Proton Transport in Maize Tonoplasts Supported by Fructose-1,6-Bisphosphate Cleavage. Pyrophosphate-Dependent Phosphofructokinase as a Pyrophosphate-Regenerating System

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The energy derived from pyrophosphate (PPi) hydrolysis is used to pump protons across the tonoplast membrane, thus forming a proton gradient. In a plant’s cytosol, the concentration of PPi varies between 10 and 800 μM, and the PPi concentration needed for one-half maximal activity of the maize (Zea mays) root tonoplast H+-pyrophosphatase is 30 μM. In this report, we show that the H+-pyrophosphatase of maize root vacuoles is able to hydrolyze PPi (Reaction 2) formed by Reaction 1, which is catalyzed by PPi-dependent phosphofructokinase (PFP):

\[
\begin{align*}
\text{Fructose-1,6-bisphosphate (F1,6BP)} & + \ P_i \leftrightarrow \ P Pi_i + \text{Fructose-6-phosphate (F6 P)} \quad \text{(reaction 1)} \\
\text{PPi} & \rightarrow 2 \ P_i \\
H^+_{\text{cyt}} & \rightarrow H^+_{\text{vac}} \quad \text{(reaction 3)} \\
\text{F1,6BP + H}^+_{\text{cyt}} & \leftrightarrow H^+_{\text{vac}} + \text{F6P} + P_i 
\end{align*}
\]

During the steady state, one-half of the inorganic phosphate released (Reaction 4) is ultimately derived from F1,6BP, whereas PFP continuously regenerates the pyrophosphate (PPi) hydrolyzed. A proton gradient (ΔpH) can be built up in tonoplast vesicles using PFP as a PPi-regenerating system. The ΔpH formed by the H+-pyrophosphatase can be dissipated by addition of 20 mM F6P, which drives Reaction 1 to the left and decreases the PPi available for the H+-pyrophosphatase. The maximal ΔpH attained by the pyrophosphatase coupled to the PFP reaction can be maintained by PFP activities far below those found in higher plants tissues.

Plants, protozoans, and certain bacteria have a membrane-bound H+-translocating inorganic pyrophosphatase (H+-PPase) that works as a proton pump. This enzyme couples the hydrolysis of PPi with electrogenic translocation of protons across the membrane (Baykov et al., 1999). In plants, this enzyme is located in the tonoplast membrane, where it supports vacuolar functions, such as preventing the harmful accumulation of Ca2+ and Na+ in the cytosol.
and allowing storage of organic metabolites that can be recovered by the cytosol when needed by the \( H^+ \)-energized tonoplast membranes. The cytosolic \( P_i \) concentration in plant cells is approximately 200 \( \mu M \) (Weiner et al., 1987; Maeshima, 2000). This concentration is sufficient to promote the maximal activity of vacuolar \( H^+ \)-PPase in plant cells (Maeshima, 2000). The \( P_i \) concentration is determined by the balance between the rates of \( P_i \) hydrolysis and synthesis. The hydrolysis of \( P_i \) is catalyzed by the \( H^+ \)-PPase (Davies et al., 1993). On the other hand, \( P_i \) is synthesized as a by-product of various biosynthetic reactions such as the synthesis of nucleic acids, polysaccharides, coenzymes, and proteins; activation of fatty acids; and isoprenoid synthesis. Under stress conditions, such as anoxia, the biosynthetic reactions are slowed down, thereby limiting the rate of \( P_i \) formation. Thus, the use of the reaction catalyzed by PFP in the direction of \( P_i \) formation may be an auxiliary mechanism to prevent a drop in the cytosolic levels of \( P_i \) under the metabolic conditions of the living cell (Davies et al., 1993). As far as we know, this proposal has never been demonstrated experimentally.

The aim of this study was to evaluate whether the maize (\textit{Zea mays}) root \( H^+ \)-PPase is able to bind and hydrolyze the \( P_i \), formed by Reaction 1 catalyzed by PFP in vitro under conditions that mimic the concentrations of \( F_1,6BP, P_i, Mg^{2+}, F_2,6BP, \) and \( F_6P \) found in the plant cell cytosol (Rebeille et al., 1983; Ukaji and Ashihara, 1987; Weiner et al., 1987; Kubota and Ashihara, 1990; Davies et al., 1993; Stitt, 1998; Table I). Here, we show that the affinity of maize root \( H^+ \)-PPase for \( P_i \) under these conditions is sufficient to shift Reaction 1 toward \( P_i \) formation, making it possible to build up a proton gradient in the tonoplast even in the presence of very low amounts of PFP.

**RESULTS**

**Cleavage of \( F_1,6BP, pH \), and \( Mg^{2+} \) Dependence**

PFP and maize root \( H^+ \)-PPase catalyzed the release of \( P_i \) from \( F_1,6BP \) (Fig. 1). There was no measurable release of \( P_i \) when the two enzymes needed to regenerate \( P_i \) or the substrates \( F_1,6BP \) and \( P_i \) were omitted from the assay medium. The rate of cleavage varied with the concentration of \( F_1,6BP \) (Fig. 1B), and the half-maximal rate was observed with 123 \( \mu M \) \( F_1,6BP \). PFP and \( H^+ \)-PPase both require \( Mg^{2+} \) for their activities (Maeshima and Yoshida, 1989; Givan, 1999; Maeshima, 2000). Magnesium was needed for the coupled reaction in Figure 1; the maximal rate of \( F_1,6BP \) cleavage was obtained in the presence of 0.6 to 1 mm \( MgCl_2 \). Higher \( MgCl_2 \) concentrations were found to be inhibitory (Fig. 1A). The findings of Figure 1 indicate that the true substrate for the maize root \( H^+ \)-PPase was the very low \( P_i \) concentration derived from Reaction 1 and that the \( P_i \) released into the medium originated from \( F_1,6BP \) by the action of PFP, which continuously regenerated the \( P_i \) hydrolyzed (Reactions 1 and 2).

### Table I. Levels of metabolites in plant cytosol; PFP and \( H^+ \)-PPase activities from different plants and experimental conditions used in this study

<table>
<thead>
<tr>
<th>Plants and Conditions</th>
<th>Metabolites in Plant Cytosol</th>
<th>Enzyme Activity Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( F_1,6BP )</td>
<td>( P_i )</td>
</tr>
<tr>
<td>Wild-type plants</td>
<td>0.6</td>
<td>5</td>
</tr>
<tr>
<td>Antisense transformant plants (lower levels)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Experimental conditions (Figs. 1, 2, and 4)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* The concentrations of \( F_1,6BP, P_i, \) and \( F_6P \) are those reported in the literature for plant cell cytosol (Rebeille et al., 1983; Ukaji and Ashihara, 1987; Weiner et al., 1987; Kubota and Ashihara, 1990; Davies et al., 1993; Stitt, 1998).  
  ** The PFP activities for wild-type and antisense transformant tobacco (\textit{Nicotiana tabacum}) plants were taken from Nielsen and Stitt (2001) for antisense transformant potato (\textit{Solanum tuberosum}) plants. Values are from Hajirezaei et al. (1994).  
  * The \( H^+ \)-PPase activities for wild-type mung bean, tomato (\textit{Lycopepsicon esculentum}) fruit, and maize leaves were taken from Maeshima and Yoshida (1989), Milner et al. (1995), and Clayton et al. (1993), respectively.  
  ** The PFP and \( H^+ \)-PPase activities shown in this report were converted to milliunits per gram fresh wt by computing the enzyme activity level (milliunits) present in 1 mL of reaction medium. This volume was converted to grams to obtain the activities shown.
The concentration of PP₁ that is attained when the PP₁-regenerating reaction reaches a steady state varied depending on the pH of the medium. The rate of F₁,₆BP cleavage was maximal in the pH range of 6.0 to 7.0 and decreased by 50% when the pH was raised from 7.0 to 8.0 (data not shown).

Transmembrane Proton Gradient

The assay in Figure 2 shows that the energy derived from the steady-state cleavage of F₁,₆BP was sufficient to form and maintain a transmembrane proton gradient in tonoplast vesicles. In the presence of PFP, the addition of F₂,₆BP, Pᵢ, and F₁,₆BP promoted a quenching of ACMA fluorescence, indicating that a proton gradient was formed across the tonoplast membrane (Fig. 2A). The gradient was abolished when 1 μM FCCP, a proton ionophore, was added to the medium (Fig. 2, A and B).

The proton gradient formed depended on the concentration of Pᵢ present in the reaction medium (Fig. 2B). In the presence of 30 μM Pᵢ, a small proton

Figure 1. Cleavage of F₁,₆BP and Mg²⁺ dependence. The F₁,₆BP cleavage was dependent on MgCl₂ (A) and F₁,₆BP (B) concentrations. The MgCl₂ dependence was measured in a medium containing: 50 mM MOPS-Tris buffer (pH 7.0), 60 milliunits mL⁻¹ mung bean (Vigna radiata) PFP, 0.1 mg mL⁻¹ maize vacuolar microsomal protein, 100 mM KCl, 2 μM F₂,₆BP, and 10 mM F₁,₆BP (A). When the F₁,₆BP dependence was measured, the MgCl₂ concentration was fixed at 0.6 mM (B). The reaction was performed at 30°C and started by addition of 0.1 mM Pᵢ. Aliquots of 0.8 mL were removed, and the reaction was stopped by the addition of 0.2 mL of 50% (w/v) trichloroacetic acid. The figure shows a representative experiment. Similar results were obtained with three different vesicle preparations.

Figure 2. Membrane proton gradient supported by PFP, F₁,₆BP, and Pᵢ. The assay conditions were identical to those described in Figure 1B, except that the 9-amino-6-chloro-2-methoxyacridine (ACMA; 3 μM) was present and F₁,₆BP, F₂,₆BP, and Pᵢ concentrations are listed below for each panel. At the end of gradient formation, 1 μM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) was added to dissipate the H⁺ gradient in A and B. In C, the addition of PFP activator, F₂,₆BP, enhanced the proton gradient formation, and the addition of 20 mM F₆P dissipated it. The arrows indicate the sequential addition of: A, 25 milliunits of PFP, 2 μM F₂,₆BP, 2 mM Pᵢ, and 0.6 mM F₁,₆BP; B, 30 μM Pᵢ was included in the reaction medium before the sequential addition of: 25 milliunits of PFP; 4 μM F₂,₆BP; 0.6 mM F₁,₆BP, and 2 mM Pᵢ; C, 10 milliunits of PFP, 2 mM Pᵢ, 0.1 mM F₁,₆BP, 4 μM F₂,₆BP, and 20 mM F₆P. The figure shows a representative experiment. Similar results were obtained in three different vesicle preparations.
gradient was observed after the addition of 0.1 mM F1,6BP (Fig. 2B). Further addition of P_i (2 mM), promoted both an increase in the initial rate of proton pumping and an enhancement of the proton gradient. This result suggests that the addition of P_i to the medium displaces Reaction 1 toward PP_i formation, increasing its steady-state concentration.

The capacity of the system to form the proton gradient was highly dependent on the presence of the PFP activator F2,6BP (Fig. 2C). A slow rate of proton pumping was measured in presence of 2 mM P_i, 0.1 mM F1,6BP, and 10 milliunits of PFP. However, when 2 μM F2,6BP was added to the medium, the rate of proton pumping was accelerated by more than 2-fold. This result confirms that the rate of the PP_i formation by PFP is stimulated by F2,6BP and that the proton gradient is also dependent on the rate of P_i formation (Fig. 2C). Finally, the transmembrane proton gradient was abolished when 20 mM F6P was added to the medium, indicating that when the equilibrium is driven toward F1,6BP formation, the steady-state concentration of P_i available to the maize root H^+-PPase is greatly reduced, a condition that led to a decrease of the H^+ gradient.

Equilibrium Concentrations of P_i Formed from F1,6BP and P_i, and P_i, Dependence of Maize Root H^+-PPase

The equilibrium concentration of P_i, formed in the reaction catalyzed by PFP varied depending on the initial concentrations of F1,6BP, P_i, and F6P. To evaluate if the range of P_i concentrations formed in our experiments was close to that needed for one-half maximal activation of the maize root H^+-PPase, the P_i concentration was calculated in two ways: by using the concentrations of substrates and products found in plant cytosol (Kubota and Ashihara, 1990; Davies et al., 1993) or by using the results obtained under the conditions of the experiments of Figure 2. In addition, we measured the apparent affinity of maize root H^+-PPase for P_i (Fig. 3). Table II shows the P_i concentrations that would be formed under these different conditions. These values were calculated using the K_eq of 0.31 (Kubota and Ashihara, 1990) for the reaction catalyzed by PFP.

The P_i concentration needed for one-half maximal rate activity of maize root H^+-PPase was 3.2 × 10^{-5} m (Fig. 3). This value is similar to that previously reported by Maeshima (2000), is 10-fold lower than the P_i equilibrium concentrations calculated in Table II (conditions A and B), and is close to the equilibrium values calculated for condition C. This means that the rate of proton pumping (Fig. 2) responds readily to the equilibrium of the reaction catalyzed by PFP. The addition of 20 mM F6P (Fig. 2C) reduces the P_i concentration to such a low level (Table II, condition D) that can no longer be used by the maize root H^+-PPase to accumulate H^+ inside the tonoplast vesicles.

PFP Dependence for Formation of the Transmembrane Proton Gradient

The rate of vacuolar proton pumping varied depending on the amount of PFP added to the reaction medium (Fig. 4). The proton pumping was blocked by 12 mM fluoride, an anion that inhibits the vacuolar maize root H^+-PPase (Fig. 4, black circles). The assay condition of 2 mM P_i, 0.5 mM F1,6BP, 2 μM F2,6BP, and 18 milliunits of PFP was sufficient to stimulate the proton pumping to a level similar to that observed using 0.3 mM P_i, a saturating concentration (Fig. 4, white circles and dotted line). The amount of PFP used in Figure 4 is very small and is close to the levels observed in antisense transformant plants in which more than 95% of the total PFP activity has been abolished (Hajirezaei et al., 1994; Nielsen and Stitt, 2001).

DISCUSSION

In this report, the capacity of maize root vacuolar H^+-PPase to use the P_i, formed at equilibrium by PFP was evaluated. It was found that in the presence of Mg^{2+}, P_i, F1,6BP and F2,6BP concentrations similar to those available in plant cytosol (Rebeille et al., 1983; Ukaji and Ashihara, 1987; Weiner et al., 1987;
Table II. PP, formation in presence of F1,6BP, P<sub>i</sub>, and F6P

<table>
<thead>
<tr>
<th>Conditions</th>
<th>F1,6BP</th>
<th>P&lt;sub&gt;i&lt;/sub&gt;</th>
<th>F6P</th>
<th>PP&lt;sub&gt;i&lt;/sub&gt; formed</th>
<th>ΔG&lt;sup&gt;1&lt;/sup&gt;</th>
<th>ΔG&lt;sup&gt;2&lt;/sup&gt;</th>
<th>ΔG&lt;sup&gt;1+2&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6×10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>5×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>1×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>3.3×10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>-0.6</td>
<td>-3.9</td>
<td>-4.5</td>
</tr>
<tr>
<td>B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6×10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>2×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>3×10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>3.5×10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>-2.1</td>
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<td>-1.3</td>
<td>-8.4</td>
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<tr>
<td>D&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1×10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>2×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>1×10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>3.0×10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>-0.02</td>
<td>-2.2</td>
<td>-2.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>The ΔG<sub>i</sub> values (kilocalories per mole) for different conditions were calculated from the equation ΔG<sub>i</sub> = ΔG<sup>°</sup> + RT ln [PP<sub>i</sub>] × [F6P]/[F1,6BP] × [P<sub>i</sub>] using the values shown in the table, and ΔG<sub>2</sub> values were calculated from the equation ΔG<sub>2</sub> = ΔG<sup>°</sup> + RT ln [P<sub>i</sub>] × [H<sup>+</sup>]<sub> vac</sub> using a ΔpH of 2 units. The final concentration of F6P derived from contamination of F1,6BP and its hydrolysis was estimated to be 5% (3×10<sup>-5</sup> M). This concentration of F6P was used to calculate the concentration of PP<sub>i</sub> formed in experiments in which extra amounts of F6P were not added. The molar concentrations of F1,6BP, P<sub>i</sub>, and F6P are those reported in Kubota and Ashihara, 1990; Davies et al., 1993; Stitt, 1998.

Kubota and Ashihara, 1990; Stitt, 1998), the P<sub>i</sub> formed by even a small amount of PFP was sufficient to activate the vacuolar H<sup>+</sup>-PPase and to form a proton gradient across the tonoplast membrane. This reaction is coupled to a decrease in the F1,6BP concentration, a compound that has a lower energy of hydrolysis than P<sub>i</sub> (de Meis, 1989, 1993). Table II shows that the free energy change (associated with Reaction 4) is markedly negative (Table II, ΔΣG<sub>1+2</sub> varying from −2.19 to −9.68 kcal mol<sup>−1</sup>), despite different levels of PP<sub>i</sub> formed under the various conditions used to calculate the equilibrium PP<sub>i</sub> concentration. Even when the PP<sub>i</sub> concentration was as low as 3 μM (Table II, condition D), the calculated ΔΣG<sub>1+2</sub> was −2.19 kcal mol<sup>−1</sup>, a value that indicates a favorable shift to PP<sub>i</sub> hydrolysis. In maize roots tips, the contribution to the steady-state level to PP<sub>i</sub> in the cytosol of only one of the reactions that produce PP<sub>i</sub>, the formation of UDP-Glc from Glic-1 P and UTP, was estimated to be 10 μM (Roberts, 1990) a value that is more than 3 times higher than that used to obtain a value of ΔΣG<sub>1+2</sub> of −2.19 kcal mol<sup>−1</sup> (Table II, condition D). This means that there are no thermodynamic constraints for the pumping of protons into the vacuole lumen using F1,6BP, P<sub>i</sub>, and 20 mM F6P to achieve the equilibrium concentration of PP<sub>i</sub> of 3 μM. The observation that the protons leak out of the vesicles when 20 mM F6P is added to the medium (Fig. 2C) is related to the decrease of the steady-state PP<sub>i</sub> concentration to a level far below that needed by the tonoplast H<sup>+</sup>-PPase (Fig. 3).

Based on reported concentrations of glycolytic intermediates of plant cells (Kubota and Ashihara, 1990), the ΔG for the formation of PP<sub>i</sub> (Reaction 1; Kubota and Ashihara, 1990) and the ΔG of PP<sub>i</sub> hydrolysis (Reactions 2 and 3; de Meis, 1984; Davies et al., 1993), it was possible to calculate the overall ΔG.
for the glycolytic conversion of F1,6BP to either pyruvate or PPi and the subsequent hydrolysis of PPi by the H+-PPase needed to maintain a ΔpH of 2 units across the tonoplast membrane. From these estimates, it becomes apparent that thermodynamic pull for the utilization of F1,6BP toward H+ pumping (ΔG1 + ΔG2 in Fig. 5) is slightly more favorable than the formation of pyruvate (ΔG3 + ΔG4 in Fig. 5). The readily reversible PFP reaction may provide an adaptive pathway for glycolysis and gluconeogenesis in higher plant cells (Black et al., 1987). The result shown in Figure 2 suggests a new role of PFP, the formation of PPi to be used for the formation of a proton gradient in tonoplast vesicles.

One of the possible limiting factors for the utilization of F1,6BP and Pi to form PPi would be the amount of PFP and/or H+-PPase present in the cells. A simultaneous up-regulation in the levels of H+-PPase and PFP have been detected during anoxia in rice (Oryza sativa) seedlings (Mertens et al., 1990; Carystinos et al., 1995; Drew, 1997). Table I shows that the levels of PFP and H+-PPase activities detected in different plants (Hajirezaei et al., 1994; Nielsen and Stitt, 2001) were sufficient in our assays to promote the formation of a H+ gradient. We found that it was possible to detect a proton gradient in tonoplast vesicles even in the presence of a PFP activity similar to that observed in antisense transformant plants (Hajirezaei et al., 1994; Nielsen and Stitt, 2001). The H+-PPase levels used in our experiments are similar to those detected in maize roots and other plants (Table I). These data suggest that, given the low ΔG values of Reactions 1 and 2 (Fig. 5), the low ratio in the activity levels of PFP to H+-PPase would limit the vacuolar proton pumping when the concentration of PFP decreases to levels below 4 milliunits g fresh weight−1. In recent reports (Hajirezaei et al., 1994; Nielsen and Stitt, 2001) using tobacco and potato plant transformants that lead up to a 95% reduction in the PFP activity (see also Table I), there were no major changes in carbon fluxes or leaf or plant growth. Nevertheless, there was a compensatory increment in the F2,6BP levels both in tobacco leaves and potato tubers (Hajirezaei et al., 1994; Nielsen and Stitt, 2001). In our study, the levels of PFP activity are similar to those detected in tobacco and potato plant transformants (Fig. 4; Table I). This raises the possibility that under steady-state conditions in the plant cytosol, different enzymes involved in energy transduction, such as maize root vacuolar H+-PPase, may use phosphate compounds with a low energy of hydrolysis (de Meis, 1989, 1993; de Meis et al., 1992a, 1992b; Montero-Lomeli and de Meis, 1992; Galina et al., 1995) to perform the work that is usually associated with the consumption of higher energy phosphate compounds such as PPi (Romero and de Meis, 1989).

**MATERIALS AND METHODS**

**Materials and Chemicals**

All chemicals were obtained from Sigma (St. Louis). PPi:Fru-6-phosphate 1-phosphotransferase was from mung bean (Vigna radiata; Sigma F-8757). FCCP was dissolved in ethanol. The amounts used were such that the

![Figure 5](https://www.plantphysiol.org/Assets/figures/76404.png)

**Figure 5.** Free-energy changes (ΔG) associated with the conversion of F1,6BP to build up a H+ gradient in tonoplast vesicles or with conversion to pyruvate by the glycolytic pathway. After Glc or Fru phosphorylation, the F1,6BP formed can be diverted to PP, by synthesis of PPi-PFP (ΔG1), and the PPi is hydrolyzed by H+-PPase to build up a proton gradient of 2 units of pH in the tonoplast vesicles (ΔG2). Alternatively, the F1,6BP is converted into triose-P by aldolase (ΔG3). The triose-P is converted by glycolytic enzymes to pyruvate (ΔG4). ΔG1 and ΔG2 were calculated as shown in Table II (condition A) using concentrations in the plant cell cytosol. The ΔG3 and ΔG4 were calculated from glycolytic reactions described by Kubota and Ashihara (1990).
ethanol concentration in the reaction mixture was never higher than 0.5% (v/v). At this concentration, ethanol had no effect on either the H⁺ gradient or PP⁺ hydrolysis.

Preparation of Plant Material and Isolation of Microsomes from Maize Root Cells

Maize (Zea mays) seeds were surface sterilized with sodium hypochlorite (approximately 10 min in a 3% [v/v] solution), then washed with sterile water and soaked in water for 24 h. Radicles were harvested for preparation of vesicles from seeds allowed to germinate for 5 d on wet filter paper in the dark at 28°C.

Vascular membrane vesicles were isolated from whole roots using differential centrifugation. Roots (approximately 100 g wet weight) were homogenized in an IKA-Euroturrax T25 basic (speed 3 for 15 s using an SS25-18G probe, Wilmington, NC) with 200 mL of an ice-cold extraction buffer containing 10% (v/v) glycerol, 0.5% (v/v) polyvinylpyrrolidone-40, 0.13% (w/v) bovine serum albumin, 5 mM EDTA, and 0.1 M Tris-HCl (pH 8.0). Just before use, 3.3 mM dithiothreitol, 150 mM KCl, and 1 mM phenylmethylsulfonil fluoride (final concentrations) were added to the buffer. The homogenate was strained through four layers of cheesecloth and centrifuged at 8,000 × g for 20 min. The supernatant was centrifuged at 100,000 × g for 20 min. The pellet was resuspended in 80 mL of the extraction buffer and centrifuged once more at 100,000 × g for 40 min. The pellet was resuspended in a small volume of ice-cold buffer containing 10 mM Tris-HCl (pH 7.6), 10% (v/v) glycerol, 1 mM EDTA, and 1 mM dithiothreitol. The vesicles were frozen under liquid N₂. Protein concentrations were determined by the method of Lowry et al. (1951).

Measurement of F1,6BP Cleavage

The cleavage of F1,6BP was measured colorimetrically at 30°C by determining the rate of liberation of P₁ (Fiske and Subbarow, 1925) in reaction media containing: 50 mM MOPS-Tris buffer (pH 7.0), 3 mM ACMA, 1 mM MgCl₂, 100 mM KCl, 2 µM F₁,6BP, 0.6 mM MgCl₂, and different F₁,6BP concentrations. When the MgCl₂ dependence was measured, the F₁,6BP was fixed at 10 mM. The pH dependence was measured, 50 mM glycyl-Gly buffer (pH 6.0–8.5), and the concentrations of MgCl₂ and F₁,6BP were 1 and 5 mM, respectively. The reaction was started by the addition of 0.1 mM P₁. Aliquots of 0.8 mL were removed and the reaction was stopped by addition of 0.2 mL of 50% (w/v) trichloroacetic acid. Reaction times were adjusted so as to limit the cleavage of F₁,6BP to <10%. Cleavage was linear with time under these conditions.

Measurement of PP₁-Dependent Proton Transport into Maize Tonoplast Vesicles

The accumulation of H⁺ by the vesicles was determined by measuring the fluorescence quenching of ACMA (Molecular Probes, Eugene, OR) using a spectrofluorimeter (model F-3010, Hitachi, Tokyo). The excitation wavelength was set at 415 nm, and the emission wavelength was set at 485 nm. The reaction was carried out in 2 mL of medium containing 10 mM MOPS-Tris (pH 7.0), 3 µM ACMA, 1 mM MgCl₂, and 100 mM KCl.

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