under particular growth conditions, such as drought or other kinds of abiotic stress. These results might deepen our understanding of the evolutionary significance of having a vacuolar membrane-bound H^+-PPase rather than a cytosolic PPase in plants.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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