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SEPARATION OF TONOPLAST VESICLES ENRICHED IN ATPASE AND PYROPHOSPHATASE ACTIVITY FROM MAIZE ROOTS

ABSTRACT: Continuous sucrose gradient (15-35%) centrifugation of maize (*Zea mays* L.) root microsomal membranes yielded two well-separated fractions of tonoplast vesicles located between 19-21% (Peak I) and 25-26% sucrose (Peak II). Marker enzyme analyses indicated that both fractions were essentially free from plasma membrane, mitochondria and Golgi contaminations. The adenosine triphosphate (ATP) supported proton transport activity was found in both Peak I and Peak II with a 70 to 30% distribution. The pyrophosphatase (PP_i) supported proton transport activity was found only in Peak II. Both hydrolytic activities assumed a bell shape pH dependency with pH optimum at 6.5-7.5 and at 6.5-8.5 for ATPase and PP_iase, respectively. The K_m of the ATPase and PP_iase, at their respective optimal pH, was found to be 1.2 mM and 0.02 mM, respectively. Both ATPase and PP_iase activities were strongly inhibited by N,N'-dicyclohexylcarbodiimide (DCCD) and diethylstilbestrol (DES) but not by molybdate. Peak I contained nitrate-sensitive and vanadate-insensitive ATP hydrolysis activity. In addition to catalyzing the nitrate and vanadate-insensitive hydrolysis of PP_i, Peak II also contained some minor ATP hydrolysis activity that was sensitive to vanadate and nitrate. The results indicate that H⁺-ATPases and H⁺-PP_iase occur different populations of tonoplast vesicles from corn roots.

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**Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

INTRODUCTION

It is well established that plant cell membranes such as plasma, tonoplast and Golgi contain electrogenic ion translocating ATPases which provide the necessary energy to support transport processes across the membranes (1,2). According to the chemiosmotic theory, these membranes utilize the hydrolysis of ATP to provide the necessary energy for transporting various ions, sugars and other molecules. For tonoplast systems, Walker and Leigh (3) first demonstrated that beet vacuoles, in addition to H⁺-ATPase, also contain a PP_iase which may function as a proton pump (4). Evidence for the PP_iase as a pump that is separated and independent from that of the H⁺-ATPase has been presented previously (5,6). Using gel filtration method, solubilized tonoplast ATPase and PP_iase could be physically separated (7,8). Recently, H⁺-translocating PP_iase has been purified from the vacuole membranes of red beet (9) and mung bean (10). Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump was described in *Arabidopsis thaliana* involved in the generation of transmembranous proton electrochemical potentials were also found in other higher plants (12) including maize roots (13). Chanson and Pail-Emile (14) showed that both ATPase and PP_iase activities of maize root microsomes were recovered in a crude tonoplast fraction obtained at the 10-35% interface of sucrose density gradients. On the basis of their study, both ATP and PP_i-dependent translocating activities could be assigned to the same enzyme complex using different substrates.

In the present study, we developed a procedure which, for the first time, separated two fractions enriched in PP_iase and ATPase activities associated with the tonoplast membranes from maize roots by continuous sucrose density gradient centrifugation. Based on physical chemical characterization, we concluded that maize tonoplast can be isolated as two different fractions, one enriched with ATPase and one with PP_iase, respectively.

MATERIAL AND METHODS

Isolation of Tonoplast Vesicles: Corn seeds (*Zea mays* L. cv. FRB 73, Illinois Foundation Seeds) were germinated and harvested as previously described (15). About 50 g of freshly excised corn roots were ground with mortar and pestle at 4°C in 3 volumes of homogenizing buffer (0.3 M sucrose, 5 mM EDTA, 5 mM

2-mercaptoethanol, 5 mM DTT, and 0.1 M Hepes, pH 7.8). The root homogenate was filtered through four layers of cheesecloth and centrifuged at 6,000 x g for 20 min. After centrifuging the supernatant at 90,000 g for 35 min, the pellet was suspended in 4 mL of homogenizing buffer and overlaid on a linear sucrose gradient (15-35%, w/w) containing 5 mM Hepes-MES, pH 7.7, 3 mM DTT, and 1 mM PMSF. The gradient was centrifuged at 4°C for 18 h at 85,000 g in a Beckman SW 28 rotor. Gradient was fractionated into 1 ml aliquots using a fraction collector equipped with a UV 280 monitor. Sucrose density was determined by reflection index. Two protein-rich peaks of tonoplast vesicles were isolated (Peak I, density at 19-21% and Peak II, density at 24-26%). and were used in current study.

Measurement of ATP Hydrolysis and PP_i Hydrolysis: ATP and PP_i hydrolysis was determined as previously described (16) by molybdate- Malachite green assay (15) of the release of inorganic phosphate. The medium consisted of 10-20 µg membrane protein, 50 mM KCl, 5 mM MgSO₄ and different substrates. The reactions were initiated by the addition of substrate after incubating for 20 min at 22°C and 37°C for ATPase and PP_iase measurement respectively.

Assay of Proton Transport Activity: The initial rate of proton pumping was measured by the change in acridine orange (AO) absorbance at 493 nm as described previously (17). Typically, 200 µl of tonoplast vesicles (20-40 µg protein) were diluted with a solution containing 17.5 mM MES-Tris (pH 6.45), 2.5 mM MgSO₄, 1 mM EGTA, and 7.5 µM AO. After equilibration at room temperature for 10 min, the reaction was started by the addition of 20 µl of 0.2 M ATP or 0.02 M of PP_i (pH adjusted to 6.45 with Tris).

Protein content of the tonoplast membrane vesicles was determined by a modified Lowry assay using BSA as a standard (18). All chemicals obtained from commercial sources were of analytical grades.

Marker Enzyme Determinations: NADH-cytochrome c reductase and cytochrome c oxidase activities were assayed as described previously [19,20] but using Triton X-100 instead of digitonin in the assay. Triton-stimulated UDPase was determined by the procedure of Nagahashi and Kane (20). L-mannopyranosidase was assayed according to the procedure of Boller and Kende (21) using PNP-β-D-glucopyranoside as the substrate.

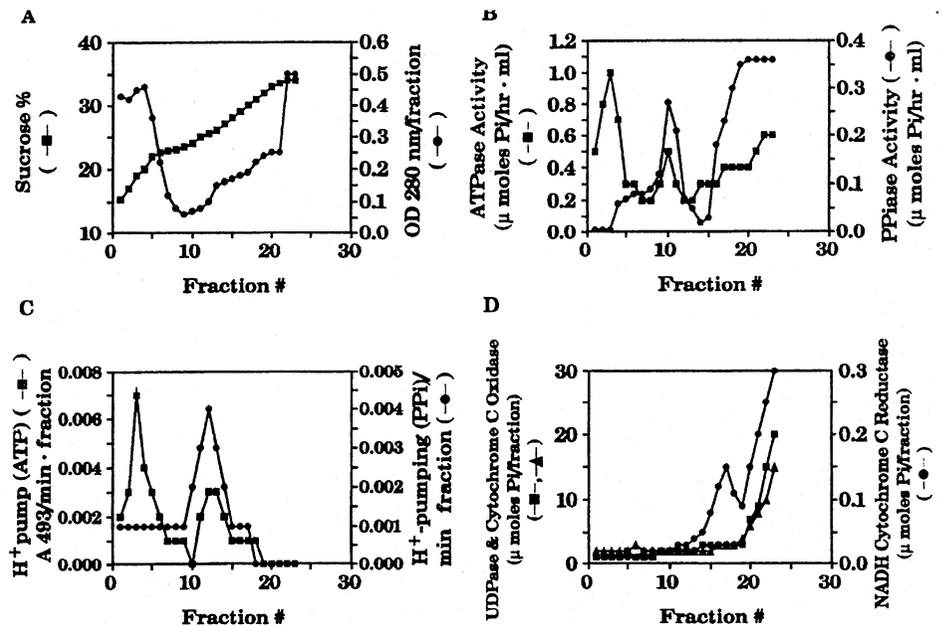


FIGURE 1. Continuous Sucrose Density Gradient Centrifugation of Microsomal Fraction. Crude Microsomal Suspension was Loaded on the Continuous Sucrose Density Gradient 15-35%, (w/w) as Described. After Overnight Centrifugation, Fractions with Volume as 1.0 ml Each were Collected and Assayed for the Following: (A) Absorbance at 280 nm (●—●) and Density in % of Sucrose (w/w) (■—■), (B) ATP Hydrolysis (■—■) in the Presence of 100 μ M Vanadate and PP_i Hydrolysis (●—●), (C) ATP-Dependent Proton Transport (■—■) and PP_i Dependent Proton Transport (●—●), (D) Cytochrome C Oxidase (▲—▲), NADH Cytochrome C Reductase (Antimycin A Insensitive, (●—●)), and Triton X-100 Stimulated UDPase (■—■).

RESULTS

Distribution of Subcellular Organelles in Continuous Sucrose Gradient: Microsomal fraction of maize roots were obtained by the procedure described by Tu et al. (15). An initial centrifugation at 6,000 \times g for 20 min removed most of the mitochondria and other cell debris. A second centrifugation of 90,000 \times g for 35 min was used to pellet microsomal membranes. These membranes were then loaded on a shallow sucrose density gradient (15-35%, w/w) (Fig. 1A). Most of the proteins were distributed at the

top and bottom of the gradient. After isopycnic centrifugation, two distinctive ATPases were localized at 19-21% and 25-26% sucrose, (Fig. 1B). In the presence of vanadate, about 80% of the total hydrolytic activity of ATPase was found at lower density Peak I (19-21% sucrose density) with the remaining 20% of the activity located at higher density Peak II (25-26% sucrose density). When PP_i (0.02 mM) was used as a substrate, the Mg^{++} -stimulated PP_i ase activity was detected only at Peak II (Fig. 10). Both Peak I and II contained ATP-dependent proton transport activity with more than 80% of the activity associated with the former. Pyrophosphate dependent proton transport activity was low and only appeared at Peak II (Fig. 1C). This PP_i -dependent proton transport activity was more sensitive to freeze-thaw treatment than the proton transport activity associated with ATP hydrolysis (data not shown). Assay of the marker enzyme for endoplasmic reticulum, antimycin-A insensitive NADH-cytochrome c reductase, indicated its presence at densities higher than 28% (Fig. 1D). Mitochondrial marker enzyme, cytochrome c oxidase, was completely pelleted with no detectable activity in the gradient. Triton X-100 stimulated UDPase (Golgi marker) was also only detected at the bottom of the gradient.

pH Dependence of the ATPase and the PP_i ase Activities: Based on the measurements of the release rate of inorganic phosphate, the pH dependence of the ATPase of Peak I and the PP_i ase of Peak II was determined (Fig. 2). The hydrolysis of ATP exhibited a bell-shaped pH dependence with a broader maximum between 6.5-7.5. The hydrolysis PP_i of Peak II also showed a bell-shaped pH profile with the maximum activity observed between pH 6.5 to 8.0.

Kinetic Parameters of the ATPase and PP_i ase: The ATPase activity of Peak I appeared to obey simple Michaelis-Menten kinetics with apparent K_m and V_{max} of 1.2 mM and 5 μ moles/mg min (Fig. 3A), respectively. The addition of PP_i up to 1.5 mM, did not change the V_{max} and K_m of the ATPase (data not shown).

The PP_i ase of Peak II also showed typical Michaelis-Menten kinetics with apparent K_m and V_{max} as 0.02 mM and 2.5 μ moles/mg min (Fig. 3B), respectively. The presence of ATP showed little, if any, effects on the values of the K_m and V_{max} of the PP_i ase. In the presence of 0.2 mM ATP, the K_m and V_{max} of the PP_i ase are only slightly increased by 10% (data not shown).

Marker Enzyme Distribution in the Tonoplast Fraction: After equilibrium density centrifugation, marker enzyme activities of various subcellular membranes in collected

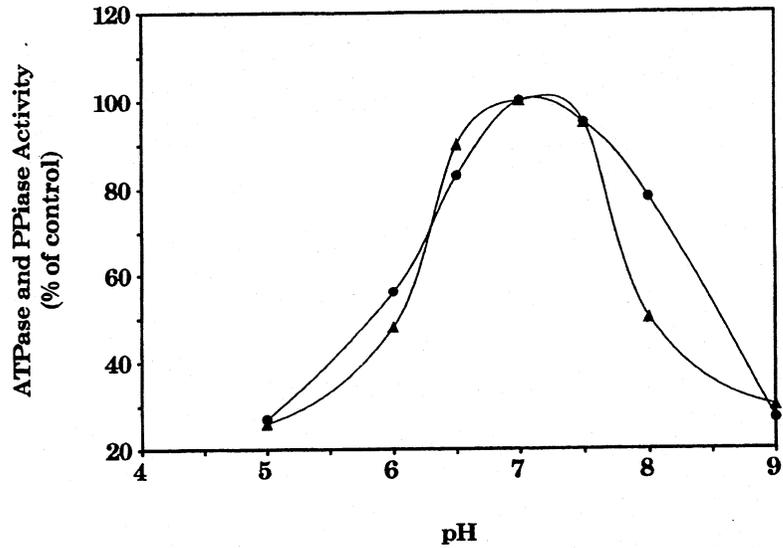


FIGURE 2. pH Profiles of the ATPase and PP_iase Activities. Peak I Protein was Assayed for ATP Hydrolysis (▲—▲), and Peak II Protein was Assayed for PP_i Hydrolysis (●—●) at Various pH (5.0, 6.0, 6.5, 7.0, 8.0, and 9.0) as Described. The Relative Rates of the Activities were Determined by Assigning the Highest Activities Determined in the pH Range as 100%.

fractions were tested. Table I summarizes the percentage of recovery of various subcellular membrane activities in Peak I and Peak II of tonoplast fraction. No activity of cytochrome c oxidase, marker enzyme of mitochondria, was detected in Peak I and II. Similarly, no activity of DPNase, marker enzyme of Golgi, was found in tonoplast fraction. The activity of antimycin-A insensitive NADH-cytochrome c reductase, marker enzyme of endoplasmic reticulum, was not found in fractions with sucrose density less than than 28%. However, activity of L-mannosidase, a vacuolar-associated hydrolytic enzyme, was completely recovered in tonoplast fraction and showed a 45-55% distribution of the activity between Peak I and Peak II. The vanadate insensitive ATP hydrolysis was found in both Peak I and Peak II with 80% and 15% distribution, respectively. ATP hydrolysis in Peak I was insensitive to vanadate but sensitive to

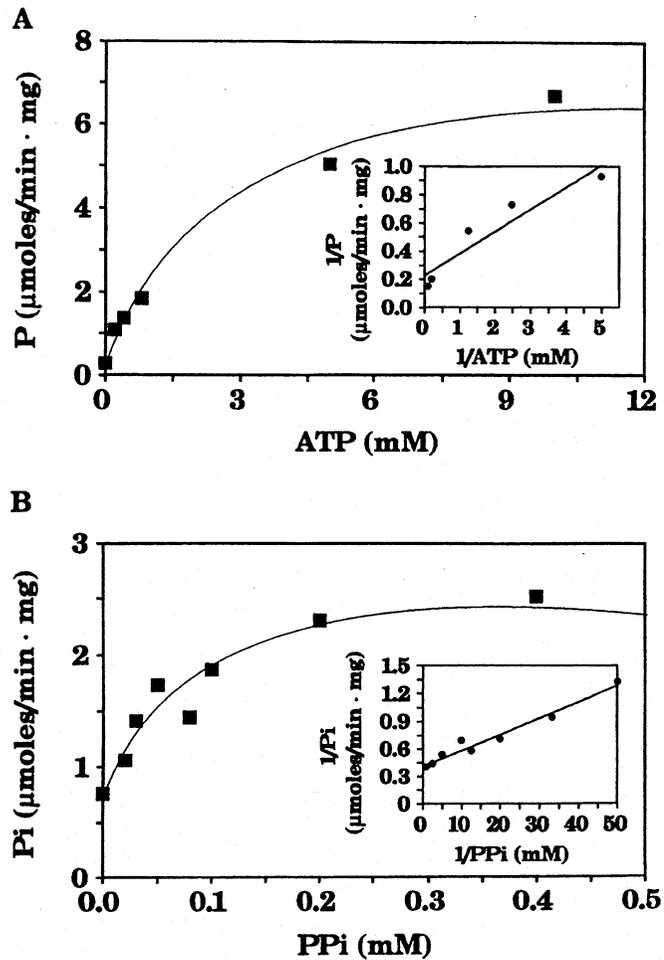


FIGURE 3. (A) Concentration Dependence of ATP on ATP Hydrolysis. The ATP Hydrolysis Rate in Peak I was Determined from the Release of Inorganic Phosphate as Described Under "Materials and Methods". Insert is the Double Reciprocal Plot of ATP Concentration and Rate of P_i Released, (B) Concentration Dependence of PP_i on PP_i ase Hydrolysis. Peak II protein was used to determine for the release of inorganic phosphate as described under "Materials and Methods". Insert is the double reciprocal plot of PP_i concentration and the rate of P_i released.

TABLE 1

The Recovery of Various Microsomal Marker Enzyme Activity in Peak I and Peak II

Marker enzymes	Peak I	Peak II
DPNase	0%	0%
Cytochrome C oxidase	0%	0%
L-mannosidase	45%	55%
NADH cytochrome C reductase	3	2%
ATPase, VO_4^- -insensitive	85%	15%
ATPase, NO_3^- -sensitive	85%	15%

The total activity, before sucrose density centrifugation, of various in microsomal fraction was assigned as 100%.

The VO_4^- sensitive or nitrate sensitive ATPase activity was assayed as described under "Material and Methods" the presence of 100 μM VO_4^- or 50 mM KNO_3 .

nitrate. ATP hydrolysis in Peak II was partially sensitive to vanadate. Without the addition of vanadate, ATPase in peak II was 20% of the total ATP hydrolysis activity. The addition of 50 mM nitrate essentially abolished ATP hydrolysis activity in Peak I, while Peak II still retained 15% total ATP hydrolysis activity. It is interesting to note that ATPase in Peak II was partially sensitive to both vanadate and nitrate.

Inhibition of the ATPase and the PP_i ase: The effect of several known membrane inhibitors was tested on the activity of the ATPase of Peak I and the PP_i ase of Peak II. As shown in Table II, 100 μM of vanadate, a potent inhibitor of plasma membrane ATPase (2), had no effect on the ATPase of Peak I and PP_i ase in Peak II. However, this level of vanadate decreased the ATPase of Peak II by about 30%. Nitrate, a specific inhibitor for H^+ -ATPase of tonoplast, completely abolished the ATPase activity of Peak I, but showed no inhibition to the PP_i ase of Peak II, and decreased the ATPase activity in Peak II only by 30%. The presence of 50 μM of DES essentially abolished both

TABLE 2
Inhibition and Stimulation of Tonoplast ATPase and PP_iase

	Total activity (% of control)		
	Peak I	Peak II	
	ATPase	PP _i ase	ATPase
Control	100	100	100
100 μ M Na ₃ VO ₄	95	95	70
50 μ M KNO ₃	5	100	70
50 μ M DES	5	5	5
50 μ M DCCD	8	10	5
1 mM molybdate	98	95	95
-KCl + NaCl*	98	20	98

Membrane vesicles of Peak I and Peak II were obtained from continuous sucrose density gradient between 19-20% and 25-26 %, respectively.

Control activity was assigned as the incubation as described under "Materials and Methods" using 50 mM KCl in the buffer.

*50 mM KCl in Control was replaced by 50 mM NaCl.

ATPase and PP_iase activities in Peak I and Peak II. The presence of 100 μ M of DCCD, a proton channel blocker, strongly inhibited both the ATPase and PP_iase. However, with the concentration of DCCD lower than 100 μ M, the ATPase of Peak I exhibited a greater sensitivity to DCCD than that of the PP_iase (data not shown). The presence of 1 mM molybdate, a non-specific phosphatase inhibitor, had no significant effect on the activity of either Peak I or Peak II. The PP_iase activity exhibited a strong K⁺ dependence as illustrated by the loss of 80% activity resulting from the replacement of KCl with NaCl. The same replacement had no effect on the ATPase activity of Peak I and Peak II.

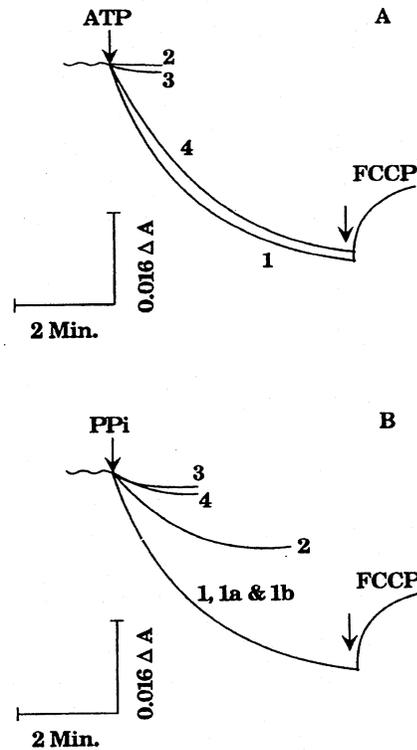


FIGURE 4. (A) ATP Dependent Proton Transport of Peak I Protein was Used to Determine the Proton Transport Induced by the Addition of 0.2 mM ATP: (1) Control with 50 mM KCl in the Assay Medium. (Line 1), (2) 0.02 mM of PP_i was Used to Replace ATP (Line 2), (3) Additional 50 mM of KNO₃ Added to the Medium (line 3), (4) 100 μM of Vanadate Included in the Medium (line 4), (B) PP_i Dependent Proton Transport. Peak II Protein was Used to Determined the Proton Transport by (1) Addition of 0.02 mM of PP_i to the Medium (Line 1) Containing 50 mM KNO₃ (Line 1a) and 100 μM Vanadate (Line 1b). (2) When PP_i was Replaced by 0.2 mM ATP (Line 2). (3) Proton Pumping (Initiated by 0.2 M ATP) Measured in the Presence of 50 mM KNO₃ (Line 3) or (4) 100 μM Vanadate (Line 4).

ATP and PP_i Dependent Proton Transport Activities: Proton transport supported by either ATP or PP_i hydrolysis of Peak I and Peak II was determined. The proton transport associated with the ATPase of Peak I (Fig. 4A, line 1), observed under different conditions, was shown in Fig. 4A. It became evident that: (1) the addition of 100 μM of vanadate did not inhibit the transport activity (Fig. 4A, line 4), (2) the addition of 0.2 mM of PP_i didn't initiate the proton pumping (Fig. 4A, line 2), and (3) the addition of nitrate completely inhibited proton transport activity (Fig. 4A, line 3). However, the vesicles of Peak II displayed both ATP and PP_i dependent proton transport. With 0.02 mM PP_i to initiate the reaction, proton transport (Fig. 4B, lines 1) was not sensitive to nitrate or vanadate (Fig. 4B, line 1a and 1b). When 0.2 mM of ATP was used to replace PP_i (Fig. 4B, line 2), the resulting proton transport was partially inhibited by nitrate and vanadate (Fig. 4B, lines 3 and 4).

DISCUSSION

The present results indicate that tonoplast fraction of corn roots contains vesicles enriched either in H⁺-ATPase or in PP_iase activity. These two types of vesicles can be separated by sucrose density gradient centrifugation reported in this study. To our knowledge, this is the first example showing that PP_iase and ATPase are separated on tonoplast vesicles. The PP_iase exhibits many properties in common with those found in yeast (22), *Rhodospirillum* (23) *Escherichia coli* (24), and a wide range of plant tissues including crude maize microsomal membranes (13,14).

As previously mentioned (12), the tonoplast H⁺-ATPase and PP_iase activities are functionally distinguishable. The criteria for this claim are: (a) different distributions in sucrose density gradient as shown by equilibrium centrifugation, (b) substrate specificity, (c) sensitivity to different inhibitors, and (d) different requirement for K⁺ to activate the enzyme activity.

As reported here, the PP_iase containing Peak II also showed some vanadate-sensitive ATPase activity. However, both the ATP hydrolysis and its supported proton transport are not as sensitive to vanadate as those associated with the plasma membrane H⁺-ATPase. In a previous study [16], we demonstrated that the lipolytic activity in crude microsomal fraction may abolish the proton transport of plasma membrane H⁺-ATPase during the 16 h centrifugation. Thus, it is unlikely that the detected H⁺-ATPase activities of Peak II are due to the cross contamination of the

plasma membrane. The exact origin of this ATPase in Peak II remains to be determined.

The physiological role of PP_i -dependent proton translocation across vacuolar membranes remains to be established. The study of Rea and Poole (7) on red beet storage tissue suggested two models for the function of PP_i ase in vacuoles of plant cells. The first model proposes that tonoplast PP_i ase spans across tonoplast and functions as an inward proton pump. The second model assumes that tonoplast PP_i ase is located on the inside of the tonoplast and catalyzes intravesicular acidification by the conversion of PP_i to P_i . The present study along with previous findings of Walker and Leigh (3), Churchill and Sze (25) and Bennett et al. (26) suggest that PP_i -dependent proton translocation may be a feature common to the tonoplast of higher plants but it is not known if a similar arrangement applies to other types of vacuolar cells.

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