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Comparison of Temperature Dependency of Tonoplast Proton Translocation between Plants Sensitive and Insensitive to Chilling[1]

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ABSTRACT

Proton transport activities in isolated tonoplast vesicles were measured as quenching of fluorescence of acridine orange. A marked difference in the temperature dependency of two types of tonoplast proton transports, *i.e.* ATP- and pyrophosphate-driven, was observed between two leguminous plants sensitive (mung bean, *Vigna radiata* [L.] Wilczek) and insensitive (pea, *Pisum sativum* L.) to chilling. In tonoplast vesicles isolated from hypcotyls of mung bean seedlings that were germinated for 3.5 days at 26°C in the dark, the total amount of fluorescence quenching at the steady state in both types of proton pumps, as a measurement of the inside-acidic pH gradient across the membrane vesicles, was markedly suppressed under temperatures below 10°C. In tonoplast vesicles isolated from epicotyls of pea seedlings, which were germinated for 7 days at 18° to 23°C in the dark, no suppression occurred in the formations of the pH gradient in either type of proton pump, even at 0°C. The cause of the low temperature-induced suppression of the proton pumps in mung bean tonoplasts seems to be not an increased permeability of the membrane vesicles to protons or accompanying anions and cations, but instead a marked inhibition in the catalytic activity of both enzymes under low temperatures.

Mung bean (*Vigna radiata* [L.] Wilczek) is a subtropical plant indigenous to Southeast Asia. The etiolated seedlings are extremely sensitive to chilling and suffer irreversible injury after exposure to 0°C in the dark more than 48 h, whereas no permanent injury occurs within the first 24 h of chilling (4). This indicates that chilling injury in mung bean seedlings proceeds in two distinct processes: an early reversible process and a later irreversible process. In the early process, *i.e.* within the first 24 h of chilling, there are reversible declines in a variety of physiological functions including respiration (15), 1-aminocyclopropane-1-carboxylic acid-dependent ethylene formation (4), and protein synthesis (7). The time courses of enzyme activities associated with various cellular membranes in mung bean hypocotyls during *in vivo* chilling at 0°C showed that the tonoplast H^+-ATPase was the most sensitive to chilling, being inactivated in the first 24 h, when membrane-bound enzymes associated with other cellular membranes remained intact (15). Given the putative role of the tonoplast H^+-ATPase in the formation of proton electrochemical potential gradient across tonoplast membranes, which may be employed for the active transport of ions and metabolites into vacuoles (12), the early decline in the H^+-ATPase activity during chilling may give rise to an alteration of the cytoplasmic environment, especially the pH and ionic concentrations. As a result, a variety of physiological functions may be disordered, eventually leading to cell death.

To gain insight into more immediate response of cells to low temperatures, we have examined and compared the temperature dependency of tonoplast proton transports *in vitro* between plants sensitive and insensitive to chilling, *i.e.* mung bean and pea (*Pisum sativum* L.), using isolated tonoplast vesicles. In mung bean tonoplast vesicles, both ATP and PPi-driven proton transports were observed to be sensitive to low temperatures, and the capability to form inside-acidic pH gradient declined abruptly below 10°C. In pea tonoplast vesicles, on the other hand, both proton transport systems were observed to function normally, even at 0°C. The marked difference in the temperature-dependency of tonoplast proton transport systems between these plants is discussed with reference to chilling sensitivity. A preliminary report of these results was presented elsewhere (16).

MATERIALS AND METHODS

Plant Materials

Seeds of mung bean (*Vigna radiata* L.) and pea (*Pisum sativum* L. cv Kinusaya) were imbibed and germinated at 26°C and 18° to 23°C in the dark, respectively. After 3½ (mung bean) or 7 d (pea) of germination, hypocotyls or epicotyls were excised from etiolated seedlings and used for experiments.

Preparation of Tonoplast Vesicles

We reported earlier (14) that a sorbitol/sucrose density gradient is useful for isolating tonoplast vesicles from etiolated mung bean seedlings in a high purity. In the present study, we modified it to obtain tonoplast vesicles with a high capacity for active proton transport. Hypocotyl tissues (mung bean) or epicotyl tissues (pea) were chopped into a homogenizing medium previously chilled at 0°C and immediately homogenized with a Polytron PT 30 at the medium speed setting for 45 s. The homogenizing medium contained 250 mM sorbitol,

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50 mM Mops-KOH (pH 7.6), 5 mM EGTA, 2 mM EDTA, 2.5 mM potassium metabisulfite, 1 mM PMSF, 0.5% (w/v) defatted BSA, 1.5% (w/v) PVP (mol wt 24,000), and 10 μg/mL butylated hydroxytoluene. The slurry was passed through four layers of gauze and subjected to differential centrifugation at 3,600*g* for 10 min followed by 156,000*g* for 20 min. The 3,600 to 156,000*g* pellets, designated a crude membrane fraction, were resuspended in 17 mL of sucrose-suspending buffer solution that contained 320 mM sucrose, 10 mM potassium phosphate buffer (pH 7.8), 1 mM EGTA, and 2 mM DTT, then put into the bottom of a centrifuge tube (25 mL) and overlayered with 7 mL of sorbitol buffer solution, which contained 250 mM sorbitol, 10 mM potassium phosphate buffer (pH 7.8), 1 mM EGTA, and 2 mM DTT. After centrifugation at 40,000 rpm for 30 min in a Hitachi RP 50.2 rotor, tonoplast vesicles were selectively floated up and banded at the interface of the sorbitol and the sucrose layers (10). The tonoplast vesicles were diluted with a sorbitol buffer solution, which contained 250 mM sorbitol, 25 mM Hepes-BTP[2] buffer (pH 7.2), and 2 mM DTT, then centrifuged at 156,000*g* for 20 min. The pellets were washed once with the sorbitol buffer solution used above. The washed tonoplast vesicles were suspended in an appropriate volume of the sorbitol suspending buffer solution (1 mg protein/mL) and freeze-stored at −80°C until use. The tonoplast vesicles thus obtained were in a high purity as assessed by analysis of membrane marker enzymes including vanadate-sensitive ATPase (plasma membrane), antimycin A-insensitive NADH cytochrome *c* reductase (endoplasmic reticulum), Triton X-100 stimulated UDPase (Golgi membranes), and cytochrome *c* oxidase (mitochondria). The tonoplast vesicles were highly enriched in NO_3-sensitive ATPase and PPase.

Measurement of Proton Transport Activity

An aliquot of the tonoplast sample (equivalent to 25 μg protein) was added to a reaction mixture containing 250 mM sorbitol, 25 mM Hepes-BTP (pH 7.2), 50 mM KCl, 1 mM PPi-Na or ATP-BTP, and 5 μM (for PPi-dependent) or 3 μM (for ATP-dependent) acridine orange in a final volume of 2.2 mL. After temperature equilibration, the reaction was started by an addition of $MgSO_4$ solution to a final concentration of 1 mM. The fluorescence decrease with time was followed with a Shimadzu spectrofluorimeter (model RF-5000) at excitation and emission wavelengths of 493 and 540 nm, respectively. The initial rate of quench ($\%\Delta F \cdot mg^{-1}\ min^{-1}$) was followed as a measurement of the rate of proton transport into the vesicles (1). The total amount of quench at the steady-state was used as a measurement of the inside-acidic pH gradient ($\%\Delta F \cdot mg^{-1}$) across the membrane vesicles (1).

Measurement of Rate of pH-Gradient Dissipation as a Function of Temperature

After initiation of ATP- or PPi-dependent pH gradient formation by tonoplast vesicles in the same reaction medium as described above, EDTA (free acid, pH adjusted to 7.2 with BTP) was added to a final concentration of 10 mM to inhibit the proton pumps (8). Then the rate of fluorescence recovery was followed as a measurement of the rate of pH-gradient dissipation. Measurements were carried out at 24° and 1°C, respectively.

Enzyme Assay

ATPase activity was assayed in a reaction mixture that contained 3 mM ATP-Na, 3 mM $MgSO_4$, 25 mM Hepes-BTP (pH 7.0), 50 mM KCl, and 0.03% (w/v) Triton X-100. PPase activity was assayed in a reaction mixture containing 1 mM PPi-Na, 1 mM $MgSO_4$, 25 mM Hepes-BTP (pH 7.4), 50 mM KCl, and 0.03% (w/v) Triton X-100 with a slight modification of the method reported elsewhere (10). Reactions were carried out at temperatures ranging from 0° to 30°C using a temperature gradient metal block. Released Pi was quantified by the Fiske-SubbaRaw (5) method. Assays of other enzymes were carried out according to the methods reported earlier (14). Protein was quantified according to the dye method of Bradford (2) using BSA as a standard.

RESULTS

Temperature Dependency of Proton Transport

Temperature dependencies of the two types of proton pumps were examined with tonoplast vesicles isolated from chilling-sensitive mung bean and chilling-insensitive pea. Figures 1 and 2 show the time courses of PPi-dependent fluorescent quench as a function of temperature. In mung bean tonoplast vesicles (Fig. 1), both the initial rate of fluorescence quench and the amount of maximum quench at the steady state were markedly reduced at the temperatures below 10°C.

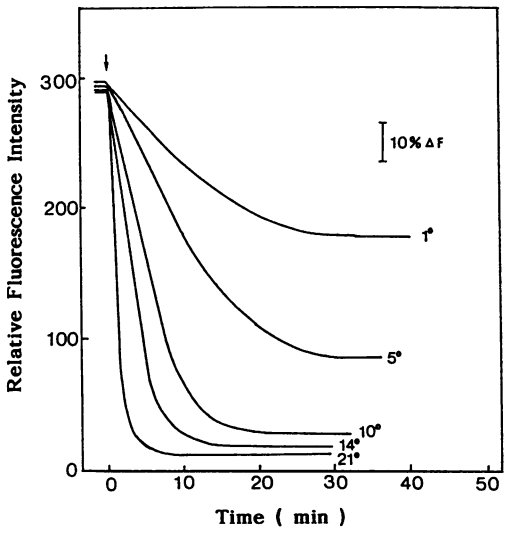

Figure 1. Time course of fluorescence quenching in mung bean tonoplast vesicles as a function of temperature. Reaction mixture contained 250 mM sorbitol, 25 mM Hepes-BTP (pH 7.2), 50 mM KCl, 1 mM PPi, 5 μM acridine orange, and an aliquot of tonoplast vesicles (equivalent to 25 μg protein). After temperature equilibration, reaction was initiated by an addition of $MgSO_4$ stock solution in a final concentration of 1 mM.

[2] Abbreviations: BTP, 1,3-bis(tris[hydroxymethyl] methylamino)propane; PPase, pyrophosphatase.

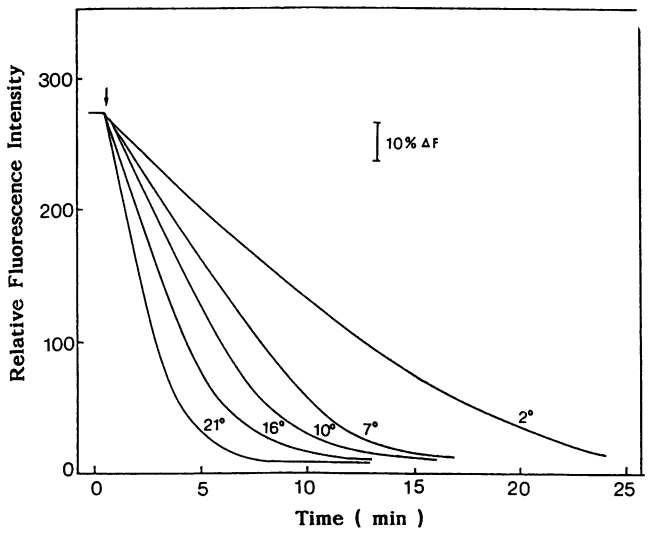

Figure 2. Time course of fluorescence quenching in pea tonoplast vesicles as a function of temperature. Experimental conditions were the same as in Figure 1.

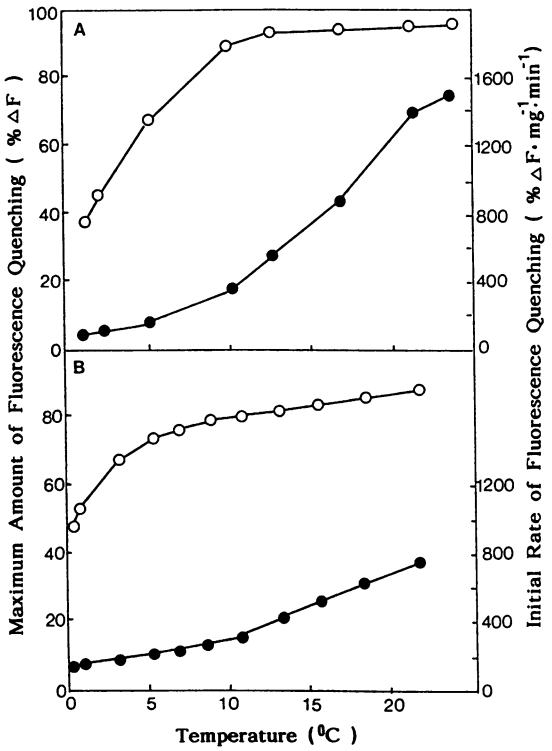

Figure 3. PPi- and ATP-dependent proton transports in mung bean tonoplast vesicles as a function of temperature. Tonoplast vesicles (equivalent to 25 μg protein) were incubated in 2.2 mL of reaction medium containing 250 mM sorbitol, 25 mM Hepes-BTP (pH 7.2), 50 mM KCl, 1 mM PPi or ATP, and 5 μM (for PPi-dependent) or 3 μM (for ATP-dependent) acridine orange at various temperatures. After temperature equilibration, reaction was initiated by an addition of $MgSO_4$ stock solution in a final concentration of 1 mM and fluorescence was followed. A, PPi as substrate; B, ATP as substrate. ○, Maximum amount of quenching; ●, initial rate of quenching.

In pea tonoplast vesicles (Fig. 2), on the other hand, the amount of maximum quench was observed to be independent of temperature, although the rate of fluorescence quench declined as temperature decreased. The time course of ATP-dependent proton transport showed nearly the same trend as observed in Figures 1 and 2 (data not shown).

Figures 3 and 4 show the initial rate of fluorescent quench and the total amount of maximum quench in two proton pumps as a function of temperature. A marked difference in the temperature profiles of the amount of the inside-acidic pH gradient was observed between mung bean (Fig. 3) and pea (Fig. 4). In mung bean tonoplast vesicles, the amount of the inside-acidic pH gradient was markedly suppressed at temperatures below 10°C (PPi-dependent) or 7°C (ATP-dependent). However, no decline in the pH gradient formation by either type of proton pump was observed in the pea tonoplast vesicles.

Figure 5 shows the Arrhenius plots of the initial rate of quenching in mung bean tonoplast vesicles. Breaks were apparently observed around 5°C in both ATP- and PPi-driven proton transport systems. No break, however, was determined in the Arrhenius plots of pea tonoplast vesicles within the temperature range from 26 to 0°C (data not shown).

Effect of Temperatures on Substrate Hydrolyzing Activities

Figure 6 shows Arrhenius plots of hydrolytic activities of H^+-ATPase and H^+-PPase. In mung bean tonoplast (Fig. 6A),

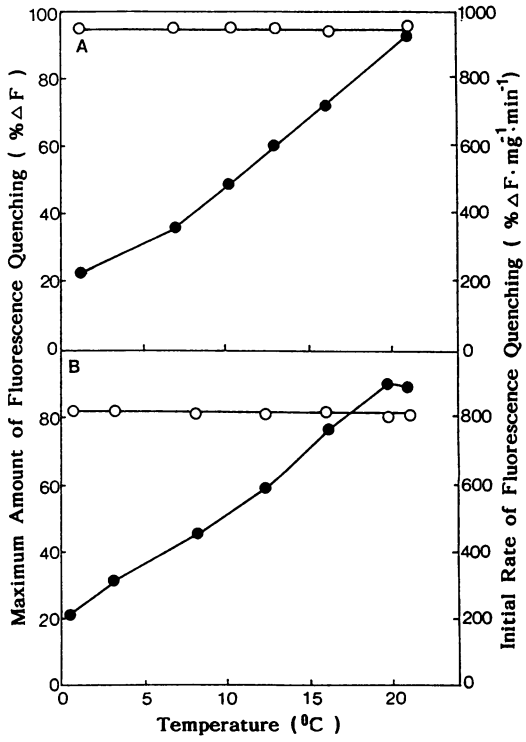

Figure 4. PPi and ATP-dependent proton transport in pea tonoplast vesicles as a function of temperature. Measurements were performed as in Figure 3. A, PPi as substrate; B, ATP as substrate. ○, Maximum amount of quenching; ●, initial rate of quenching.

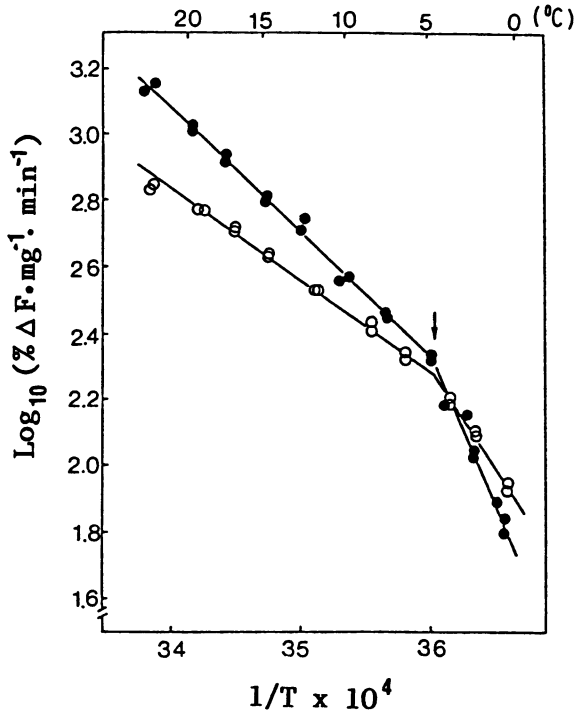

Figure 5. Arrhenius plots of initial rate of fluorescence quenching in mung bean tonoplast vesicles. Fluorescence quenching was measured as in Figure 3. O, ATP dependent; ●, PPi dependent.

breaks were observed around 7°C in both cases. The activation energies were apparently higher in H^+-PPase than H^+-ATPase throughout the temperature range tested. In pea tonoplast (Fig. 6B), however, no break was observed in the Arrhenius plots of hydrolytic activities of H^+-ATPase between 0° and 25°C.

Effect of Temperatures on Dissipation of pH Gradient

To assess effects of temperature on the rate of pH-gradient dissipation in tonoplast vesicles, ATP or PPi-dependent proton transport was initiated at different temperatures and stopped by the addition of EDTA into the reaction mixture. Figure 7 represents the time course of fluorescence changes associated with formation of pH gradient via H^+-PPase and collapse of the gradient upon the addition of 10 mM EDTA at 24° and 1°C, respectively. The rate of fluorescence recovery was dependent on the amount of pH gradient when measurements were carried out 24°C (Fig. 7, A and B). The rate of pH-gradient dissipation was rapid at 24°C, *i.e.* 1386 ± 147 $\%\Delta F \cdot mg^{-1}$ min^{-1}, and much slower at 1°C, *i.e.* 176 ± 39 $\%\Delta F \cdot mg^{-1}$ min^{-1}, when measured at a comparable starting pH gradient (Fig. 7, A and C). These results suggest that membrane permeability to protons as well as accompanying anions and cations is lower at lower temperatures.

DISCUSSION

In the present study, temperature-dependency of proton transports in isolated tonoplast vesicles was examined using acridine orange as a fluorescent dye with special reference to chilling sensitivity of plants. Pope and Leigh (11) discussed various artifacts that occurred when acridine orange was used to measure pH gradient formation in tonoplast vesicles isolated from beet storage tissues. They postulated that the protonated acridine orange forms an anion complex permeable through the membrane and collapses the pH gradient. In our tonoplast preparations, however, an addition of KNO_3 into the reaction mixture for PPi-driven proton transport system did not inhibit the formation of pH gradient, but rather stimulated the proton translocation, suggesting no formation of the permeable anion-dye complex. When quinacrine was used as the fluorescent dye, we obtained nearly the same results as in acridine orange (data not shown).

A marked difference in the temperature-dependency of the two types of tonoplast proton pumps was observed between plants sensitive and insensitive to chilling. In chilling-sensitive mung bean, the formation of inside-acidic pH gradient showed a marked decline at temperatures below 10°C in both transport systems. On the contrary, no decline in the formation of pH gradient by both proton pumps was observed even at 0°C in the tonoplast vesicles isolated from chilling-insensitive pea seedlings. As reported earlier (16), tonoplast H^+-PPase in mung bean hypocotyls was observed to be more stable than H^+-ATPase during chilling *in vivo,* and no inactivation occurred within the first 24 h of chilling. In mung bean hypocotyls, it has been suggested that the tonoplast H^+-PPase may also be actively involved in the generation of proton electrochemical potential gradient across tonoplast membranes, because of the continuous *de novo* synthesis of the enzyme and presence of sufficient amounts of the substrate throughout growth (9). Therefore, one may consider that the H^+-PPase can be substituted for the chill-induced inactivation of H^+-ATPase during the early process of chilling, provided that the H^+-PPase can function normally under low temperatures. However, present results show that this assumption is quite unlikely.

As reported by DuPont and Mudd (3), suspension-cultured cells of tomato (*Lycopersicon hirsutum*) that originated from a high altitude in the Andes showed a great tolerance to chilling. They measured ATP-dependent proton transport as a function of temperatures using isolated tonoplast vesicles and found no reduction in the formation of inside-acidic pH

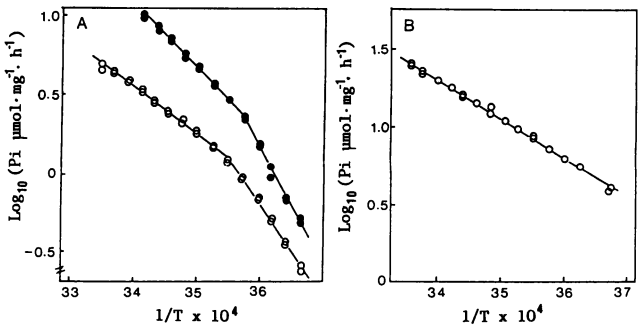

Figure 6. Arrhenius plots of H^+-ATPase and H^+-PPase in tonoplast vesicles isolated from mung bean hypocotyls and pea epicotyls. A, Mung bean; B, pea. O, ATPase; ●, PPase.

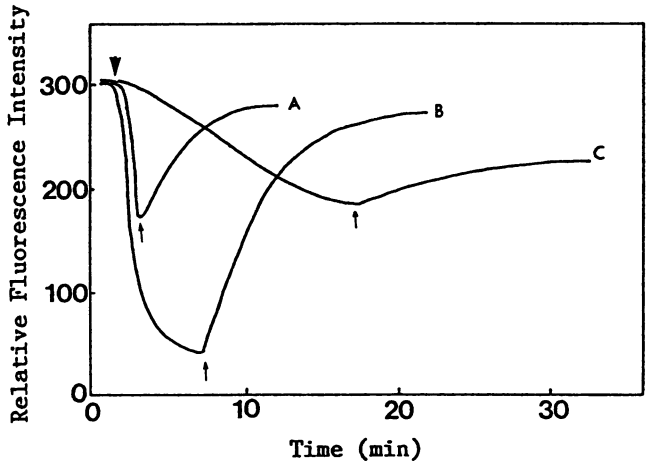

Figure 7. Effects of temperature on the rate of pH-gradient dissipation in mung bean tonoplast vesicles. Pyrophosphate-dependent proton transport was initiated by the addition of $MgSO_4$ (arrowhead) to a final concentration of 1 mM. The assay condition was the same as in Figure 1. The fluorescence changes were followed at different temperatures (A and B, at 24°C; C, at 1°C). After reaching a comparable (A and C) or a maximum pH-gradient (B), EDTA was added to a final concentration of 10 mM (arrows) to inhibit the proton pump.

gradient across the membranes at temperatures down to 5°C. After preculture of cells at 9°C, the temperature optimum for the proton transport broadened and shifted to a lower temperature range, suggesting a temperature acclimation of the proton pump. Joyce *et al.* (6) have examined the effects of temperatures on ATP-dependent transports of protons and calcium in tonoplast-enriched fractions isolated from green tomato fruits (*L. esculentum* Mill. cv Castlemart) and red beet roots. They found no difference in the temperature profiles of the initial rate of proton transport and the maximum amount of inside-acidic pH gradient at the steady-state between these plants. However, the temperature profile of ATP-dependent transport of calcium mediated by a H^+/Ca^{2+} antiporter was much different between these plants, with a sharp increase in activation energy at temperatures below 10° to 12°C in tomato membrane vesicles that was not observed in red beet membrane vesicles. They postulated that low temperature directly affects the H^+/Ca^{2+} antiporter molecules or indirectly affects lipid interactions with the antiporter. These results may suggest that not only proton transport but also calcium transport in tonoplasts has an important role in temperature acclimation phenomena in plants.

The inside-acidic pH gradient created in the tonoplast vesicles is assumed to represent the steady-state in which the rate of proton influx via proton pumps equals the rate of passive proton efflux through the membranes (1, 8). Therefore, the decrease in the steady-state pH gradient in mung bean tonoplast vesicles under the low temperatures may be interpreted either as a function of decrease in proton influx or an increase in proton efflux. However, the process might be rather complex, dependent not only on proton flux but also on fluxes of accompanying anions and cations (13). To assess the effects of temperature on the membrane permeability, we measured the rate of pH-gradient dissipation at different temperatures upon inhibition of the proton pumps. In mung bean tonoplast vesicles, PPi-induced pH gradient was rapidly dissipated upon addition of EDTA (10 mM) at 24°C, but very slowly at 1°C. This was also true in the case of ATP-induced proton transport system (data not shown). These results indicate that the low temperature-induced inhibition of the pH-gradient formation may not be directly related to an increased membrane permeability. Arrhenius plots of substrate hydrolyzing activities and the initial rate of proton translocation across the tonoplast vesicles showed an apparent "break" around 7°C in both proton transport systems, suggesting that the low temperature-induced suppression of the formation of inside-acidic pH gradient is probably due to the marked reduction in the catalytic activities of the enzymes and/or a partial uncoupling of proton transport under such a low temperature. To gain more insight into the mechanisms, it is necessary to perform experiments using a reconstituted membrane system from purified enzymes.

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