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Chilling-Induced Inactivation and Its Recovery of Tonoplast H^+-ATPase in Mung Bean Cell Suspension Cultures[1,2]

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ABSTRACT

The processes involved in adaptation to cold temperature were examined by growing suspension cultured cells of mung bean (*Vigna radiata* [L.] Wilczek) at 2°C for various periods of time and assaying the activities of various membrane-bound enzymes *in vitro*. The tonoplast H^+-ATPase activity and the ATP-proton transport extracted from cells incubated at 2°C declined rapidly and reached a minimum level after 10 hours. The inactivation was reversible within 24 hours of chilling. The recovery of the cold-inactivated H^+-ATPase was found to proceed in two steps, a faster recovery of ATP hydrolysis activity and a slower recovery of the proton transport. The recovery was markedly inhibited by the presence of azide, but not affected by 0.578 millimolar cycloheximide. This suggested the involvement of an energy process that had no requirement for *de novo* synthesis of protein. The cold-induced inactivation of the H^+-ATPase may be due to a structural alteration of the enzyme. The slower recovery of proton transport relative to ATP hydrolysis during warming suggests that the protogenic domains in the enzyme may be affected differently by chilling.

Vacuoles are thought to be one of the primary cellular sites that respond to chilling stress in mung bean seedlings (*Vigna radiata* [L.] Wilczek) (18). The activities of two types of proton pumps located on tonoplast membranes are markedly suppressed with temperatures below 10°C *in vitro* (17). The H^+-ATPase activity extracted from cells subjected to chilling treatment decreased rapidly with a loss of 50% within 48 h (18). Given the important role of the primary proton pumps in the secondary transport of ions and metabolites, it can be presumed that low temperatures may result in an alteration of the homeostasis of the intracellular environment, including cytoplasmic pH and concentrations of ions and metabolites. Suspension cultures of mung bean would serve as a good system for getting more insight into cellular responses to chilling *in vivo*, especially the biochemical changes occurring in tonoplast membranes, because both the chemical and physical environments can be more easily controlled in cultured cells than intact whole plants.

In the present study, cold-induced inactivation of the tonoplast H^+-ATPase *in vivo* and its repair after transfer to a warm condition were investigated using chilling-sensitive cultures of mung bean and chilling-resistant cultures of bromegrass (*Bromus inermis* Leyss).

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MATERIALS AND METHODS

Plant Materials

Callus was initiated from young roots of sterile seedlings of mung bean (*Vigna radiata* [L.] Wilczek). The culture medium for callus and the cell suspension contained Murashige and Skoog's major and minor salts, and vitamins, except that thiamine concentration was increased 10-fold, supplemented with 3% sucrose, 0.5% glucose, 0.1 mg/L, 2,4-D, and 0.05 mg/L kinetin. Cell suspension cultures of bromegrass (*Bromus inermis* Leyss) were obtained from the Department of Crop Science, University of Saskatchewan, and grown in Erickson's medium containing B_5 micronutrients and vitamin solutions supplemented with 0.5 mg/L 2,4-D as reported elsewhere (12). All stock cultures were grown in 50 mL of liquid medium at 26°C in 200 mL Erlenmeyer flasks on a reciprocating shaker (60 cycles/min) under continuous illumination with fluorescent light and subcultured at 12-d intervals.

Low Temperature Treatment

Cell suspension cultures grown for 8 d at 26°C were transferred to a cold room at 2 ± 0.5°C in the dark and kept on a rotatory shaker (60 cycles/min). After cold treatment for various periods, cells were collected on Miracloth, washed with distilled water at 0°C, and blotted on filter paper. Non-chilled control cells were collected as described above at 25°C. Cell suspension cultures previously chilled for various periods were transferred to a warm condition at 26°C in the light and kept there for various periods. Cells were then harvested as described above.

Preparation of Crude Microsomal Fraction

Cells (5–6 g fresh weight) were disrupted in 10 mL of a homogenizing medium with a glass tissue grinder (Kontes K-885450, 15 mL). The homogenizing medium contained 250 mM sorbitol, 30 mM Mops-KOH (pH 7.6), 5 mM EGTA, 1 mM EDTA, 0.5% BSA, 1.5% PVP, 1 mM PMSF, 10 μg/mL butylated hydroxytoluene, and 2.5 mM potassium metabisulfite. The slurry was squeezed through one layer of Miracloth and the filtrate was centrifuged at 10,000*g* for 10 min followed by 156,000*g* for 20 min. The 10,000 to 156,000*g* pellets

(designated crude microsomal fraction) were suspended in 250 mM sorbitol, 25 mM Hepes-BTP[3] (pH 7.2), and 2 mM DTT, centrifuged at 156,000*g* for 20 min, and resuspended in the pH 7.2 Hepes-BTP medium described above.

Enzyme Assay

ATPase activity was assayed in a reaction mixture containing 3 mM ATP, 3 mM $MgSO_4$, 25 mM Hepes-BTP (pH 7.0), 50 mM KCl, 1 mM DTT, 1 mM sodium molybdate, 5 mM NaN_3, 0.03% Triton X-100 (w/v) and with either 100 μM sodium orthovanadate plus 150 mM KNO_3 (*a*), 100 μM sodium orthovanadate (*b*), or 150 mM KNO_3 (*c*). ATPases in plasma membranes (19) and tonoplast membranes (20) were effectively inhibited by these concentrations of inhibitors. Reaction was performed at 30°C. Tonoplast H^+-ATPase (NO_3-sensitive) was evaluated by subtracting activity value in (*a*) from activity in (*b*). Plasma membrane ATPase (vanadate-sensitive) was evaluated by subtracting activity in (*a*) from activity in (*c*). In these ways, plasma membrane and tonoplast ATPases in a crude microsomal fraction could be determined more accurately than was described in our previous study (18). PPase activity was assayed as reported by us (7) with a slight modification. The reaction mixture consisted of 1 mM sodium pyrophosphate, 1 mM $MgSO_4$, 25 mM Hepes-BTP, pH 7.4, 50 mM KCl, 1 mM sodium molybdate, 1 mM DTT, and 0.03% (w/v) Triton X-100. Reaction was performed at 30°C. Released Pi was determined as reported elsewhere (18, 19). Antimycin A-insensitive NADH Cyt *c* reductase was assayed as reported elsewhere (18).

Proton Transport Assay

An aliquot of microsomes (equivalent to 75 μg protein) was added to a reaction mixture consisting of 250 mM sorbitol, 25 mM, Hepes-BTP (pH 7.2), 50 mM KCl, 2 mM NaN_3, 100 μM sodium orthovanadate, 1 mM ATP-BTP, and 2 μM acridine orange in a final volume of 2.2 mL. After temperature equilibration at 25°C for 3 min, reaction was started by addition of $MgSO_4$ stock solution to a final concentration of 1 mM. The fluorescence decrease with time was followed with Shimadzu spectrofluorimeter, model F-5000, using excitation and emission wavelengths of 493 and 540 nm, respectively. The fluorescence quench was completely inhibited by the addition of 25 mM KNO_3 into the reaction mixture, indicating proton transport via tonoplast H^+-ATPase. The initial rate of quench (% $\Delta F \cdot mg^{-1}\ min^{-1}$) was used as the measurement of the initial rate of proton transport.

Measurement of Cell Viability

Cell viability after chilling treatment was evaluated by TTC-reduction test (14) and growth recovery after transfer to a warm condition. For the TTC-reduction test, 300 mg (fresh weight) of cells were incubated with 3 mL of reaction mixture containing 50 mM potassium phosphate buffer, pH 7.3, and 0.6% (w/v) TTC at 26°C in the dark under N_2 gas. After incubation for 15 h, cells were collected on Miracloth, blotted, and extracted with 7 mL of hot 95% ethanol for 15 min. Absorbance of the ethanol extracts was measured at 540 nm against 95% ethanol as a blank. The absorbance measured with non-chilled control cells was taken as 100%. To test for growth, an aliquot of the cell suspension cultures containing 0.3 mL packed cell volume was transferred to a flask containing 20 mL of fresh culture medium and cultured at 26°C in the light. After culture for 8 d, growth rate was evaluated by measurement of the packed cell volume. The packed cell volume of control cultures was taken as 100%.

RESULTS

Changes in the TTC reduction rate and regrowth capacity of cell suspension cultures of mung bean as a function of chilling period were measured as estimates of cell viability (Fig. 1). The regrowth capacity showed a marked decline after chilling more than 48 h, but the change was negligible with chilling for 24 h. The TTC reduction rate, however, declined linearly up to 48 h of chilling, then reached a steady level. The lower values of the TTC reduction relative to the regrowth capacity up to 48 h of chilling may indicate a recovery of cells from a partially injured state following periods of culture at 26°C. Changes in membrane-bound enzyme activities as a function of chilling period were assessed (Fig. 2A). Activities of vanadate-sensitive ATPase (plasma membrane) and PPase (tonoplast) showed no significant inhibition up to 48 h at 2°C. Both activities were slightly stimulated during the first 12 h. Activity of antimycin A-insensitive NADH Cyt *c* reductase

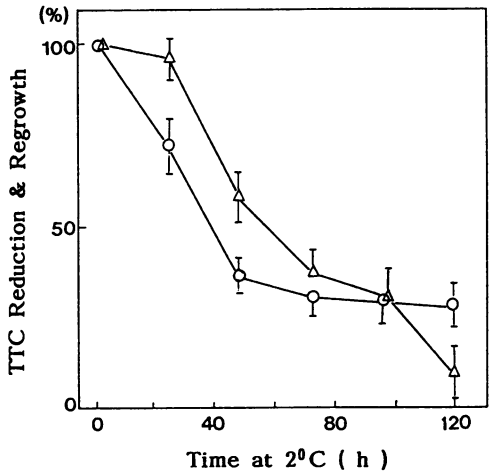

Figure 1. Effects of chilling on TTC-reduction rate and growth recovery in mung bean suspension cultures. After culturing for 8 d at 26°C, cells were grown at 2°C in the dark for indicated periods. A sample of 300 mg fresh weight cells was tested for TTC reduction. An aliquot of cell samples (0.3 mL packed cell volume) was transferred to a new culture medium and cultured at 26°C for 8 d to follow growth recovery. The TTC-reduction rate and the packed cell volume in nonchilled control cells were taken as 100%, respectively. Experimental details are described in "Materials and Methods." Each point represent the mean ± SE (n = 3). (○), TTC-reduction rate; (△), packed cell volume.

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

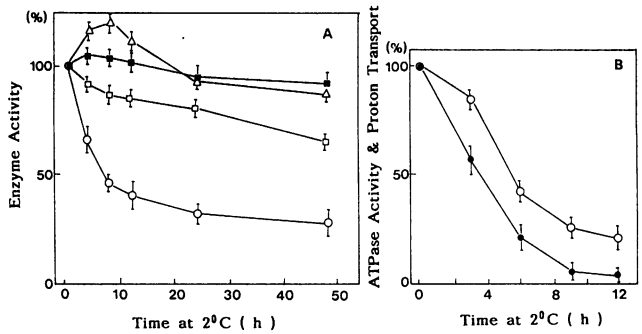

Figure 2. Changes in membrane-bound enzyme activities (A) and ATP-dependent proton transport (B) during chilling of mung bean suspension cultures. After culturing for 8 d at 26°C, cells were exposed to 2°C in the dark. After the chilling treatment for indicated periods, cells were disrupted and microsomal fractions were prepared. Assays for enzyme activities and ATP-proton transport were carried out as described in "Materials and Methods." A and B were separate experiments. Activities in nonchilled cells were taken as 100%. Activities of enzymes and proton transport in nonchilled control cells are: NO_3-sensitive ATPase, 5.2 (A) and 5.9 (B) Pi μmol · mg^{-1} h^{-1}; ATP-proton transport, 22.6% ΔF · mg^{-1} min^{-1}; vanadate-sensitive ATPase, 7.3 Pi μmol · mg^{-1} h^{-1}; PPase, 8.8 Pi μmol · mg^{-1} h^{-1}; antimycin A-insensitive NADH Cyt *c* reductase, 3.3 μmol · mg^{-1} min^{-1}. Each point represents the mean ± SE (n = 3). A: (○), NO_3-sensitive ATPase; (■), vanadate-sensitive ATPase; (△), PPase; (□) NADH Cyt *c* reductase. B: (○), NO_3-sensitive ATPase; (●), ATP-dependent proton transport.

(endoplasmic reticulum) decreased linearly as a function of the chilling period. NO_3-sensitive ATPase activity (tonoplast H^+-ATPase) was affected more severely with chilling, decreasing abruptly during the first 12 h, attained a minimum level after 24 to 48 h. In another set of experiments (Fig. 2B), proton transport by tonoplast H^+-ATPase decreased rapidly after the onset of chilling, proportionally with decreases in ATP hydrolysis. It is of interest to note that the proton transport decreased faster than the hydrolysis activity during chilling.

Table I shows effect of chilling on tonoplast H^+-ATPase activity and the proton transport in chilling resistant cell suspension cultures of bromegrass. In contrast with mung bean cell suspension cultures, no decline was observed in either ATP hydrolysis or proton transport during chilling for 24 h.

The chilling-induced inactivation of tonoplast H^+-ATPase in mung bean cultured cells was found to be completely reversible within 24 h of chilling (Fig. 3). ATP hydrolysis by H^+-ATPase recovered very fast and reached nearly the same level of the nonchilled control cells after 40 min of warming. However, the proton transport activity recovered more slowly than ATP hydrolysis and reached the control level after 60 min of warming. NADH Cyt *c* reductase activity (antimycin A-insensitive) recovered from 75 (at zero time of warming) to 100% of the control level after warming for 60 min (data not shown). Plasma membrane ATPase activity decreased in the first 20 min of warming from 93 (at zero time of warming) to 70% of nonchilled control value, then recovered nearly to the nonchilled control level in the subsequent 40-min incubation (data not shown).

The effects of cycloheximide and azide on the recovery of chill-induced inactivation of tonoplast H^+-ATPase and the proton transport activities following warming were assessed (Table II). After chilling for 24 h, the cultured cells were transferred to 26°C and incubated with or without addition of inhibitors. Cycloheximide at 576 μM had no effect for the recovery of tonoplast H^+-ATPase activity. However, azide was observed to be inhibitory to the recovery of ATP hydrolysis activity and proton transport. The recovery of NADH Cyt *c* reductase activity (antimycin A-insensitive) was hardly affected by the addition of cycloheximide, but slightly inhibited by the addition of azide during warming (data not shown). A transient decline of plasma membrane ATPase activity during warming was further enhanced by the addition of inhibitors (data not shown).

DISCUSSION

In the present study, the processes of cold-induced inactivation of tonoplast H^+-ATPase and its recovery after warming were investigated using suspension cultured cells of mung bean. Similar to the intact seedlings (18), suspension cultured cells of mung bean were highly sensitive to chilling and sustained an irreversible injury after exposure to 2°C for more than 48 h. However, no permanent injury occurred within the first 24 h as assessed by regrowth test (Fig. 1). Upon exposure to low temperature, both activities of the tonoplast H^+-ATPase, substrate hydrolysis and proton transport declined rapidly, reaching a minimum within 10 h. This effect appears far earlier than irreversible cell injury. On the contrary, no cold-induced inactivation was observed in the tonoplast H^+-ATPase in suspension cultured cells of bromegrass (Table I), which are reported to be capable of cold hardening (12, 13). Therefore, there seems to be a marked difference in the cold stability of tonoplast H^+-ATPase *in vivo* between chilling-sensitive and -resistant cultured cells. A correlation between chilling sensitivity of cells and the instability of tonoplast H^+-ATPase against cold environment was also reported in suspension cultured cells of rice plants (6). In cell suspension cultures of chilling tolerant tomato which originated from a high altitude in Andes, tonoplast H^+-ATPase is reported to exhibit an adaptation to cold after preculture at low temperature (3). The temperature-activity profile of the ATP-dependent proton transport changed markedly after the

Table I. *Effects of Chilling in Vivo on Tonoplast H^+ -ATPase Activity and Proton Transport in Cold Tolerant Suspension Cultures of Bromegrass*

After chilling of the cultured cells (12-d-old) at 2°C for various periods, microsomal fractions were prepared and used for analysis. Values in parentheses are percent control. Values ± SE.

Duration Time of Chilling	NO_3-Sensitive ATPase Activity	ATP-Dependent Proton Transport
h	*Pi μmol·mg^{-1} h^{-1}*	*%ΔF·mg^{-1} min^{-1}*
0	2.5 ± 0.1 (100)	38.3 ± 2.5 (100)
3	2.2 ± 0.2 (88)	36.1 ± 1.7 (94)
9	2.5 ± 0.2 (100)	43.1 ± 2.1 (112)
24	2.6 ± 0.1 (104)	35.3 ± 1.8 (92)

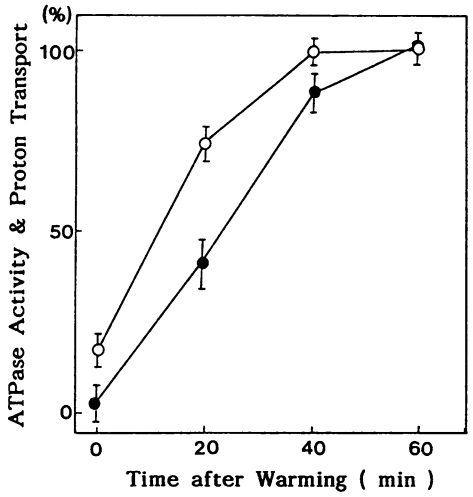

Figure 3. Recovery of NO_3-sensitive ATPase activity and ATP-dependent proton transport after warming. Mung bean cultured cells (8-d-old) were chilled at 2°C for 24 h, then transferred to 26°C. After incubation at 26°C for indicated periods, cells were disrupted and microsomal fractions were obtained. Assays for NO_3-sensitive ATPase activity and ATP-dependent proton transport activity were carried out as described in "Materials and Methods." Activities in nonchilled control cells were: NO_3-sensitive ATPase, 5.0 Pi μmol · mg^{-1} h^{-1}; ATP-dependent proton transport, 65% Δ F · mg^{-1} min^{-1}, and these values were taken as 100%. Each point represents the mean ± SE (n = 3). (○), NO_3-sensitive ATPase; (●), ATP-dependent proton transport.

low temperature conditioning, resulting in a broad optimum temperature, shifted to a lower temperature. These results may indicate the importance of the vacuolar proton transport systems for cold adaptation of plants (3). In the present study, transient increases of tonoplast H^+-PPase activity (Fig. 2A) and the proton transport (data not shown) were observed after the onset of chilling treatment, although the detailed mechanisms have not been clarified. At present, it is uncertain whether or not the changes in H^+-PPase are related to the adaptation process of cells.

In suspension cultured cells of mung bean, the cold-induced inactivation of tonoplast H^+-ATPase was reversible after 24 h of chilling but became irreversible after prolonged chilling (data not shown). Upon warming of the cultured cells following chilling for 24 h, both ATP-hydrolyzing activity and ATP-proton pumping activity returned quickly to the normal level within 60 min. The recovery of the cold-inactivated H^+-ATPase was markedly inhibited by the presence of azide (5.8 mM). The presence of 576 μM of cycloheximide, sufficiently enough to inhibit protein synthesis (S Yoshida, unpublished data), had little effect on recovery. These results suggest an involvement of an energy-dependent process that had no requirement of *de novo* protein synthesis. From these observations, one could postulate that the cold-induced inactivation of the H^+-ATPase may be due to a structural alteration in the enzyme complex, including a modification of the intermolecular association of the subunits.

Recently, tonoplast H^+-ATPase has been solubilized and purified from mung bean hypocotyls in our laboratory (9). The enzyme consists of nine subunits which include major subunits of 68 and 57 kD, a dicyclohexylcarbodiimide-binding subunit of 16 kD, and six other minor polypeptides. Thus the structure of the mung bean tonoplast H^+-ATPase resembles the structure of other vacuolar type H^+-ATPases (1, 4, 11). Except for the 68 and 57 kD polypeptides and the dicyclohexylcarbodiimide-binding subunits, nothing is known about the physiological functions of the remaining subunits. The vacuolar type H^+-ATPases from chromaffin granule membranes (10) and clathrin coated vesicles (1) are reported to be cold sensitive, especially in the presence of chaotropic anions and ATP, resulting in dissociation of the peripheral sectors that include the ATP hydrolyzing subunits.

According to Moriyama and Nelson (10), the H^+-ATPases from plant vacuolar membranes are also irreversibly cold-inactivated *in vitro* by a similar mechanism. Taking into consideration these results, one possible explanation for the *in vivo* cold inactivation of mung bean tonoplast H^+-ATPases as presented in this report seems likely. The enzyme complex, especially the ATP hydrolysis sectors, may either dissociate or, at least, attain a "loosened state" by the chilling treatment. In plant cells, a large amount of Cl^- and NO_3^- are accumulated in the vacuoles (5, 8). After cell exposure to low temperature, tonoplast membranes may become leaky to these chaotropic anions. These anions would then be released into the cytosol. Under these circumstances, the tonoplast H^+-ATPase may encountered nearly the same condition as in the *in vitro* experiments with the H^+-ATPases from chromaffin granules (10) and coated vesicles (1). However, in mung bean cells, no experimental evidence on the effects of anions on cold stability of the ATPase is available to date.

Based on the differential inhibition of catalytic activity and proton translocation of tonoplast H^+-ATPase by nitrate (15) and mercury (16), it has been suggested that ATP hydrolysis and proton transport are indirectly coupled. Accordingly, these inhibitors are suggested to interact with the protogenic domain of the enzyme. The recovery of the cold-inactivated tonoplast H^+-ATPase in mung bean cell cultures was observed to proceed in two steps, a fast recovery of the ATP-hydrolyzing activity and a slower recovery of the ATP-proton transport activity. Furthermore, the decrease of ATP-proton transport

Table II. *Effects of cycloheximide and Sodium Azide on the Recovery of Tonoplast H^+ -ATPase Activity in Vivo During Warming*

Mung bean cultured cells (8-d-old) were previously chilled at 2°C for 24 h, then incubated for 2 h at 26°C with or without addition of inhibitors. After the treatments, micromsomal fractions were prepared and used for analysis as described in "Materials and Methods." Values in parentheses are percent control. Values ± SE.

Treatment	NO_3-Sensitive ATPase Activity	ATP-Dependent Proton Transport
	Pi μol·mg^{-1} h^{-1}	*%ΔF·mg^{-1} min^{-1}*
Nonchilled	7.5 ± 0.4 (100)	64.3 ± 7.3 (100)
Chilled	2.9 ± 0.5 (39)	10.0 ± 1.2 (16)
Warmed after chilling		
No addition	6.9 ± 0.2 (92)	78.4 ± 2.7 (122)
Plus cycloheximide (576 μM)	6.0 ± 0.1 (80)	75.7 ± 1.4 (117)
Plus sodium azide (5.8 mM)	3.9 ± 0.5 (52)	24.7 ± 2.7 (38)

was faster than that of the ATP-hydrolysis activity during chilling (Fig. 2B). Taken together, these results suggest that the protogenic domain of the tonoplast H^+-ATPase was structurally altered by chilling.

When isolated mung bean tonoplast vesicles were incubated at 0°C in an appropriate buffer solution containing 250 mM sorbitol and 1 mM DTT, no detectable inactivation occurred after several hours. Furthermore, even freeze-thawing of the tonoplast suspension at −80°C did not cause any detectable loss of activity (data not shown). Based on these facts, it seems probable that the *in vivo* inactivation of the H^+-ATPase by cold treatment is closely related to cytosolic alterations that might be produced by chilling. Further detailed studies are required to specify the related cytosolic factors and to elucidate the molecular mechanisms of the cold-induced inactivation of tonoplast H^+-ATPase in chilling sensitive plant cells.

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