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Chill-Induced Changes in the Activity and Abundance of the Vacuolar Proton-Pumping Pyrophosphatase from Mung Bean Hypocotyls[1]

Catherine P. Darley, Julia M. Davies*, and Dale Sanders

Plant Laboratory, Department of Biology, University of York, P.O. Box 373, York YO1 5YW, United Kingdom

Changes in the properties of extractable vacuolar H^+-pumping pyrophosphatase (V-PPase) and vacuolar ATPase activities in chilling-sensitive seedlings of mung bean (*Vigna radiata*) were investigated. Following chilling at 4°C for 48 h, both hydrolytic and proton-pumping activities of the V-PPase increased 1.5- to 2-fold over controls and remained elevated even after 72 h at low temperatures. Vacuolar ATPase levels did not change significantly throughout the chilling regime. However a large increase in alcohol dehydrogenase activity during chilling suggests a shift toward fermentative metabolism, which can be expected to decrease ATPase activity in situ. Western blotting of vacuolar membrane-enriched fractions from control and treated plants has confirmed that the changes in V-PPase activity are mirrored by increases in the amount of pump protein. Results suggest a specific role for the V-PPase in protecting chill-sensitive plants from the injurious effects of low temperatures via the maintenance of the proton gradient across the vacuolar membrane.

The vacuole is the dominant organelle in the mature plant cell, in which it characteristically occupies more than 90% of the total intracellular volume. Correspondingly, the vacuole plays a prominent role in many important physiological processes, including metabolite storage, pH, and ionic homeostasis (Boller and Weimken, 1986; Taiz, 1992). Two distinct primary proton-translocating enzymes, V-ATPase and V-PPase, are located on the vacuolar membrane. Although each enzyme is specific in its use of respective substrate (Rea et al., 1992), both catalyze the electrogenic H^+ translocation from the cytosol to the vacuolar lumen to generate an inside-acid pH and a cytosol-negative electrical potential difference. This proton motive force provides energy for the secondary transport of various ions and metabolites across the membrane (Rea et al., 1992; Sze et al., 1992). Unlike the V-ATPase, which is ubiquitous at the non-energy-coupling membranes of eukaryotes, the V-PPase is unique to plant vacuoles. Although the V-PPase has been well characterized at both biochemical and molecular levels, its H^+-pumping role in vivo remains obscure (Rea and Poole, 1993). Is there a specific role for the V-PPase other than that of an auxiliary H^+-pump scavenging the free energy released during PPi hydrolysis?

Damage to chill-sensitive plant species caused by low, nonfreezing temperatures has traditionally been thought to result from temperature-dependent changes in the physical state of the lipid bilayers causing electrolyte leakage from the cellular compartments (Lyons, 1973). However, a number of recent studies have shown that many more, wider-ranging biochemical changes occur in advance of irreversible membrane damage. Principal among such changes is oxidative stress, which occurs within hours and is marked by the de novo synthesis of enzymes associated with fermentative metabolism (Christie et al., 1991), as well as decreases in both cytoplasmic pH (Yoshida, 1994) and the P:O ratio (Yoshida et al., 1989). Chilling has also been reported to have specific effects on the integrity of the V-ATPase (Moriyama and Nelson, 1988; Yoshida et al., 1989). In mung bean (*Vigna radiata* L.) hypocotyls irreversible decrease in specific activity of the V-ATPase (to approximately 50% of control values) has been reported to occur within 48 h of chilling at 0°C (Matsuura-Endo et al., 1992). This decrease in activity is believed to result from cold-induced dissociation of specific subunits from the enzyme complex. Thus decreases in V-ATPase activity that occur because of lack of substrate following the onset of fermentative metabolism may be augmented by enzyme dysfunction.

Unlike ATP levels, cellular PPi concentrations remain stable during marked changes in respiratory states (Weiner et al., 1987; Dancer and ap Rees, 1989). Together with the likely reduction in V-ATPase activity this raises the possibility that the V-PPase may assume increased importance under low-temperature stress, both by combating the tendency for cytoplasmic acidosis and by maintaining a proton motive force across the vacuolar membrane to drive secondary transport of solutes.

To test this hypothesis seedlings of the mung bean were incubated at 4°C for up to 72 h. Cold-induced changes in V-PPase and V-ATPase hydrolytic and proton-pumping activities were examined following isolation of vacuolar vesicles. Using antibodies raised against each enzyme, we also investigated the specific amounts of pump protein.

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* Corresponding author; e-mail jmd3@unix.york.ac.uk; fax 44–1904–432860.

Abbreviations: ADH, alcohol dehydrogenase; ΔpH, pH gradient across the vacuolar membrane; TBS, Tris-buffered saline (10 mM Tris, 0.15 M NaCl); V-ATPase, vacuolar H^+-ATPase; V-PPase, vacuolar H^+-pumping pyrophosphatase.

ADH activity was monitored throughout the chilling regime as a marker for the onset of fermentative metabolism.

MATERIALS AND METHODS

Plant Material

Seeds of mung bean (*Vigna radiata* L. Wilczek) were sown directly into moist vermiculite and grown in the dark at 22°C for 7 d. Chill-treated plants were then generated by transfer to 4°C and incubation in the dark for up to 72 h. Seven-day-old seedlings without chilling treatment were used as control plants. Both control and chill-treated plants were watered daily. Hypocotyls approximately 8 cm in length were excised from seedlings and used for the preparation of vacuolar membranes and ADH extraction.

Growth Relations

Fifty intact seedlings were taken from chill-treated and control plants at 24-h intervals throughout the growth period. Dry weight was measured and is reported for each batch of 50 plants.

Isolation of Vacuolar Membrane-Enriched Vesicles from Mung Bean

Vacuolar membrane-enriched vesicles were prepared by a combination of the methods of Maeshima and Yoshida (1989) and Rea and Poole (1985).

Etiolated hypocotyls from control or chilled plants were harvested with a razor blade and washed in ice-cold distilled water. For each membrane preparation 250 to 350 g fresh weight of tissue were used and homogenized in 1.33 mL/g homogenization medium using a domestic food liquidizer. Homogenization medium consisted of 10% (v/v) glycerol, 5 mM Tris-EGTA, 1.5% (w/v) PVP-40, and 1% (w/v) ascorbic acid, buffered to pH 7.6 with solid Tris.

The homogenate was then filtered through two and then four layers of muslin and centrifuged at 3,600*g* (Sorvall SS-34 rotor) for 10 min at 4°C. The supernatant was then decanted and centrifuged at 100,000*g* (Beckman type-35 rotor) for 30 min at 4°C. The resultant pellet was resuspended in suspension medium (10% [v/v] glycerol, 1 mM Tris-EGTA, and 2 mM DTT, buffered to pH 7.6 with solid Mes) and dispersed using a Dounce homogenizer. One milliliter of the suspension was layered onto a 10/23% (w/v) Suc step gradient prepared in suspension medium and then centrifuged at 100,000*g* (Beckman SW 41 rotor) for 2 h at 4°C. Vacuolar membrane-enriched vesicles were carefully removed from the 10/23% interface, pelleted at 100,000*g* (Beckman Ti60 rotor) for 30 min at 4°C and then resuspended in 1.6 mL of suspension medium. If not used immediately, 200-μL aliquots of the membrane preparation were frozen in liquid nitrogen and stored at −80°C.

Protein Estimation

Protein concentration was determined using an assay kit from Bio-Rad, based on a modification of the dye-binding method of Bradford (1976) using bovine γ-globulin as the standard. For protein samples containing added detergent (western blotting), a BCA protein assay kit was used (Pierce), and BSA was used as a standard in this assay.

V-PPase and V-ATPase Hydrolytic Activity

V-PPase and V-ATPase hydrolytic activities were measured as the liberation of free Pi from ATP or PPi estimated by the method of Ames (1966).

For V-PPase hydrolysis, the reaction mixture contained 50 mM KCl, 1.3 mM $MgSO_4$, 5 μM gramicidin D, 1 mM sodium molybdate, and 300 μM Tris-PPi buffered to pH 8.0 with 30 mM Tris-Mes. Tonoplast protein (10–15 μg) was made up to a final volume of 100 μL with water, added to 200-μL aliquots of reaction mixture, and incubated for 20 min at 37°C. The reaction was stopped by the addition of 0.9 mL of Ames reagent (6 parts 0.4% [w/v] ammonium molybdate in 0.5 M H_2SO_4 to 1 part 10% [w/v] ascorbic acid) and allowed to stand for 30 min at room temperature. A_{820} was measured in 3-mL cuvettes (Shimadzu spectrophotometer) against a reagent blank. To compare V-PPase and V-ATPase activities on a mole/mole basis, V-PPase activity was calculated as one-half the rate of Pi liberation (= nmol PPi consumed per unit time).

V-ATPase activity was estimated as described above but in the presence of 3 mM Tris-ATP and 3 mM $MgSO_4$ as substrates. Reaction mixtures including an inhibitor of mitochondrial ATPase (3 mM sodium azide), plasma membrane H^+-ATPase (0.25 mM sodium vanadate), or V-ATPase (100 nM bafilomycin A_1) were used to determine specific V-ATPase activity and the degree of contamination from nonvacuolar membranes. Routinely, V-ATPase activity was calculated as the bafilomycin-sensitive fraction from total ATPase activity (Bowman et al., 1988).

Sodium salts of ATP and PPi were converted to Tris salts by cation exchange with Dowex 50W-H^+ followed by titration to pH 7.6 with a concentrated Tris solution.

Measurement of PPi- and ATP-Dependent H^+ Translocation

PPi- and ATP-dependent proton pumping were measured fluorimetrically using a Perkin-Elmer Cetus LS-5 dual-wavelength fluorescence spectrophotometer. Intravesicular acidification was monitored by following the fluorescence quench of the amine dye acridine orange using the method of Rea and Turner (1990). This technique makes use of the property of fluorescent monoamines (i.e. acridine orange) to accumulate intravesicularly in response to an inside-acid pH gradient resulting in a concentration-dependent decrease in fluorescence signal through dye stacking.

Excitation and emission wavelengths were set at 495 and 540 nm, respectively, with a slit width of 5 nm. Membrane protein (88 μg) was preincubated for 5 min at room temperature in 3 mL of fluorescence buffer (100 mM KCl, 1 mM Tris-EGTA, 10% [v/v] glycerol, 2.5 μM acridine orange, 300 μM Tris-PPi, or 3 mM Tris-ATP, buffered to pH 7.0 with 50 mM Tris-Mes) prior to the initiation of proton pumping by

the addition of 1.3 mM $MgSO_4$ (PPi-dependent assays) or 3 mM $MgSO_4$ (ATP-dependent assays).

After the ΔpH had reached steady-state, the rate of passive H^+ leakage was estimated through relaxation of the fluorescence quench on addition of 3 mM Na_4-EDTA to chelate all Mg^{2+}. Any remaining ΔpH was dissipated by subsequent addition of 5 μM gramicidin-D. Initial rates were calculated as the zero-time derivative of an exponential function fitted to the data using a nonlinear least squares method (Marquardt, 1963; Jennings et al., 1988).

Western Blotting

Vacuolar membrane-enriched vesicle preparations containing approximately 3 mg of protein were centrifuged at 100,000*g* (Sorvall Ti60 rotor). The resultant pellet was then resuspended in 5% (w/v) SDS buffered to pH 7.5 with 50 mM Tris-Mes to a final concentration of approximately 100 μg of protein in 15 μL. The exact protein concentration of this suspension was then determined after the sample was diluted 1:10 and 1:20 with water. Interference of the assay by SDS was assessed using BSA standards with or without 0.5% SDS.

The solubilized protein samples were diluted in SDS reducing buffer containing 20 μL of mercaptoethanol in 400 μL of denaturation buffer (10% [v/v] glycerol, 10% [w/v] SDS, 0.1% [w/v] bromphenol blue buffered to pH 6.8 with 0.5 M Tris-HCl) such that 10 μL contained exactly 20 μg of protein. The sample was then denatured at 80°C for 3 min.

Proteins were resolved by SDS-PAGE on 10% gels at a constant 200 V using a Bio-Rad minigel system. Proteins were transferred to nitrocellulose (Schleicher & Schuell, 0.45-μm pore size) in a Bio-Rad transblot system with Tris/Gly/methanol as transfer buffer under standard conditions of 250 mA and for 75 min. Nonspecific binding sites on the nitrocellulose blots were blocked by shaking at room temperature with 5% (w/v) Marvel dried skim milk powder (Premier Beverages, Stafford, UK) in TBS containing 0.2% (v/v) Tween 20, for 1 h. Blots were then incubated overnight (18 h) with antibodies raised against a synthetic oligopeptide corresponding to the putative cytosolic loop of the V-PPase or the 67-kD subunit of the V-ATPase from *Beta vulgaris*, diluted 1:1,000 or 1:800, respectively, in 1% Marvel TBS-Tween. The blots were subsequently washed three times in TBS-Tween and incubated with anti-rabbit IgG/peroxidase conjugate (Sigma) diluted 1:10,000 in 1% Marvel TBS-Tween and stained for peroxidase activity using the ECL light detection assay (Amersham). Levels of antigen on the nitrocellulose filter were quantified by densiometric scanning. Control blots were performed using preimmune serum and secondary antibody only.

ADH Assay

Enzyme extraction from the hypocotyls of chill-treated and control plants was performed using a modified method of Rivoal et al. (1991) and Hwang and Van-Toai (1991). Hypocotyls were harvested as described previously and ground in a mortar and pestle in 50 mM Na_3PO_4, 1 mM PMSF (5 mL/g fresh weight of tissue), and 0.3 g/mL insoluble PVP. The brei was centrifuged at 1200*g* for 20 min. The supernatant was then decanted and protein content was estimated using the Pierce BCA method. The remaining sample was used immediately for the ADH assay. Recovery of ADH activity following extraction was verified in triplicate samples via the addition of purified ADH enzyme (Sigma) at the start of the extraction process.

ADH activity was measured spectrophotometrically in the ethanol→acetaldehyde direction by recording ethanol-dependent NAD reduction at 340 nm. The assay mixture contained 100 mM Tris-HCl (pH 8.0), 0.1 mM ethanol, 5 mM KCN, and 0.1 mM NAD. For mung bean samples aliquots equivalent to 50 μg of protein were used to start the reaction. Samples (5 ng) of pure ADH were used as positive controls. The activity of the samples was then measured against a standard curve constructed with a series of NADH concentrations.

RESULTS

Effect of Chill Stress on Growth

Growth curves for untreated plants and plants subjected to 3 d at 4°C (from d 7) and then returned to 22°C are shown in Figure 1. Exposure to the lower temperature effectively prevented growth of the mung bean. However, the effects of exposure did not result in permanent damage, since growth resumed after the plant was returned to 22°C.

Further biochemical analysis was carried out on 7-d-old plants subjected to variable lengths of time at 4°C. Controls were 7-d-old plants not subjected to the 4°C treatment; since growth was shown to have been arrested by exposure to the low temperature, all plants were judged to be at equivalent developmental stages with respect to growth physiology.

Chill-Induced Changes in Hydrolytic Activity

Changes in V-ATPase- and V-PPase-extractable hydrolytic activities from the 10/23% interface of the Suc gradi-

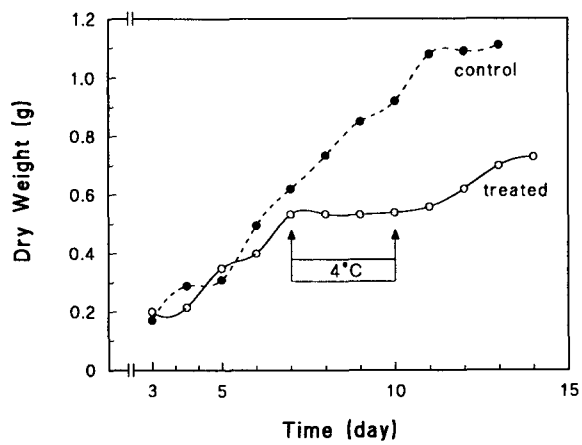

Figure 1. Effects of low temperature on the growth characteristics of mung bean. Growth curves are shown for plants treated at 4°C (○) and control plants (●) and were based on the total dry weight (g) of 50 plants harvested every 24 h. Arrows indicates the duration of the period at 4°C.

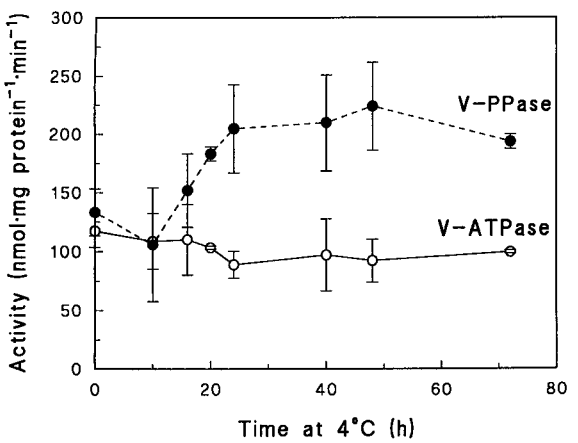

Figure 2. Comparative hydrolytic activities of the V-PPase (●) and the V-ATPase (○) from mung bean hypocotyls throughout a 72-h chilling regime. Activities were measured and calculated as described in "Materials and Methods." Results are the means ± SE of three separate preparations at each time point.

ent were assessed as a function of time spent at 4°C (Fig. 2). The percentage of recovery of the V-PPase from the supernatant of the initial low-speed spin was 13.6% and that of the V-ATPase was 4.3%: In neither case were these values affected by the chilling regime.

The activity of the V-ATPase showed no significant decline during the chilling regime. Indeed after 72 h of exposure the V-ATPase activity was approximately 90% of the control value. This insensitivity of the V-ATPase contrasts with the findings of Matsuura-Endo et al. (1992), and a possible explanation for the discrepancy is advanced in "Discussion."

In comparison, the activity of the V-PPase increased markedly as a function of the chilling period. After a lag period of 12 h, V-PPase increased approximately 1.5-fold over nonchilled control values, reaching a peak after 48 h and maintaining elevated levels for 72 h following incubation at 4°C.

Changes in H^+ Pumping

To investigate whether this differential increase in the hydrolytic activity of the V-PPase was accompanied by an increase in H^+ translocation, V-ATPase- and V-PPase-dependent proton pumping was monitored fluorimetrically as described in "Materials and Methods." Figure 3, A and B, shows the typical rates and extents of PPi- and ATP-dependent fluorescence quenches established by vacuolar membrane-enriched vesicles isolated from control, 48-, and 72-h chill-treated plants. Figure 3C represents the corresponding mean initial rates of quench from treated and control plants.

In control plants both with respect to the initial rate and the overall extent of fluorescence quench, the V-PPase generated approximately 30% greater quench than that for the V-ATPase. This greater transport efficacy of the V-PPase has been reported previously (Britten, 1992). After 48 and 72 h at 4°C, the rate and extent of ATP-dependent H^+ translocation remained constant. By contrast the initial rate of quench generated by the V-PPase increased 2-fold over control values (0.19% fluorescence $min^{-1}\mu g^{-1}$ protein for control plants; 0.34% fluorescence $min^{-1}\mu g^{-1}$ protein for 72-h chill-treated plants; Fig. 3C). This was reflected in a similar increase in the magnitude of steady-state PPi-dependent fluorescence quench from chill-treated plants (Fig. 3A).

It is possible that in vivo chilling increases the leakiness of the membrane to ions and that the measured increase in PPi-driven H^+ translocation is simply a reflection of the lack of capacity of the vesicle membrane to sustain a ΔpH

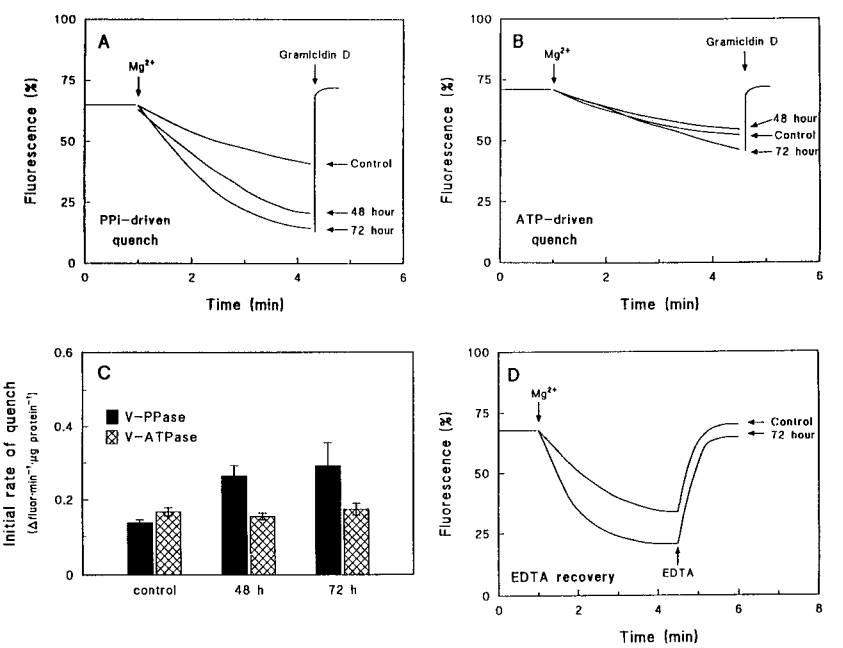

Figure 3. H^+ pumping in vacuolar membrane-enriched vesicles from control and chill-stressed mung beans. H^+ pump-dependent quench of acridine orange fluorescence was initiated by the addition of 1.3 mM $MgSO_4$ for the V-PPase with 0.3 mM Tris-PPi in the assay medium or 3 mM $MgSO_4$ for ATPase with 3 mM Tris-ATP in the assay medium. All assays contained 88 μg of protein in 3 mL of reaction volume. Typical traces of PPi-driven (A) and ATP-driven (B) quench from control and 48- and 72-h chill-treated plants. Gramicidin (5 μM) was used to recover quench. C, Comparative initial rates of ATP-driven and PPi-driven quench from treated and control plants. Results represent change in fluorescence (Δfluor) μg^{-1} protein min^{-1} (±SE) from three separate experiments for each time point. D, Comparative recovery of quench from control and 72-h chill-treated plants following the addition of 3 mM EDTA.

rather than changes in V-PPase per se. To assess passive H^+ permeability, 3 mM Na_4EDTA was added to the reaction following establishment of steady-state ΔpH to chelate free Mg^{2+} and inactivate the H^+ pump. Any change in the rate of recovery of fluorescence quench between vesicles derived from control or treated plants would indicate changes in membrane permeability.

However, the EDTA-dependent rates of recovery showed no difference between control and treated samples (Fig. 3D). This evidence, in conjunction with differential effects of low temperature on pump hydrolytic activities, indicates that the hydrolytic and transport activities of the two enzymes are tightly coupled and that low temperature has no discernible effect on the passive H^+ leakiness of vacuolar membranes. The present results, therefore, confirm the findings from hydrolytic assays by demonstrating a 1.5- to 2.0-fold increase in V-PPase activity during the first 48 h of chilling.

Abundance of the V-PPase and V-ATPase

To investigate whether the observed increases in hydrolysis and H^+-pumping functions of the V-PPase were due to increases in the turnover number of V-PPase or genuine differences in its abundance, the vacuolar membrane-enriched fractions from chilled and control plants were probed with polyclonal antibodies raised against the V-PPase and the 65-kD subunit of the V-ATPase. A constant amount (10 μg of V-PPase; 20 μg of V-ATPase) of vacuolar protein was loaded onto a gel. Chilling-dependent differences in signal after immmunoblotting were quantified via densiometric scanning, on the basis of signal per microgram of protein loaded. Figure 4 displays typical results from a series of experiments. Increases observed in both hydrolytic and proton-pumping assays were reflected in a clear and visually striking increase in signal of the V-PPase

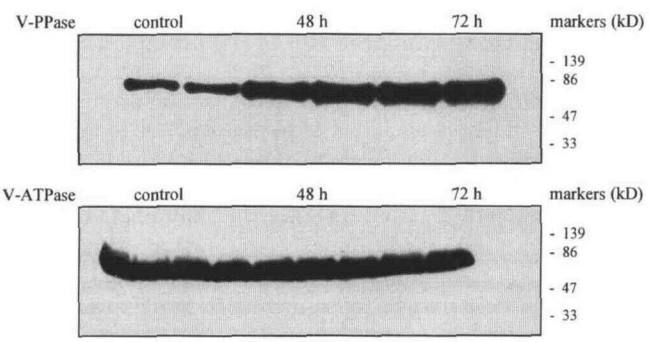

Figure 4. Western blots probed with anti-V-PPase (top) or anti-V-ATPase (bottom) from control and 48- and 72-h chill-treated plants. Vacuolar protein (10 μg of V-PPase; 20 μg of V-ATPase) was loaded in each lane and separated by SDS-PAGE as described in "Materials and Methods." Electrophoretic transfer to nitrocellulose was followed by probing with antiserum to an oligopeptide representing a putative cytoplasmic loop of the mung bean V-PPase (two replicate lanes per time point) or the 67-kD subunit of the V-ATPase (three replicate lanes per time point) before incubation with the peroxide-labeled anti-rabbit IgG. Control blots (not shown) were carried out with preimmune serum and secondary antibody only, and no cross-reactivity was observed. Molecular mass of markers is given in kD.

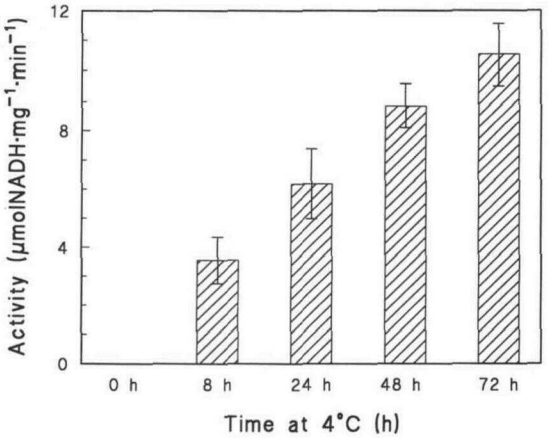

Figure 5. Extractable ADH activity from mung bean hypocotyls subjected to chilling in vivo at 4°C. Activity was measured as reduction of NAD mg^{-1} protein min^{-1}. Results represent the means ± SE of three experiments.

protein following 48 and 72 h of chilling in vivo, because V-PPase signal increased approximately 2-fold over control values after 48 h and remained elevated even after 72 h. The specific content of the V-ATPase did not change during the chilling regime. Linearity of the amount of protein on the gel to the intensity of signal was verified by loading a series of protein standards onto the gel and measuring the signal after blotting. The amount of protein loaded was found to be directly proportional to the signal (data not shown). Therefore, increases in signal must be due to corresponding increases in protein.

ADH Activity

To determine whether the changes in V-PPase level were reflected in changes in the activity of other enzymes that have been previously shown to respond to stress in vivo, a simple assay for ADH was developed. ADH was chosen because detection of changes in its activity can be used as a marker to indicate shifts toward fermentative metabolism. Indication of the metabolic state of the cell is important in evaluating possible stimuli for the induction of enhanced V-PPase expression following chilling.

ADH activity was not detected in control plants (detection limit, 5 μmol of NADH). However, following 8 h of incubation at 4°C, activity was detected that continued to increase as a function of time spent at 4°C (Fig. 5). Given the involvement of ADH in anaerobic respiration, these results suggest that there is a shift toward fermentative metabolism and concomitant changes in the cytoplasmic environment as discussed previously

DISCUSSION

The present work has focused on the effects of low nonfreezing temperature on the specific activity and specific content of the V-PPase and V-ATPase. It has been shown that both the extractable hydrolytic and proton-pumping activities of the V-PPase from mung bean hypocotyls increased by 1.5- to 2-fold in response to chilling at

Table I. *Summary of chill-induced changes in V-PPase and V-ATPase activity and abundance*

All data represent the means ± SE from three separate membrane preparations. Δfluor, Change in fluorescence.

Time at 4°C	Extractable Hydrolytic Activity		Proton-Pumping Activity		Immunoexpression	
	V-ATPase	V-PPase	V-ATPase	V-PPase	V-ATPase	V-PPase
h	*nmol mg^{-1} protein min^{-1}*		*initial rate of quench Δfluor μg^{-1} protein min^{-1}*		*signal/μg protein loaded*	
0	118.2 ± 13.6	144.8 ± 19.9	0.16 ± 0.01	0.19 ± 0.01	0.205 ± 0.059	0.185 ± 0.068
48	95.7 ± 18.2	223.3 ± 37.5	0.11 ± 0.04	0.48 ± 0.01	0.215 ± 0.012	0.320 ± 0.098
72	99.4 ± 15.6	193.2 ± 6.25	0.13 ± 0.03	0.38 ± 0.02	0.208 ± 0.024	0.305 ± 0.045

4°C. Moreover, this increase in activity was reflected by a corresponding increment in signal following western blotting. Thus it appears that chilling induces the expression and hence the abundance of the V-PPase, resulting in the observed changes in activity. These changes in V-PPase level are all the more remarkable given the failure of extractable ATPase activity or level to respond to low-temperature treatment. The results of the present study are summarized in Table I.

Although these results firmly indicate that the V-PPase responds to low temperatures in vivo, its actual role in protecting cell function can only be speculated. For example, when assayed in vitro at 4°C the activity of the V-PPase is only 10 to 15% of that of control values from mung beans grown at 24°C (Yoshida and Matsuura-Endo, 1991). Therefore, a 2-fold restorative increase in the amount of the enzyme may not appear to be of great physiological significance. However, in nature plants are often subjected to transient low temperatures, and it might be suggested that the up-regulation of the V-PPase may assist recovery of cytoplasmic homeostasis. In addition the differential in activities of the V-PPase and ATPase from chilled plants measured at 37°C (Fig. 1; see "Materials and Methods") is sustained or even increased when activities are measured at 4°C (data not shown), suggesting that the V-PPase continues to play a role in maintaining the energization of transport at the vacuolar membrane at low temperatures.

It has previously been reported that the activity of the V-ATPase decreases to 47% of control values in response to chilling in vivo at 0°C for 48 h (Matsuura-Endo et al., 1992). However, in the present study V-ATPase activity appeared to be unaffected by the chill treatment at 4°C. The insensitivity of the V-ATPase may be due to the different growth conditions used throughout these investigations. For example, as assessed by regrowth when plants were returned to 22°C (Fig. 1), we observed that even after 3 d at 4°C permanent damage to the plant did not occur. The more severe chill treatment (0°C) used by Matsuura-Endo et al. (1992) resulted in unrecoverable damage after 3 d of exposure. These differences in growth characteristics are likely to be reflected in different intracellular changes during chilling; hence, subunit dissociation of the V-ATPase may be negligible, membrane disruption could be less profound, and signaling mechanisms may be stimulated, serving to protect the plant at nonfreezing temperatures.

A shift toward fermentative metabolism was indicated by the increases in ADH activity in chill-treated tissue. Cytosolic changes resulting from the onset of fermentative metabolism have been defined by a number of workers (Sachs et al., 1990; Yoshida, 1994), and it is important to consider the manner by which such changes might elicit a differential response by the vacuolar H^+ pumps and give insights into their respective physiological roles.

In response to chilling or anaerobiosis a decrease in cytosolic pH of between 0.4 and 0.8 pH unit is characteristically observed (Roberts et al., 1984; Yoshida, 1994), largely as a result of lactic acid production. This pH decrease is then believed to stimulate ethanolic fermentation, preventing further cell acidosis (Rivoal et al., 1991). Since oxidative phosphorylation can no longer continue, cytosolic ATP levels are rapidly reduced. Thus, although the present study showed that V-ATPase activity is unaffected by chilling, its actual function as a H^+ pump in vivo is likely to be diminished as a consequence of this decrease in substrate supply. On the other hand, PPi levels do not decline significantly even during sustained inhibition of respiration (Weiner et al., 1987), and, therefore, substrate availability is unlikely to constrain V-PPase function. Furthermore it has been calculated that the potential for PPi to substitute for ATP as a phosphoryl donor is favored by low pH, which would predominate as a result of fermentative metabolism (Davies et al., 1993). Therefore, given the likelihood that cold treatment induces a shift toward fermentative metabolism, there appear to be considerable energetic advantages to the V-PPase acting as the predominant H^+ pump in response to low temperature. It is tempting to speculate that similar mechanisms (e.g. lowered cytosolic pH), which have been shown to trigger the induction of the enzymes of fermentative metabolism, may also stimulate the observed increase in the expression of the V-PPase.

The data presented in this study provide strong evidence that the V-PPase may be instrumental in protecting against chilling damage by sustaining energization of the vacuolar membrane. Furthermore, given the similarity of the intracellular changes that occur following chilling and anaerobiosis, the V-PPase may also safeguard the plant from the injurious effects of oxidative stress, in general. The present work has not examined whether transcript levels increase in mung bean after chilling; however, work published during the preparation of this paper showed a 10-fold increase in V-PPase transcript in rice following chilling or anoxia (Carystinos et al., 1995). Thus, accumulating evidence suggests that the V-PPase may represent a crucial link between metabolism and ion transport.

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