The flip side of the Arabidopsis type I proton-pumping pyrophosphatase (AVP1): Using a transmembrane H⁺ gradient to synthesize pyrophosphate

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Energy partitioning and plant growth are mediated in part by a type I H⁺-pumping pyrophosphatase (H⁺-PPase). A canonical role for this transporter has been demonstrated at the tonoplast where it serves a job-sharing role with V-ATPase in vacuolar acidification. Here, we investigated whether the plant H⁺-PPase from Arabidopsis also functions in “reverse mode” to synthesize PPi using the transmembrane H⁺ gradient. Using patch-clamp recordings on Arabidopsis vacuoles, we observed inward currents upon application to the cytosolic side. These currents were strongly reduced in vacuoles from two independent H⁺-PPase mutant lines (vhp1-1 and fugu5-1) lacking the classical PPi-induced outward currents related to H⁺ pumping, whereas they were significantly larger in vacuoles with engineered heightened expression of the H⁺-PPase. Current amplitudes related to reverse-mode H⁺ transport depended on the membrane potential, cytosolic P$i$ concentration, and magnitude of the pH gradient across the tonoplast. Of note, experiments on vacuolar membrane–enriched vesicles isolated from yeast expressing the Arabidopsis H⁺-PPase (AVP1) demonstrated P$i$-dependent PPi synthase activity in the presence of a pH gradient. Our work establishes that a plant H⁺-PPase can operate as a PPi synthase beyond its canonical role in vacuolar acidification and cytosolic PPi scavenging. We propose that the PPi synthase activity of H⁺-PPase contributes to a cascade of events that energize plant growth.

H⁺-Pyrophosphatases are hydrophobic proteins thought to function in the generation of proton gradients across membranes using the energy of the phosphoanhydride bond of pyrophosphate (PPi) molecules (1, 2). Plant type I H⁺-pumping pyrophosphatase (H⁺-PPases) were isolated from tonoplasts and considered to be vacuolar markers (V-PPase)(4). Vacular functions require significant fluxes of ions and solutes across the tonoplast that are presumed to be energized by the proton gradient and membrane potential created by the combined activity of the vacuolar H⁺-ATPase (V-ATPase) and the V-PPase (3, 4). However, studies with gold-conjugated H⁺-PPase–specific antibodies show a dual localization of the Arabidopsis H⁺-PPase (AVP1; At1g15690; also referred to as AtVHP1 and VHP1) at the tonoplast and at the plasma membrane (PM) of vascular cells (5) supported by proteomic approaches (6, 7).

The type I H⁺-PPase is a single-subunit enzyme highly conserved among plant species and has been amenable for enhanced expression in a variety of crops (8, 9). Up-regulation of H⁺-PPases in Arabidopsis, rice, corn, barley, wheat, tomato, lettuce, cotton, and finger millet contributes to improved crop yield and nutrient acquisition (9–11). Although H⁺-PPase expression improves crops and some of these traits may be due to enhanced vacuolar acidification and PPi scavenging, the field remains largely puzzled regarding the mechanistic basis of the beneficial traits achieved by enhancing H⁺-PPase expression. Several models have been proposed, including greater vacuole sequestration, enhanced heterotrophic growth, and increased transport of sucrose from source to sink tissues (12).

The role of H⁺-PPase in vacuolar acidification has been studied using a variety of approaches (13, 14). Heterologous expression of AVP1 in yeast has firmly established PPi hydrolysis and P$i$-dependent H⁺ translocation (15). However, the plant studies are less straightforward (12). A fugu5 mutant plant, which lacks functional PPase activity of AVP1, had little to no change in measured vacuole pH and cell sap pH compared with WT plants (3, 16). Stable AVP1 overexpression in Arabidopsis had no effect on vacuolar pH (3, 17), whereas transient overexpression of NbVHP1 in tobacco leaves hyperacidified mesophyll vacuoles (18), possibly due to differences in the respective protein levels.

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4 The abbreviations used are: V-PPase, vacuolar pyrophosphatase; BTP, bistris propane (1,3-bis[tris(hydroxymethyl)methylamino]propane); H⁺-PPase, H⁺-pumping pyrophosphatase; PM, plasma membrane; SE-CC, sieve element–companion cell; Suc, sucrose; V-ATPase, vacuolar H⁺-ATPase; pf, picofarad; vac, vacuole; cyt, cytosol; KF, potassium fluoride; ACMA, 9-amino-6-chloro-2-methoxyacridine; CAX1, cation exchanger 1; Col-0, Columbia-0.
In *Arabidopsis*, AVP1 appears to regulate PP₄-dependent metabolic pathways in some plant tissues. A *fugu5* mutant has 60% fewer and 175% larger cells than WT and has 2.5-fold higher levels of PP₄ per seedling and 50% less sucrose per seedling (16). These phenotypes are suppressed by sucrose or glucose in the media or when plants are engineered to express a cytosolic soluble inorganic pyrophosphatase (Ipp1) from yeast. Additionally, *fugu5* lines expressing uncoupling mutants of the H⁺-PPase that are able to hydrolyze cytosolic PP₄, without pumping protons grow in a manner indistinguishable from WT (8). Recent studies show that in *fugu5-1* lines H⁺-PPase is essential for maintaining adequate PP₄ levels and together with cytosolic pyrophosphatase (PPα) isozymes prevents increases in PP₄ concentrations to toxic levels (14). These studies support a robust model that in metabolically active tissues such as the mesophyll AVP1 plays an important role in PP₄ homeostasis.

Independent studies have suggested that H⁺-PPases can also function in reverse and drive PP₄ synthesis (19–22). Under steep trans-tonoplast H⁺ gradients, tonoplast fractions isolated from orange fruit cells effectively synthesize PP₄ (19). However, the identity of the polypeptides directly contributing to this activity has not been determined. The PP₄ synthase model is supported by the phenotypes of plants in which up-regulation of the H⁺-PPase localized to the PM of the sieve element–companion cell (SE-CC) complex increases sucrose loading and transport from source to sink tissues, presumably through enhanced PP₄ supply (20, 23, 24). The phloem-specific expression of AVP1 phenocopies the higher reduced carbon translocation capacity seen when AVP1 is constitutively expressed using the CaMV35S promoter (24). This synthase model is further buttressed by the thermodynamical feasibility for H⁺-PPases to synthesize PP₄ if a steep proton gradient is present (25). As mentioned above, PP₄ synthesis has been shown within plant tissues (19, 22). Furthermore, PP₄ synthesis is theoretically possible based on the physical structure of the H⁺-PPase (21, 26). However, to date, no studies have directly ascribed PP₄ synthase activity to AVP1 or any other plant H⁺-PPase (12).

By using patch-clamp recordings on *Arabidopsis* vacuoles, we demonstrated here inward currents upon PP₄ application, consistent with the reverse function of the type I H⁺-PPase. Furthermore, experiments on vacuolar membrane–enriched vesicles isolated from yeast expressing *Arabidopsis* H⁺-PPase demonstrated that this gene encodes both the canonical PP₄, hydrolysis–driven H⁺-pumping and synthase activities. The synthase activity associated with AVP1 is indistinguishable from the synthase activity identified previously on the vacuolar membrane of plant cells. We propose that these functions, modulated by different environmental and spatial cues, contribute to the cascade of events that energize plant growth.

**Results**

The canonical function of vacuolar H⁺-PPases as PP₄, hydrolysis–driven H⁺ pumps (“forward” mode) is well-established, and the related outward currents are easily resolvable in whole-vacuole patch-clamp experiments (18, 27, 28). Here, we investigated the electrical activity related to the PP₄ synthesis activity or “reverse” mode of vacuolar H⁺-PPases. With an imposed gradient of 4 pH units (acidic inside), whole-vacuolar membrane currents in *Arabidopsis thaliana* Columbia-0 (Col-0) WT vacuoles were recorded in response to repeated stimulation with a 200-ms voltage ramp ranging from −80 to +80 mV (Fig. 1A, top). Upon perfusion of the recording chamber with a bath solution containing 5 mM P₄ (as a H⁺-PPase substrate), small inward currents were elicited exclusively at negative membrane potentials (Fig. 1A, top, blue trace). Current activation was fully reversible after washout (Fig. 1A, bottom). The appearance of inward currents in response to P₄ application is consistent with cytosol-directed H⁺ flux (see sign convention under “Experimental procedures”). To test whether these inward currents are related to H⁺-PPase operating in reverse, we determined the amplitudes of P₄-induced currents (obtained by subtraction of background currents in control conditions) in several *Arabidopsis* lines differing in their H⁺-PPase expression. Compared with Col-0 WT vacuoles, P₄-induced current densities were strongly reduced in two different AVP1 mutant plants (Fig. 1, B and C), *vhp1-1* (a T-DNA insertion line) and *fugu5-1* (carrying the A709T substitution) (16, 48). Vacuoles from both lines lack PP₄ hydrolysis activity (3, 16) and PP₄-dependent H⁺ currents (*fugu5-1* data shown in Fig. 3B; *vhp1-1* data not shown). By contrast, P₄-induced current densities were distinctly higher in two independent *UBQ10::AVP1* overexpressor lines (Fig. 1, B and C), which have been shown to exhibit 2–3-fold higher V-PPase activity compared with Col-0 plants (3). Moreover, P₄-induced current densities in knockout plants for the major vacuolar phosphate transporter that contributes to vacuolar P₄ sequestration, VPT1 (29), were comparable with those in Col-0 vacuoles (Fig. 1C), arguing against a significant contribution of vacuole-directed P₄ flux in the observed vacuolar inward currents.

When K⁺ and Mg²⁺ in the pipette solution were substituted by the large impermeable cation bistris propane (BTP), similar P₄-induced currents were observed in *UBQ10::AVP1* vacuoles (−0.94 ± 0.13 pA/pF (n = 6) at −73 mV (corrected for liquid junction potential) and pH₅₀ 3.2), further supporting the idea of H⁺ acting as the principal charge carrier of P₄-dependent inward currents.

Based on the observation that inward currents in Col-0 and *UBQ10::AVP1* vacuoles activated predominantly at negative membrane potentials (Fig. 1, A and B), we further explored their voltage dependence. P₄-induced currents at −160 mV were about twice as large as those determined at −80 mV (Fig. 1, D and E), indicating that negative membrane potentials strongly support AVP1-mediated cytosol-directed H⁺ fluxes.

To investigate whether these currents depend on the cytosolic P₄ concentration, we successively exposed the same vacuole to different bath solutions containing increasing P₄ amounts. P₄-induced currents were small at 0.5 mM P₄, but strongly increased at 2 mM and increased further at 5 and 20 mM (Fig. 2, A and B). Similar experiments on *fugu5-1* mutant vacuoles showed low current densities at 5 mM P₄ (compare Fig. 1C) and only slightly higher values at 20 mM P₄ (Fig. 2B). Dose-response analyses on normalized P₄-induced currents recorded from individual vacuoles (Fig. 2C) revealed a half-maximal P₄ con-
**PP_i synthase function of Arabidopsis type I H^+-PPase**

**Figure 1. Cytosolic P_i elicits AVP1-dependent inward currents.** A, whole-vacuolar membrane currents (top) and time course of current amplitudes at $-80$ mV (bottom) recorded in an Arabidopsis Col-0 vacuole, switching from control bath solution (black trace) to bath solution containing 5 mM P_i (blue trace) and back to control solution. Currents were elicited using the voltage protocol shown below (x axis, time in ms; y axis, V in mV). Voltage stimulation was applied every 10 s; current traces correspond to the indicated time points. Pipette solution was adjusted to pH 3.2; bath solution was adjusted to pH 7.2. B, whole-vacuolar membrane currents recorded in vacuoles isolated from *fugu5-1* (top) and UBQ::AVP1 18-4 plants (bottom). Experimental conditions and scaling were as in A. The zero current level is indicated by dashed lines. C, summary plot of P_i-induced current amplitudes (obtained by subtraction of background currents in control conditions) at $-80$ mV, determined from recordings as in A and B and normalized to the vacuolar membrane capacitance (in pA/pF). D, whole-vacuolar membrane currents recorded in an UBQ::AVP1 18-4 vacuole in control bath solution (black trace) and in bath solution containing 5 mM P_i (blue trace). Currents were elicited by a voltage ramp ranging from $+80$ to $-160$ mV and plotted versus the applied membrane potential. P_i-dependent current amplitudes at $-80$ and $-160$ mV are marked by red vertical lines. Pipette solution was adjusted to pH 4.5. E, summary plot of P_i-induced current amplitudes at the test potentials $-80$ and $-160$ mV, determined from recordings on the same vacuole (pH_vac 4.5) as in D and normalized to the vacuolar membrane capacitance (in pA/pF), in vacuoles isolated from UBQ::AVP1 overexpressor plants ($n = 6$). Gray bars represent mean values, error bars show S.D., and circles represent individual data points. Asterisks (**) indicate statistical significance with $p < 0.001$; *, $p < 0.05$, using Student’s unpaired t test or Mann–Whitney U test versus Col-0; ns indicates not significant. D, current-voltage relationships of whole-vacuolar currents recorded from an UBQ::AVP1 18-4 vacuole in control bath solution (black trace) and in bath solution containing 5 mM P_i (blue trace). Currents were elicited by a voltage ramp ranging from $+80$ to $-160$ mV and plotted versus the applied membrane potential. P_i-dependent current amplitudes at $-80$ and $-160$ mV are marked by red vertical lines. Pipette solution was adjusted to pH 4.5. E, summary plot of P_i-induced current amplitudes at the test potentials $-80$ and $-160$ mV, determined from recordings on the same vacuole (pH_vac 4.5) as in D and normalized to the vacuolar membrane capacitance (in pA/pF), in vacuoles isolated from UBQ::AVP1 overexpressor plants ($n = 6$). Gray bars represent mean values, error bars show S.D., and circles represent individual data points. Asterisks (**) indicate statistical significance with $p < 0.001$ (Student’s paired t test).

**Figure 2. P_i-elicited inward currents depend on the cytosolic P_i concentration.** A, time course of current amplitudes (at $V = -80$ mV) in response to 0.5, 2, 5, and 20 mM P_i in the bath solution as indicated. Whole-vacuolar membrane currents were recorded from an UBQ::AVP1 18-4 vacuole. Voltage stimulation was applied every 10 s. B, average P_i-induced current amplitudes (in pA/pF) determined at different cytosolic P_i in UBQ::AVP1 18-4 vacuoles ($n = 4$ for 0.5/2 mM, $n = 6$ for 5/20 mM) and *fugu5-1* vacuoles ($n = 3$). Asterisks (**) indicate statistical significance with $p < 0.01$ (Mann–Whitney U test). C, semilogarithmic plot of normalized P_i-induced currents in UBQ::AVP1 18-4 vacuoles ($n = 4$). For each individual vacuole, current amplitudes measured at different cytosolic P_i were normalized to the value determined at 5 mM P_i and subjected to a fit with a Hill function, resulting in $K_{0.5} = 3.1 \pm 0.2$ mM, $h = 1.27 \pm 0.07$, and $h_{max} = 1.61 \pm 0.08$. In A–C, pipette solution was adjusted to pH 3.2, and bath solution was adjusted to pH 7.2. Data in B and C represent mean values, and error bars show S.E.

PP_i synthesis by vacuolar H^+-PPases is supposed to be energized by the trans-tonoplast H^+ gradient. Indeed, measured P_i-induced current densities of AVP1 overexpresser vacuoles were larger at pH_vac 3.2 (Fig. 1C) than at pH_vac 4.5 (Fig. 1E). To
investigate the pH dependence of H\(^+\)-PPase in more detail, we quantified both forward-mode H\(^+\) pump activity and reverse-mode currents at varying vacuolar pH values.

Bath application of 100 \(\mu\)M PPi elicited small outward currents in \textit{UBQ10}:AVP1 vacuoles (Fig. 3A), consistent with H\(^+\)-pumping activity into the vacuolar lumen (see sign convention under “Experimental procedures”). PPi-dependent current amplitudes recorded at pH\(_{\text{vac}}\) 7.2 were on average +3.86 ± 0.21 pA/pF (\(n = 8\)), about twice the value measured in Col-0 vacuoles (+2.09 ± 0.22 pA/pF; \(n = 18\)), which is in good agreement with the increase in V-PPase activity determined in \textit{UBQ10}:AVP1 plants (14). H\(^+\) currents at pH\(_{\text{cyt}}\) 7.2 (\(\Delta pH = pH_{\text{cyt}} - pH_{\text{vac}} = 0\)) were only moderately voltage-dependent, whereas those recorded at pH\(_{\text{vac}}\) 3.2 (\(\Delta pH = 4\)) were highly sensitive to the applied membrane potential in the ±80-mV range (Fig. 3A). This is consistent with the prediction that the reversal potential of the pump current gradually shifts in the positive direction as \(\Delta pH\) increases and vacuolar pH becomes more acidic (the diffusional force \(E_{\text{d}}\) changes from 0 mV at pH\(_{\text{vac}}\) 7.2 to +237 mV at pH\(_{\text{vac}}\) 3.2). PPi-induced outward current densities determined at 0 mV were maximal at pH\(_{\text{vac}}\) 7.2 and progressively decreased at more acidic pH\(_{\text{vac}}\) values. At pH\(_{\text{vac}}\) 2.9 (\(\Delta pH = 4.3\)), there was still measurable H\(^+\) pump activity, which was about 6 times lower than the maximum (Fig. 3B). No PPi-dependent currents were detected in \textit{fugu5-1} vacuoles (Fig. 3B).

Conversely, inward currents elicited by 5 mM Pi were completely absent at pH\(_{\text{vac}}\) 7.2 (\(\Delta pH = 0\)) in \textit{UBQ10}:AVP1 vacuoles (Fig. 3C) but activated in the presence of a trans-tonoplast H\(^+\) gradient, steadily increasing in amplitude with more acidic pH\(_{\text{vac}}\) values (Fig. 3, C and D). PPi-dependent currents recorded in \textit{fugu5-1} vacuoles were very small both at pH\(_{\text{vac}}\) 5.5 and pH\(_{\text{vac}}\) 3.2 (Fig. 3D) and possibly reflect residual reverse-mode H\(^+\) flux mediated by the mutant protein (21) and/or vacuolar background activity due to lumen-directed Pi flux.

These data collectively demonstrate that (i) cytosol-directed H\(^+\) currents related to vacuolar H\(^+\)-PPase working in reverse mode can be resolved in Col-0 WT vacuoles and clearly dominate the PPi-dependent inward currents in \textit{UBQ10}:AVP1 vacuoles, (ii) the prevailing pH gradient across the tonoplast has a strong impact on the direction and voltage dependence of H\(^+\)-PPase activity, and (iii) reverse-mode PPi synthesis by vacuolar H\(^+\)-PPase may potentially be active in the modestly acidic conditions present inside \textit{Arabidopsis} vacuoles (vacuolar pH 5.8 versus cytosolic pH 7.3), requiring cytosolic Pi and a trans-tonoplast H\(^+\) gradient as sole prerequisites.

To provide direct evidence of the ability of the \textit{Arabidopsis} proton-pumping pyrophosphatase AVP1 to synthesize Pi, we expressed the fusion protein TcGFP-AVP1 in yeast mutants defective in vacuolar proteases (\textit{pep4 prb1}). Heterologous expression of AVP1 in these strains yields consistent V-PPase activity (15, 31, 32). Also, translational fusions using the N-terminal signal sequence of the H\(^+\)-PPase from the protist \textit{Trypanosoma cruzi} (33) enhance the expression levels of AVP1 and accumulation in intracellular acidic vesicles (34). Vacuole vesicles isolated after a 60-min copper induction in the \textit{pep4 prb1} strain expressing the tagged AVP1 confirmed expression of TcGFP-AVP1 (Fig. 4A) and displayed Pi hydrolysis and PPi-dependent H\(^+\) translocation demonstrated in previous yeast-based expression studies (Fig. 4, B–D, and Fig. S1, A and B) (15, 32). Specifically, cells transformed with pTcGFP-AVP1 showed higher PPi, hydrolytic activity than control vacuoles that was sensitive to potassium fluoride (KF), a well-known inhibitor of soluble and membrane-bound pyrophosphatases. This residual and fluoride-sensitive activity of control vacuoles was attributed to contaminating soluble yeast pyrophosphatase (Fig. 4B and Fig. S1A). The AVP1 PPi-dependent pumping activity was measured by a characteristic trace of 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence quenching that depended on the addition of PPi. This quenching was reversed by the addition of the ionophore gramicidin, which proves that this activity depends on intravesicular H\(^+\) accumulation. Vacuoles from cells transformed with the empty vector (control) did not yield PPi-dependent ACMA quenching (Fig. 4, C and D, and Fig. S1B), as expected.

As our patch-clamp data showed that AVP1 reverse mode requires an acidic pH (Fig. 5D), we incubated our yeast vesicles in the presence of ATP as we verified that the yeast V-ATPase generates a pH gradient (Fig. 5A) necessary to drive TcGFP-AVP1 activity in the reverse direction. In the presence of 2 mM P, levels of PPi were below the detection limit of our assays. However, in the presence of both ATP and P, TcGFP-AVP1–expressing vesicles displayed PPi synthase activity (Fig. 5B and Fig. S1C), suggesting that an acidification of the vacuoles is needed for AVP1-mediated PPi synthesis. Variability in activity was observed among different yeast cells transformed with the
AVP1 plasmid due to different TcGFP-AVP1 expression levels. In every case, yeast strains expressing TcGFP-AVP1 displayed at least 2-fold higher PPi synthase activity than controls (Fig. S1). Our preliminary data also suggest that untagged versions of AVP1 exhibit synthase activity. To validate that this PPi synthesis activity was mediated by the heterologous expression of this specific plant enzyme, we evaluated PPi synthesis levels in a vacuolar protease–deficient strain expressing the Arabidopsis cation exchanger 1 (CAX1). Although there were background levels of PPi synthase function of Arabidopsis type I H\textsuperscript{+}-PPase

Figure 4. Characterization of AVP1 pyrophosphatase activity from yeast vacuolar membranes. A, Western blot analysis of membranes purified from cells of \textit{S. cerevisiae}. \textit{Lane 1}, vacuolar membrane vesicles (10 \mu g) isolated from an empty vector expressing pep4 \textit{prb1} strain after a 60-min copper induction; \textit{lane 2}, vacuolar membrane vesicles (10 \mu g) isolated from a TcGFP-AVP1–expressing pep4 \textit{prb1} strain after a 60-min copper induction. The positions of the molecular mass markers (in kDa) are indicated. The amount of protein present in each sample is shown in the Ponceau S–stained (PonS) filter. ALP, alkaline phosphatase. B, specific PP\textsubscript{i} hydrolysis associated with vacuolar membranes obtained from strains indicated in A. PP\textsubscript{i} hydrolysis was calculated as the release of Pi in the absence (KF) or presence of KF (+KF). Background levels were subtracted from the measurements. The bars represent the average value for three technical replicates representative of three independent experiments with different biological replicates. The circles represent each individual data point, and the error bars show the S.D. Asterisks (***) indicate statistical significance (\textit{t} test) with a p value \textless 0.001 at the 20-min time point.

Figure 5. Characterization of the AVP1-PP\textsubscript{i} synthesis activity from yeast vacuolar membranes. A, proton translocation activity was measured by recording the ATP-dependent ACMA fluorescence quenching in the PP\textsubscript{i} synthesis activity reaction conditions in vacuolar membrane vesicles isolated from W303 pep4 \textit{prb1} mutant strain transformed with a multicopy copper-inducible plasmid containing a TcGFP-AVP1 chimeric fusion or with an empty plasmid (Control), both after a 60-min exposure to copper. This experiment is a graphical representation of spectrofluorometric data reading from a typical experiment. AU, arbitrary units. B, PP\textsubscript{i} formation by 10 \mu g of vacuoles in the presence of 1.5 mM ATP and 2 mM of Pi (ATP/Pi) from strains indicated in A. Control samples contained boiled membrane aliquots (Boiled TcGFP-AVP1 (ATP/Pi)). Data are a graphical representation of spectrophotometric output and converted to nmol. These data are the average of three technical replicates representative of three different biological replicates. The S.D. is 0–1\% for boiled TcGFP-AVP1, 0–2\% for control (ATP/Pi) and 0–6\% for TcGFP-AVP1 (ATP/Pi). Asterisks (***) indicate statistical significance (\textit{t} test) with a p value \textless 0.001 at the 20-min time point.

AVP1 plasmid due to different TcGFP-AVP1 expression levels. In every case, yeast strains expressing TcGFP-AVP1 displayed at least 2-fold higher PP\textsubscript{i} synthase activity than controls (Fig. S1C). Our preliminary data also suggest that untagged versions of AVP1 exhibit synthase activity. To validate that this PP\textsubscript{i} synthesis activity was mediated by the heterologous expression of this specific plant enzyme, we evaluated PP\textsubscript{i} synthesis levels in a vacuolar protease–deficient strain expressing the Arabidopsis cation exchanger 1 (CAX1). Although there were background biological replicates. D, graphical representation of the average rate of PP\textsubscript{i}–dependent ACMA quenching of three technical replicates representative of three independent experiments with different biological replicates. The circles represent each individual data point, and error bars show the S.D. Asterisks (**) indicate statistical significance (\textit{t} test) with a p value \textless 0.01.
levels of PP, synthesis activity apparent in the CAX1-expressing vacuolar vesicles, the levels were comparable with vector controls and did not show the magnitude of PP, synthesis activity displayed in the TcGFP-AVP1—expressing lines (data not shown). Taken together, the yeast results suggest that Arabidopsis AVP1 possesses both pyrophosphatase and PP, synthase activities.

Discussion

Nothing in the idea of a PP, synthase function of H⁺-PPases is in conflict with thermodynamic laws (22, 40, 41). The question is therefore not whether it works but rather in which circumstances and for what purpose. For the plant cell, the PP, synthase makes sense as it allows dynamic conversion of ATP-derived energy stored in transmembrane H⁺ gradients back into a biochemically accessible form. This may be particularly important during times when anaerobic respiration becomes more prominent (19, 35, 36).

In this study, we demonstrated PP, synthase activity of AVP1 using two complementary experimental approaches: (i) patch-clamp recordings on isolated Arabidopsis vacuoles and (ii) detection of PP, production in membrane vesicles derived from AVP1-expressing yeast cells. Patch-clamp recordings of the switchable real-time electrical activity (cytosol-directed H⁺ flux) related to the reverse-mode PP, synthase function provided unique information on AVP1 transport rates in a native membrane environment and under defined conditions of substrate concentration and membrane potential. In this study, experiments were designed to determine forward-mode and reverse-mode currents independently, i.e., by exclusive application of one of the two substrates at near-saturating concentrations combined with continuous bath perfusion, preventing a significant accumulation of the respective products. Consequently, PP, exclusively elicited outward currents, P, exclusively elicited inward currents, and no current reversal could be observed in either condition. Reversibility of vacuolar H⁺-PPase has been reported by early patch-clamp work on red beet and Chenopodium vacuoles (37, 38), solely based on the observation of P, elicited inward currents (according to the sign convention used in the present study). Here, genetic precepts allowed us to ascribe the P,-dependent currents directly to AVP1 activity and to exclude the contribution of alternative ion fluxes. Notably, P, dependent currents in Arabidopsis vacuoles lacked the strict vacuolar K⁺ dependence reported in these earlier studies, supporting the idea of cytosol-directed H⁺ acting as the principal charge carrier.

Although this study demonstrates PP, synthase activity at the vacuolar membrane, we are left to infer the same activity at the PM. Notably, our finding that reverse-mode currents were strongly dependent on the voltage amplitude in the negative voltage range indicates that the proposed PP, synthase function at the PM of the SE-CC complex may be greatly favored by the steep electrical potential difference of -150 to -200 mV across the plant PM (39). Combined with transient expression assays, future work will specifically address questions on AVP1 reversibility in mixed substrate conditions as well as structure-function relations for the two activities, which can be determined independently for the same vacuolar AVP1 protein population.

The main limitation of the patch-clamp approach, i.e., lack of direct information on AVP1-mediated PP, production, was overcome by heterologous expression in bakers’ yeast. In this system, AVP1 expression has to be carefully modulated to maintain viability of the cells. Here, a vacuolar protease–deficient yeast strain, copper-inducible expression system, and tagged versions of AVP1 were used to improve stability/expressions (34). Addition of ATP to vesicles from TcGFP-AVP1—expressing cells resulted in greater synthesis of PP, than in control vacuoles as we postulate that the V-ATPase is able to generate a pH gradient to drive AVP1 activity in the reverse direction (Fig. 5). In the absence of ATP, no activity was detected. Future work will identify yeast strains that display minimal endogenous PP, formation and allow maximal AVP1-mediated synthase activity. Techniques to improve the spatial resolution and kinetics of PP, formation will also allow kinetic measurement for this synthase.

This work reinforces other studies that have demonstrated the multiple roles of AVP1 in different tissue and cell types throughout the life cycle of the plant (12). We favor a model where there are dynamic fluctuations in H⁺-PPase activity based on environmental and spatial cues (9, 23). By directly demonstrating synthase activity, this work helps galvanize the model where the H⁺-Phs syntheses activity plays a role in phloem sucrose (Suc) loading and transport (20, 24). Phloem loading of Suc in the SE-CC complex of plants that load from the apoplast requires Suc/proton symporters. P-type H⁺-ATPases energize this process, establishing a proton motive force. The apoplastic pH in loading phloem can thereby reach values of 5.0 (40). To generate the required ATP, a portion of the loaded Suc is oxidized in companion cells. Suc oxidation via the P, dependent Suc synthase pathway is essential. The PP, synthase activity of the H⁺-PPase localized to the PM of the SE-CC complex may increase the PP, supply, facilitating Suc loading and transport from source to sink tissues (23, 41).

The cellular PP, content, unlike that of ATP, remains relatively stable during changes in respiratory state. It is well documented that under anoxic conditions flood-resistant plants (i.e., rice) induce a PP, dependent glycolytic pathway (35). The induction of PP, dependent phosphofructokinase and pyruvate phosphate dikinase is analogous to the pathway shown by eukaryotic protists (42). A recent model shows that in flood-tolerant plants PP, can be generated by the above-mentioned enzymes if the direction of the pathway is sucrose biosynthesis; however, if the pathway goes in the catabolic direction from sucrose to pyruvate the model fails to provide a source for PP, (35). It is tempting to speculate that a tonoplast-localized H⁺-PPase could be the source of PP, under these scenarios: the synthase function may become active whenever PP, falls below threshold, maintaining a minimum level of cytosolic PP, at the expense of the transmembrane H⁺ gradient. In keeping with this hypothesis, H⁺-PPases in rice have been shown to be induced under anoxia (43).

The assignment of precise roles to AVP1 synthase activity will be difficult to dissect due to the two, albeit intriguing, characteristics of PP, utilizing reactions (44). First, each PP, utilizing protein operates in parallel with an ATP-utilizing protein: in this case, the tonoplast energization can be driven by the
**PP\textsubscript{i} synthase function of Arabidopsis type I H\textsuperscript{+}-PP\textsubscript{ase}

V-ATPase. Second, *in vivo* the PP\textsubscript{i}-consuming reactions are close to equilibrium. Likewise, the operational direction of AVP\textsubscript{1} depends on the respective concentrations of its ligands in a given physiological situation, which may be difficult to ascertain. In spite of the experimental challenges, it will be interesting to discern how AVP\textsubscript{1} expression is regulated, both in stress conditions and nonstressed conditions, at specific membranes and tissues where PP\textsubscript{i}, synthase is thermodynamically and energetically favored (12).

The importance of H\textsuperscript{+}-PP\textsubscript{ase}–mediated synthase activity may extend beyond plants (45). Many protozoa such as *Trypanosoma brucei* and *Plasmodium falciparum*, the causative agents of sleeping sickness and malaria, also contain H\textsuperscript{+}-PP\textsubscript{ase} (33). Is the synthase activity important for pathogenesis of these organisms, and can specific inhibitors of this activity be used in the development of novel therapeutic compounds?

The potential for H\textsuperscript{+}-PP\textsubscript{ase} to function as a PP\textsubscript{i} synthase has been largely overlooked by plant scientists. Pyrophosphatase and pyrophosphate synthase activities of H\textsuperscript{+}-PP\textsubscript{ase} were originally described in chromatophores from the photosynthetic bacterium *Rhodospirillum rubrum* (46, 47). The patch-clamp and yeast-based assays used here now offer the potential to further unravel structure-function relationships among synthase activity, proton pumping, and PP\textsubscript{ase} activity. Additionally, allelic variants in plant H\textsuperscript{+}-PP\textsubscript{ases} can be tested for correlations between plant growth and synthase activity.

**Experimental procedures**

**Plant material, growth conditions, and protoplast isolation**

Plants of *A. thaliana* Col-0 WT, the AVP\textsubscript{1} mutant lines *vhp\textsubscript{1}-1* and *fugu\textsubscript{5}-I (16, 48), the *UBQ\textsubscript{10}::AVP\textsubscript{1} overexpressor lines 13-4 and 18-4 (3), and the *vpt\textsubscript{1} knockout line (29)* were grown on soil at 22 °C under an 8-h light/16-h dark regime. Mesophyll tissue of 7–12-week-old plants was enzymatically grown on soil at 22 °C under an 8-h light/16-h dark regime.

**Patch-clamp recordings and data analysis**

For patch-clamp experiments, mesophyll protoplasts were allowed to settle on the bottom of the recording chamber, and vacuoles were freshly released by bath perfusion with VR solution (49) containing 100 mM malic acid, 155 mM BTP, 5 mM EGTA, and 3 mM MgCl\textsubscript{2}, pH 7.5, adjusted to 460 mosm with D-sorbitol.

Recordings were performed in the whole-vacuole configuration using an EPC-7 patch clamp amplifier (HEKA Elektronik, Germany). High-resistance membrane seals were generally formed in VR solution. Starting at 2–3 min after the establishment of the whole-vacuole configuration, vacuoles were kept under continuous bath perfusion for the entire duration of the experiment by means of a gravity-driven perfusion system coupled to a peristaltic pump. Membrane currents were allowed to stabilize in standard bath solution for at least 15 min. Patch pipettes pulled from thin-walled borosilicate glass (Clark Electrochemical Instruments, UK) had resistances of 3–3.5 megaohms when filled with standard pipette solution (100 mM KCl, 3 mM MgCl\textsubscript{2}, osmolality adjusted to 460 mosm with D-sorbitol). Pipette solutions were buffered at pH 7.2 with 20 mM HEPES, at pH 5.5 with 20 mM MES, and at pH 2.9/3.2/3.7/4.5 with 5 mM citrate; pH was adjusted with KOH. BTP-based pipette solution was prepared using 100 mM HCl, 5 mM citric acid, 46 mM BTP, pH 3.2, and D-sorbitol to an osmolality of 460 mosm. The standard bath solution contained 100 mM potassium gluconate, 2 mM EGTA, 3 mM MgSO\textsubscript{4}, and 20 mM HEPES, adjusted to pH 7.2 (with KOH) and 480 mosm (with D-sorbitol). P\textsubscript{i}-containing bath solutions had the same composition except for the addition of 5 or 20 mM KH\textsubscript{2}PO\textsubscript{4} and a corresponding reduction of [potassium gluconate] after pH adjustment to match the total [K\textsuperscript{+}] of the standard bath solution. For P\textsubscript{i} dose-response experiments, the desired P\textsubscript{i} concentration was reached by mixing appropriate volumes of standard bath solution and 20 mM P\textsubscript{i} bath solution. PP\textsubscript{i} (potassium salt) was added by dilution of a 100 mM aqueous stock solution. Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich.

Membrane currents were elicited by 200-ms voltage ramps ranging from −80 to +80 mV or by 240-ms voltage ramps ranging from +80 to −160 mV at a holding potential of 0 mV. Tonoplast voltage is given as $V_{\text{cytosol}} - V_{\text{vacuole}}$. Negative (or inward) currents represent cation flow toward the cytosol (or anion flow toward the vacuolar lumen). The measured liquid junction potential between KCl-based pipette solutions and VR bath solution (<2 mV) was left uncorrected. In experiments using BTP-based pipette solution, applied membrane potentials were corrected offline for the measured liquid junction potential of −7 mV. The experimental conditions were chosen to obtain an intrinsically low current background in *Arabidopsis* vacuoles (50). Therefore, only recordings with low background currents and low interpulse variability were considered for data analysis. P\textsubscript{i}-induced current amplitudes at −80 or −160 mV were determined within a 40-ms time interval at the respective test potential, immediately adjacent to the voltage ramp. PP\textsubscript{i}-dependent H\textsuperscript{+} pump activity was quantified using the mean current amplitude determined within a 50-ms interval at 0 mV. For group averages, current amplitudes were normalized to the vacuolar membrane capacitance. Current amplitudes recorded at different cytosolic P\textsubscript{i} concentrations were normalized to the value determined at 5 mM P\textsubscript{i} and fitted to the Hill equation: $I = I_{\text{max}}/(1 + (K_{0.5}/[P_i])^n)$.

**Construction of yeast expression vectors**

The entire reading frame of the chimeric fusion TcGFP-AVP\textsubscript{1} (32), where the sequence coding for yeast enhanced GFP is placed between the coding sequence of N-terminal signal peptide of the H\textsuperscript{+}-PP\textsubscript{ase} from the protist *T. cruzi* and the K\textsuperscript{+}-dependent H\textsuperscript{+}-PP\textsubscript{ase} from the higher plant *A. thaliana* AVP\textsubscript{1}, was amplified by PCR using High Phusion polymerase (New England Biolabs) according to the manufacturer’s recommendations. The PCR primers were engineered to contain ClaI 5‘-GAATTCATCGATATGGGTTACATGAAGAGGT-3‘ and XhoI 5‘-GAATTCCTCGAGTTAGAAGTACTTGAAAAGG-
PPase activity

V-PPase activities were measured colorimetrically following the production of free P$_1$ (58). PPase activity was assayed in a reaction medium containing 300 μM Tris-PP$_1$, 1.3 mM MgSO$_4$, 50 mM KCl, 5 μM gramicidin, and 30 mM Tris-MES (pH 7.6). Where indicated, DF was added as a concentration of 250 μM.

Proton pumping assay

The formation of a pH gradient across vesicle membranes obtained from the Ficoll gradient was measured on a SpectraMax M2 fluorometer using the ACMA quenching assay (59). Portions (20–40 μg) of vacuolar membranes were added to 1 ml of transport buffer (100 mM KCl, 50 mM NaCl, and 20 mM HEPES, pH 7) containing 1 μM ACMA. Pumping was initiated by adding a solution containing 1.5 mM ATP and 3.0 mM MgSO$_4$. Fluorescence intensity was monitored continuously at an excitation wavelength of 410 nm and an emission wavelength of 490 nm. Where indicated, concanamycin A was added to a final concentration of 0.1 μM. For the PP$_i$ pumping assays, ATP was replaced by 1 mM PP$_i$. MgSO$_4$ was present to a final concentration of 3 mM. Inhibition of membrane-bound PP$_i$ase activity was achieved by adding gramicidin to a final concentration of 10 μM in the transport buffer. Reactions were carried out at 25 °C.

PP$_i$, synthesis and determination

Determination of PP$_i$ was achieved by coupling its synthesis with the production of fructose-1,6-P$_2$ by phosphofructokinase and ensuing triose phosphates and following the continuous change in NADH absorbance at 340 nm by utilizing a PP$_i$ enzymatic determination kit following the manufacturer’s instructions (Sigma-Aldrich). To study the synthesis of PP$_i$, 10–20 μg of tonoplast vesicles were incubated for 1 min in reaction buffer containing 100 mM KCl, 50 mM NaCl, 20 mM HEPES buffered to pH 7, 3 mM MgSO$_4$, and 2 mM PP$_i$. When indicated, 1.5 mM ATP was added to the reaction buffer (ATP + P$_i$). Then, continuous PP$_i$ measurements were performed in the PP$_i$ reagent for 20 min. Spectrophotometric output was converted to nmol.

Western blotting and total protein estimation

Immunoblot analyses were performed after electrophoresis (55). All lanes contained 10 μg of tonoplast protein. Proteins were transferred to a cellulose nitrate membrane and immunostained with an mAb against GFP (Covance). The alkaline phosphatase antibody was a generous gift from Dr. Kane (State University of New York Upstate Medical University) and was used as a vascular marker. The enhanced chemiluminescence method (GE Healthcare) was used to detect binding.

The total protein concentration was estimated by a modification of the dye binding method (56) in which membrane protein was partially solubilized for 10 min at 70 °C with 0.025% SDS before the addition of the dye reagent concentrate (Bio-Rad). BSA was used as the protein standard.

Purification of vacuolar vesicles and activity assays

Vacuolar vesicles were isolated as described previously (57). 3 liters of cells were grown overnight to log phase (A$_{600}$ of 0.4–0.6) in yeast extract–peptone–dextrose medium. After induction for 60 min with 100 μM CuSO$_4$, cells were harvested (A$_{600}$ of 0.8–1). Cells were washed and subsequently resuspended in 1.2 M sorbitol containing 1 mM DTT and treated with T100 Zymolyase for removal of the cell wall. Spheroplasts were washed and resuspended in growth medium containing 1.2 M sorbitol. The spheroplasts were glucose-recovered for 20 min at 30 °C. Subsequent cell lysis and isolation of vacuoles by flotation on Ficoll gradients were performed identically for all samples. To improve vacuolar yield, this procedure was shortened by subjecting the spheroplast homogenate to only one cycle of flotation centrifugation (12% Ficoll containing 0.1 mM phenylmethylene sulfonyl fluoride, 1 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 μg/ml pepstatin) (22,000 rpm for 50 min at 4 °C). After centrifugation, the white layer (which was enriched for vacuoles) at the top of the tube was vesiculated for the transport assays or prepared directly for electrophoresis.
tions were carried out in a SpectraMax M2 spectrophotometer at 30 °C.

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