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Low Temperature-Induced Cytoplasmic Acidosis in Cultured Mung Bean (*Vigna radiata* [L.] Wilczek) Cells[1]

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Cold-induced changes in vivo in the cytoplasmic pH of suspension-cultured cells of mung bean (*Vigna radiata* [L.] Wilczek) were investigated by fluorescence-ratio imaging cryomicroscopy with special reference to the variations in the chilling sensitivity of cells during the growth cycle. Because of the preferential localization of the fluorophore in the cytoplasm under specified conditions and the ideal response of fluorescence to pH, fluorescein diacetate allows measurements to be made of temporal changes in cytoplasmic pH at low temperature. A remarkable difference was demonstrated in the cold-induced changes in cytoplasmic pH between cells at the early and late stages of exponential growth. The cells at the early stage of exponential growth were most sensitive to chilling, and the cytoplasmic pH decreased dramatically within a short period of incubation at 0°C, decreasing from 7.4 to 6.8 after 4 h and to 6.3 after 18 h. The cells at the late stage of exponential growth were chilling tolerant, and no significant decrease in the cytoplasmic pH was observed during the incubation at 0°C for 24 h or even longer. From the results presented here, it appears that cold-induced cytoplasmic acidosis is characteristic of chilling-sensitive mung bean suspension-cultured cells.

Plants face a number of pH-perturbing processes associated with their metabolism, the transport of solutes (Raven, 1985a, 1985b, 1988), and the effect of various environmental factors (Kurkdjian and Guern, 1989, and refs. therein). Because many vital biochemical reactions occur at narrowly defined pH optima, generally at a weak alkaline pH, cells must have the regulatory systems to maintain the homeostasis of the intracellular pH. As discussed by Smith and Raven (1979), control of the intracellular pH is achieved by a combination of membrane transport between intracellular compartments and between the cell and its surroundings. Alterations in the homeostasis of the intracellular pH in response to environmental stress, such as anaerobiosis, hypoxia, salt stress, and light-to-dark transitions, have been reported in some plant species (Roberts et al., 1984; Katsuhara et al., 1989; Kuchitsu et al., 1989; refs. in Kurkdjian and Guern, 1989; Saint-Ges et al., 1991).

It has long been thought that a physical disturbance in cellular compartments containing ions and metabolites, which is caused by temperature-dependent changes in the physical state of lipid bilayers, may be a cause of chilling injury in chilling-sensitive plants (Lyons, 1973). Even though this may be the case, no information has been available regarding the rapidity and the extent of the cellular disturbance that may be brought about in cells by exposure to a chilling environment. Although the role of temperature in controlling pH-dependent phenomena in living cells has already been investigated for heterothermic animals (Roos and Boron, 1981), the response of intracellular pH to temperature variations in plant cells is still uncertain.

Vacuoles in higher plants accumulate large amounts of salts and metabolites, sustain turgor pressure, and are thought to maintain the homeostasis of the cytoplasmic environment (Sze, 1985; Rea and Sanders, 1987). Two distinct proton-translocating enzymes, namely H^+-ATPase and proton-translocating inorganic pyrophosphatase, are located on the tonoplast membranes and acidify the vacuolar lumen. The inside-acidic pH gradient provides an energy source for the secondary transport of various ions and metabolites across the vacuolar membrane. In etiolated young seedlings of mung bean (*Vigna radiata* [L.] Wilczek), which are highly susceptible to chilling (Etani and Yoshida, 1987), vacuoles are thought to be among the main cellular components that respond to chilling stress (Yoshida et al., 1989). The activities of both types of vacuolar proton pumps are markedly depressed in vitro upon lowering of the temperature to less than 10°C as a result of the high-temperature dependency of the enzymes (Yoshida and Endo-Matsuura, 1991). Among various membrane-associated enzymes in the hypocotyls, the vacuolar H^+-ATPase is the most sensitive to cold and may be preferentially inactivated during chilling in vivo at 0°C (Yoshida et al., 1989; Matsuura-Endo et al., 1992). Such cold inactivation occurs far earlier than the appearance of injury to cells and the general decreases in the activities of enzymes associated with plasma membranes, the ER, and mitochondria. Thus, damage to the tonoplast proton-transport system is one of the primary cellular reactions that is directly induced upon exposure to low temperature.

As reported previously (Yoshida et al., 1993), chilling sensitivity in suspension-cultured cells of mung bean changes markedly during the growth cycle, and this is closely associated with changes in the cold stability of the tonoplast H^+-ATPase: both cells and the enzyme are most sensitive to cold at the early stage of exponential growth. Given the important role of the vacuolar proton pumps in maintaining homeosta-

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Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; FDA, fluorescein diacetate.

sis of the cytoplasmic environment, the cold-induced depression and/or inactivation of the vacuolar proton-translocating enzymes, and of H^+-ATPase in particular, would be expected to cause a change in the cytoplasmic environment, most notably in the pH.

To test this hypothesis, the effects of chilling stress on the cytoplasmic pH were investigated in suspension-cultured cells of mung bean. Fluorescence-ratio imaging cryomicroscopy was used to measure cold-induced alterations in the cytoplasmic pH with FDA as the fluorescent indicator of pH. The results indicate that the cold-induced acidosis of the cytoplasm is a characteristic of these cells at the most chilling-sensitive phase of growth.

MATERIALS AND METHODS

Plant Materials

Suspension-cultured cells of mung bean (*Vigna radiata* [L.] Wilczek), derived from young roots as described previously (Yoshida, 1991), were used in the present study. The culture medium contained Murashige and Skoog major and minor inorganic nutrients and vitamins, with the exception that the concentration of thiamine was increased 10-fold, plus 3% Suc, 0.5% Glc, 0.1 mg/L 2,4-D, and 0.05 mg/L kinetin. Stock cultures were grown in 50 mL of liquid medium at 26°C in 200-mL Erlenmeyer flasks on a reciprocating shaker (90 cycles/min) under continuous illumination with fluorescent light. Stock cultures were maintained by subculturing at 12-d intervals. For every subculture, a 2.0-mL aliquot of the 12-d-old suspension of cells was introduced into 50 mL of fresh culture medium.

Isolation of Protoplasts

Cells were collected by filtration on Miracloth and suspended in cell wall-digesting solution, as described below. After the digestion of cell walls at 26°C for 2.0 to 2.5 h, the released protoplasts were filtered through a sheet of nylon mesh with 40-μm pores and pelleted by centrifugation at 400*g* for 10 min. Protoplasts were washed three times with isotonic suspension solution. The cell wall-digesting solution consisted of 3% cellulase Onozuka R-10 (Yakult Pharmaceutical Co., Ltd., Tokyo, Japan), 1% macerozyme R-10 (Yakult Pharmaceutical Co.), 0.1% pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo, Japan), 25 mM Glc, 0.5% (w/v) BSA, 2 mM Mes, the major inorganic nutrients of Murashige and Skoog medium at 50% of the normal concentration, and either 500 or 350 mM of sorbitol for cells at the early or the late stage of exponential growth, respectively. The pH was adjusted to 5.7 with a solution of KOH. Enzymes were desalted by gel filtration on a column of Sephadex G-25 (medium; Sigma) prior to the preparation of the enzyme solution. The protoplast-suspension solution consisted of 500 or 350 mM sorbitol for cells at the early or late stage of exponential growth, respectively, 25 mM Glc, 0.2% (w/v) BSA, and Murashige and Skoog major inorganic nutrients at 50% of the normal concentration. The pH was adjusted to 6.8 with a solution of KOH. The protoplasts were viable at room temperature for at least 24 h in the suspension solution.

Incubation of Protoplasts at Low Temperature and Loading of Fluorescein

A 1-mL aliquot of the suspension of protoplasts was put into a test tube (10 mm i.d. × 100 mm) and incubated at 0°C with occasional shaking. After cold incubation for various periods, protoplasts were pelleted by centrifugation and incubated at 0°C for 30 min with a 10-μM solution of FDA made up in isotonic suspension solution. Then protoplasts were washed with the suspension solution without added fluorophore.

The fluorescein-loaded protoplasts were mounted on the cold stage of a fluorescence cryomicroscope, the temperature of specimens was maintained at 2 ± 0.2°C, and the cytoplasmic pH was measured by fluorescence-ratio imaging as described below. Cold treatment and the measurement of pH of intact cells were also carried out in the same way as in the case of protoplasts, with the exception that cells were loaded with fluorescein and suspended in the same culture medium as that in which the cells had been growing.

Cryomicroscopy System

The cryomicroscopy system was designed as described by Steponkus et al. (1984) and is shown schematically in Figure 1. The system consisted of an inverted microscope (Diaphoto TMD; Nikon, Tokyo, Japan), a cold chamber, a system for the supply of vaporized liquid nitrogen gas with a flow-rate controller, and a heat exchanger for a dry nitrogen gas curtain, a digital multithermometer (TR 2114; Takeda Riken Kogyou, Tokyo, Japan), and a programmable temperature-controller (EC1003; Ohkura Electric Ltd., Tokyo, Japan). The cold chamber (100 mm in diameter, 29 mm in thickness; Korett Kogyou, Tokyo, Japan) was constructed as depicted schematically in Figure 2. It consisted of a basal port with a cold substage on which a transparent electrical resistance heater was attached and an upper lid with a glass window (20 mm in diameter), positioned in the center and facing a condenser. A glass coverslip (22 mm in diameter, 170 μm in thickness), the underside of which had been coated with indium trioxide (1000-Å thickness; Geomatic Ltd., Yokohama, Japan), was positioned over the circular edge of the copper cold sink of the cold substage and served as an electrical resistance heater. The glass resistance heater was connected electrically to the programmable temperature controller at two opposite edges on the lower surface that had been coated with indium trioxide. The temperature-measuring junction of a copper-constantan foil thermocouple (5 μm thick; Rdf Corp., Hudson, NH) was positioned at the center of the viewing region of the upper surface of the resistance heater. The thermocouple signal was conditioned and linearized through the digital multithermometer. The electrical current was controlled by a proportional integrated differential control system such that it was proportional to the difference in temperature between the specimen and the reference. Vaporized liquid nitrogen gas was introduced into the copper tubing of the heat exchanger in the cold substage and served as the coolant for the resistance heater. The flow rate of vaporized liquid nitrogen gas was controlled and depended on experimental conditions, namely rates of cooling and warming and the range of temperature to be used.

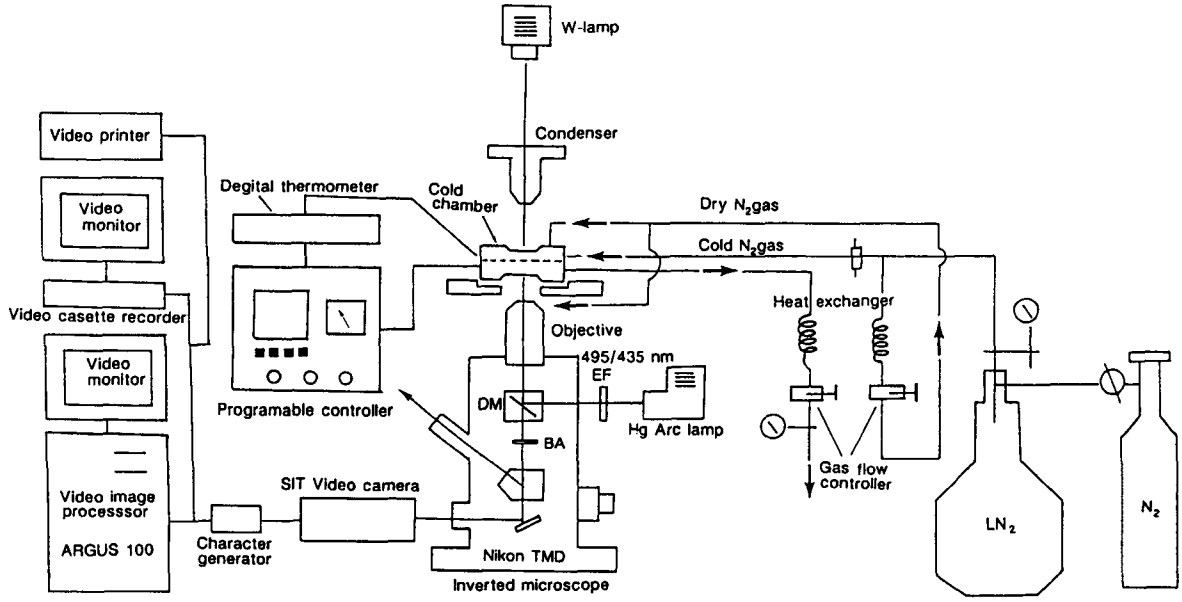

Figure 1. Schematic diagram of the cryomicroscopy system and the fluorescence-ratio imaging system. W-lamp, Tungsten lamp; DM, dichroic mirror; BA, barrier filter; EF, excitation filters; LN_2, liquid nitrogen; N_2, nitrogen gas.

Vaporized liquid nitrogen gas was subjected to heat exchange and discharged onto both the upper surface of the glass window in the upper lid of the cold chamber and onto the underside of the resistance heater to prevent condensation of dew during observations. With this system, the specimen temperature could be controlled with an accuracy of ±0.2°C over a range of temperature from −40 to +40°C.

Fluorescence-Ratio Imaging of Cellular pH

Video Image-Processing System

The video-imaging system is illustrated on the left side of Figure 1. It consisted of a silicon-intensifier target (SIT)-video camera (C2400–08H; Hamamatsu Photonics, Shizuoka, Japan), a character generator (time/temperature; UTG-33/TG-160; FOR. A, Ltd., Tokyo, Japan), video image processor (ARGUS-100/pH; Hamamatsu Photonics), a video cassette recorder (model BR-S611; Victor, Ltd., Tokyo, Japan), video monitors, and a color video printer (model VY-P1; Hitachi Co., Ltd., Tokyo, Japan).

Optical System

Fluorescein-loaded cells were mounted on the resistance heater in the cold chamber. The specimens were excited alternatively at 495 and 435 nm by a 100-W mercury arc lamp with 10-nm band-pass interference filters (Asahi Optics, Tokyo, Japan). The levels of excitation light at 495 and 435 nm were adjusted, by use of neutral density filters, such that emission ratios ranged from 1.0 to 8.0 in the pH range of interest (pH 5.5–8.0). To eliminate photo-bleaching, the total level of excitation light was minimized by use of a neutral density filter (ND32; Nikon) positioned in front of the shutter in the light path. The excitation filters, 495 and 435 nm, were placed in a two-position aluminum holder constructed in a Nikon dichroic mirror cassette with a broadband emission filter of 520 to 560 nm (Nikon). Opening of the shutter and the exchange of excitation filters were performed manually.

Image Processing and Data Analysis

Fluorescence images were focused on an SIT video camera through a zoom lens (×1.0–×2.5) and were recorded with

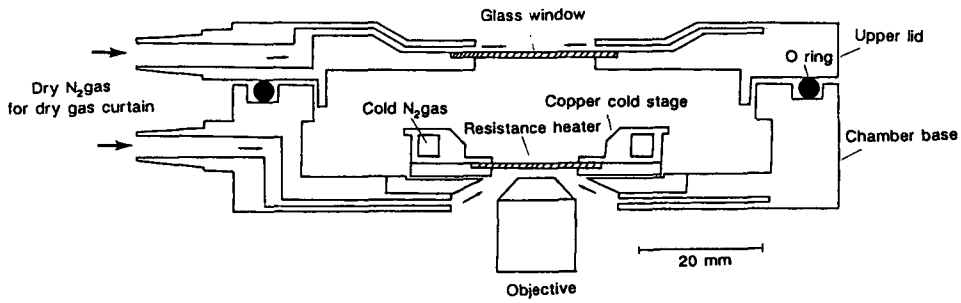

Figure 2. Schematic diagram of the cold chamber.

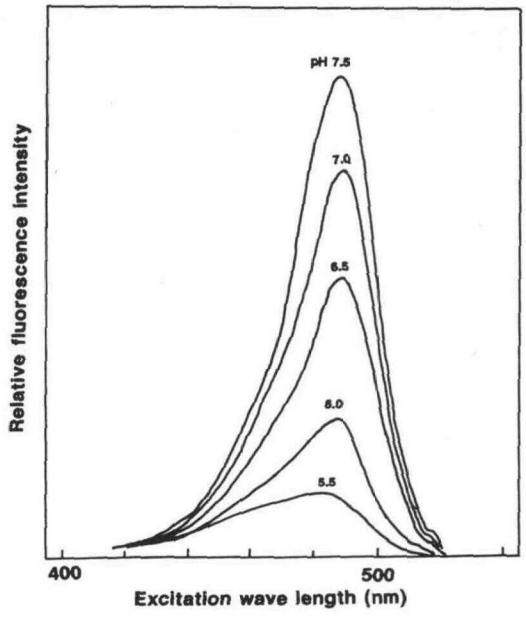

Figure 3. Excitation spectra of free fluorescein at various pH values. Free fluorescein (3 μM) was dissolved in 50 mM Mes or Hepes buffer solution, and the pH was adjusted to the indicated values by the addition of BTP. The spectra were recorded at 25°C.

the image processor that averaged 16 video frames. The "ratio image" (495/435 nm) was calculated, after subtraction of the dark signal, and displayed on a TV monitor. The ratios for a selected cell area were calculated by reference to pixel windows.

pH-Ratio Calibration in Situ and in Vitro

For pH-ratio calibration in situ, protoplasts isolated from cells at the early exponential stage of growth were incubated with a 10-μM solution of FDA made up in the protoplast-suspension solution for loading with the fluorophore. After incubation at 0°C for 30 min, protoplasts were washed twice with the suspension solution. This condition minimized permeation of fluorescein into vacuoles. The cytoplasmic pH was equilibrated to that of the external buffer solution by the partitioning effect of weak acid and weak base exchange, as reported by Heiple and Taylor (1980), with slight modification. The buffer solution for pH equilibration contained 50 mM Hepes (pH 6.6–7.8) or Mes (pH 5.5–6.6), 50 mM ammonium acetate, and 450 mM sorbitol. The pH of each buffer solution was adjusted to the desired value by the addition of BTP. The fluorescein-loaded protoplasts were mounted on the glass resistance heater in the cold chamber, and the fluorescence ratio was allowed to reach a new and stable value at 2°C. A time-course study showed that the fluorescence ratio stabilized at the new value within 20 min at 2°C. The mean ratio values were obtained by measuring more than 20 cell samples at a fixed pixel window (15 μm^2) and were analyzed statistically. The mean ratio values were plotted against pH.

For pH-ratio calibration with buffer alone, a small droplet (10 μL) of the pH-equilibration buffer containing 3 μM of free fluorescein was sandwiched between the glass resistance heater and a coverslip with a plastic spacer ring (about 50 μm thick), and the mean ratio values were determined as described above.

RESULTS

Fluorescence Properties of Free Fluorescein

Figure 3 shows changes in the excitation spectra of free fluorescein as a function of pH. The fluorophore (3 μM) was dissolved in 50 mM Hepes-BTP or Mes-BTP buffer at various pH values, and the spectra were recorded with a spectrofluorometer (Shimadzu; F5000). The maximum fluorescence intensity at an excitation wavelength of 495 nm varied with pH. When the fluorescence ratio (495/435 nm) was plotted against pH, linear and nonlinear relationships were obtained from pH 5.6 to 7.4 and above pH 7.5, respectively (Fig. 5, bottom). At a given pH, the ratio was not affected by the fluorescence intensity, provided that the fluorescence intensity did not exceed extremely low or high values.

Cellular Distribution of Free Fluorescein

Figure 4 shows a fluorescence image of protoplasts that had been isolated from cells at the early exponential stage of growth, namely 6 d after subculturing, after incubation for 6 min at 25°C with 10 μM FDA. A strong fluorescent signal was recorded from the cytoplasm-enriched portion centered around the nucleus. Vacuolar portions appeared dark against the fluorescent background of the cytoplasm. This result suggested either limited localization of the fluorophore in the cytoplasm or the quenching of the fluorescent signal by the acidic pH in the vacuolar lumen. When the vacuolar pH was brought into equilibration with that of cytoplasm by treatment of cells with nigericin (10 μg/mL) and KCl (50 mM) (Bright et al., 1987), no fluorescence was observed in the vacuoles within 10 min after loading of the dye at room temperature, indicating that the fluorophore was restricted to the cytoplasm under these conditions. However, prolonged

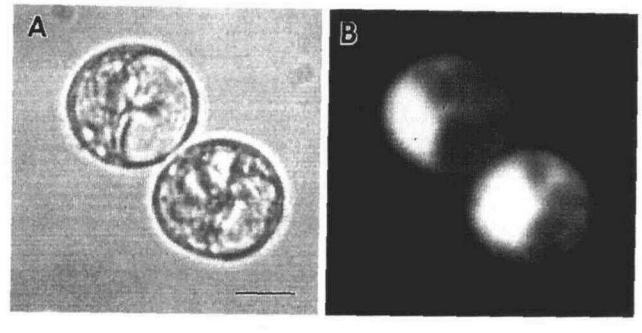

Figure 4. Cellular distribution of fluorescence after loading of cells with FDA. Protoplasts isolated from cells at the early exponential stage of growth were incubated at 25°C for 6 min in a 10-μM solution of FDA made up in isotonic suspension solution, as described in the text. A, Bright-field image; B, fluorescence image. Excitation and emission wavelengths were 495 and 520–560 nm, respectively. Bar, 10 μm.

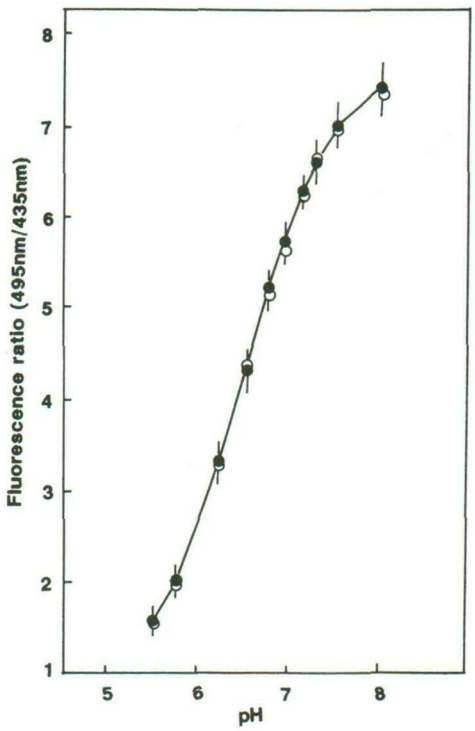

Figure 5. In situ and in vitro pH-ratio calibration curves. Before measurement of the fluorescence ratio (495/435 nm) in situ under the cryomicroscope, the fluorescein-loaded protoplasts were preincubated in pH-equilibration buffer that contained 50 mM Mes/BTP or Hepes/BTP, 50 mM ammonium acetate, and 450 mM sorbitol of various pH values. Loading of fluorescein, equilibration of pH, and measurement of the fluorescence ratio were performed at 0°C. The in vitro pH-ratio calibration curve was generated using the pH-equilibration buffers that contained 3 μM free fluorescein. Experimental details are given in the text. ○, In situ; ●, in vitro.

incubation under warm conditions resulted in the gradual penetration of the fluorophore into the vacuoles. The rate of entry of the fluorophore into the vacuoles decreased markedly at temperatures below 5°C, and no detectable entry of the fluorophore into vacuoles was observed during 2 h of incubation at 0°C. These characteristics allow use of this fluorophore for monitoring the cytoplasmic pH without any disturbance by background fluorescence from vacuoles, in particular, at low temperature.

pH Calibration under the Microscope

Measurements of intracellular pH by fluorescence-ratio imaging require calibration of the measured fluorescence ratios against pH. One problem that greatly complicates the relationship between fluorescence properties and pH is that the pH calibration curves obtained in situ and in vitro are reported often to be not superimposable (Heiple and Taylor, 1980). In the present studies fluorescein-loaded protoplasts were preincubated in the weak acid and weak base buffer solution for equilibration of the cellular pH with the external pH.

Figure 5 shows the pH calibration curves obtained in situ and in vitro. In mung bean cultured cells, significant differences were not observed in the calibration curves between the experimental conditions defined as in situ and in vitro. In some plants, in contrast, the pH-ratio calibration curves differ significantly between in situ and in vitro conditions. The reason for these differences has not been determined.

Changes in Cytoplasmic pH in Response to Incubation at Low Temperature

To determine changes in cytoplasmic pH in the cold, protoplasts were first incubated at 0°C for various periods and then loaded with fluorophore for 30 min at 0°C. The fluorescein-loaded protoplasts were mounted on a glass resistance heater in the cold chamber, and the fluorescence ratio was measured. The temperature of cell specimens was maintained at 2 ± 0.2°C throughout the recording of fluorescent images. Protracted exposure of cells to low temperature in the cold chamber after loading with fluorescein was avoided to eliminate any effects of changes in the osmotic pressure of the external solution due to evaporation and a limited supply of oxygen.

In the present study, mung bean cultured cells at different stages of growth, namely the early and the late stages of exponential growth, were used for a comparison of the changes in cytoplasmic pH in response to cold treatment because of their marked difference in chilling sensitivity, as reported previously (Yoshida et al., 1993). Figure 6 shows the time-dependent changes in the images during cold incubation at 0°C of protoplasts that had been isolated from cells at the early stage of exponential growth. In the images of nonchilled control cells, cytoplasm was bright red (A), indicating a weakly alkaline pH. After the cells were chilled for 4 h, the color changed abruptly to yellowish green or light blue (B), although some cells remained red. The color changed to dark

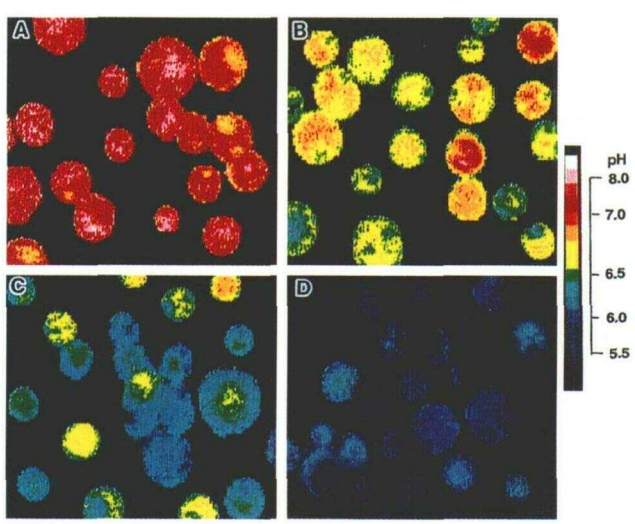

Figure 6. Changes in pH-ratio images of protoplasts isolated from cells at the early stage of exponential growth, as a function of the duration of incubation at 0°C. A, Untreated control; B, incubated at 0°C for 4 h; C, incubated at 0°C for 8 h; D, incubated at 0°C for 18 h. Experimental details are given in the text.

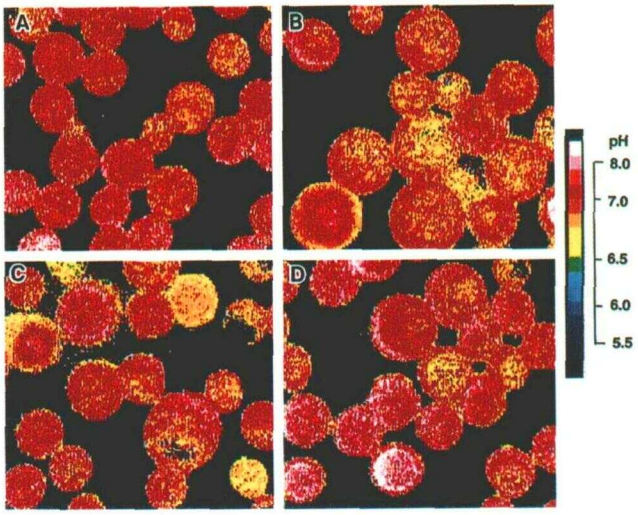

Figure 7. Changes in fluorescence-ratio images of protoplasts isolated from cells at the late stage of exponential growth as a function of the duration of incubation. A, Control; B, incubated at 0°C for 4 h; C, incubated at 0°C for 8 h; D, incubated at 0°C for 24 h. Experimental details are given in the text.

blue or violet (D) after protracted exposure to chilling temperature for up to 18 h, indicating the marked acidification of the cytoplasm. The cold-induced acidification of the cytoplasm was, however, completely or almost completely reversible, and the normal pH was observed after rewarming to 26°C for 2 h unless the duration of cold incubation exceeded 18 h (Fig. 9, bottom). After protoplasts were incubated in the cold for 48 h, the reversal of the acidification of the cytoplasm was only partial, suggesting an irreversible process (data not shown). The reversibility of cellular pH was reflected in cell viability as assessed by the triphenyltetrazolium chloride-reduction test, as reported previously (Yoshida et al., 1993). In control protoplasts, which were kept in the suspension medium at 20°C with occasional shaking, no change in the ratio image was observed within 16 h.

Figure 7 shows cold-induced changes in the ratio images of protoplasts that had been isolated from cells at the late stage of exponential growth, i.e. 14 d after subculturing. In contrast to the cells at the early stage of exponential growth (Fig. 6), significant changes in the images were not observed in the cells at the late stage of exponential growth, at least within 24 h of incubation at 0°C, suggesting that the cytoplasmic pH remained stable. This result is consistent with the increased chilling tolerance of the cells at the late stage of exponential growth.

During preparation of protoplasts, cells are exposed to artificial conditions and are subject to physical (osmotic) and chemical (nutrient) stress, which might, in turn, differentially affect cellular responses to cold. To examine this possibility, the cold-induced acidification of the cytoplasm was monitored in intact cultured cells of mung bean. The cells at the early stage of exponential growth were incubated in the cold in the culture medium in which they had been growing. Figure 8 shows the images of cells after such incubation for various periods. During incubation in the cold, cytoplasmic acidosis occurred to nearly the same extent as it did in isolated protoplasts. As indicated by the inset in Figure 8C, the cytoplasm that had become acidified after incubation at 0°C for 8 h returned to the normal pH upon warming to 26°C for 2 h. The results confirm that the cold-induced cytoplasmic acidosis of protoplasts is unlikely to be due to artifactual phenomena.

Cytoplasmic pH values were determined by sampling more than 20 cells with a fixed pixel window size of 15 μm^2, and values were averaged. In Figure 9, the cytoplasmic pH is plotted against the duration of chilling. In nonchilled cells, the cytoplasmic pH values were 7.42 ± 0.19 and 7.30 ± 0.18 for cells at the early and late stages of exponential growth, respectively. In the cells at the early stage of exponential growth, the pH decreased to 6.82 ± 0.22 after incubation at 0°C for 4 h. Protracted chilling for up to 24 h resulted in a further reduction in the pH to 6.31 ± 0.16. As indicated by arrows, the cold-induced reduction in pH was reversible unless the duration of chilling exceeded 18 h. In cells at the late stage of exponential growth, in contrast, no cytoplasmic acidosis occurred during incubation at 0°C for 24 h. Upon further protracted incubation at 0°C for up to 96 h, the cytoplasmic pH gradually decreased to 6.75 ± 0.23 (data not shown). Thus, there was a marked difference in the cold-induced changes in cytoplasmic pH between cells at the different stages of growth.

DISCUSSION

In the present study we attempted to obtain insight into the low temperature-induced perturbation of cytoplasmic pH in chilling-sensitive cultured cells of mung bean. Cold-induced changes in cytoplasmic pH in mung bean cultured cells were quantitated with a fluorescence cryomicroscope with a video image-processing system. For measurement of cellular pH by fluorescence-ratio image processing, selection of the appropriate fluorophore is critically important. 2′,7′-

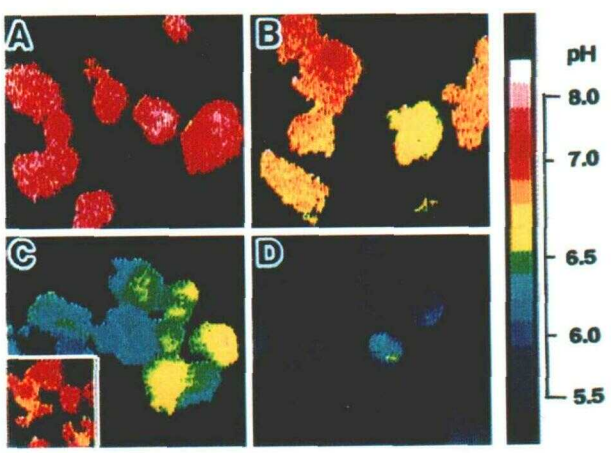

Figure 8. Changes in pH-ratio images of intact cultured cells at the early stage of exponential growth as a function of the duration of incubation at 0°C. A, Untreated control; B, incubated at 0°C for 4 h; C, incubated at 0°C for 8 h (inset, after warming at 26°C); D, incubated at 0°C for 24 h. Experimental details are given in the text.

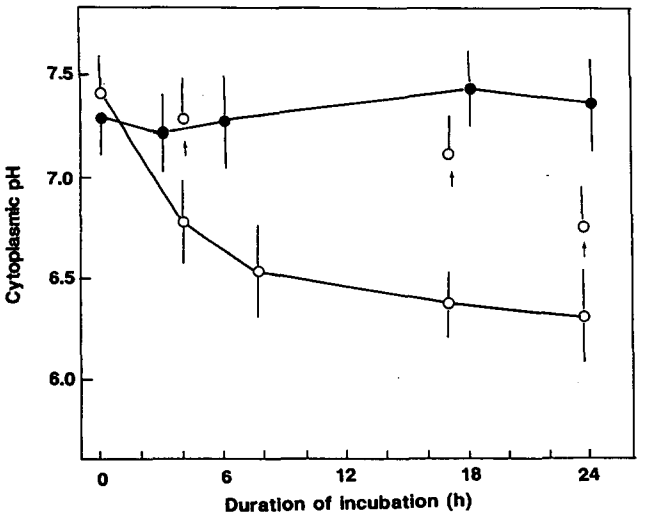

Figure 9. Changes in the cytoplasmic pH of isolated protoplasts as a function of the duration of incubation at 0°C. The fluorescence ratios in Figures 6 and 7 were converted to pH values by reference to the pH calibration curve (Fig. 5). The pH values were determined for more than 20 protoplasts and averaged. O, Protoplasts isolated from cells at the early exponential stage; ●, protoplasts isolated from cells at the late exponential stage. Arrows indicate recovery of the cytoplasmic pH after rewarming to 26°C for 2 h. Experimental details are given in the text.

Bis-(2-carboxyethyl)-5 (and 6-)carboxyfluorescein and its acetoxymethyl ester are widely used for measurement of cellular pH in mammalian cells (Thomas et al., 1979; Rogers et al., 1983; Bright et al., 1987; Davis et al., 1987) and in plant cells (Irving et al., 1992) primarily because of the pK_a value, which is ideally suited to the physiological range of pH values in most cells. In our preliminary experiments, however, the acetoxymethyl ester of 2′,7′-bis-(2-carboxyethyl)-5 (and 6-) carboxyfluorescein was not suitable for use with protoplasts isolated from mung bean cultures because of the insufficient intensity of fluorescence even after lengthy loading.

FDA has been used for the determination of cell viability (Widholm, 1972) and for the measurement of intracellular pH in yeast cells (Slavik, 1982). In most plant cells, entry and enzymic cleavage of the ester in the cells occur almost instantaneously. The fluorescence signal of free fluorescein is highly sensitive to the surrounding pH, and the fluorescence ratio (495/435 nm) is almost directly proportional to the change in pH over the range from pH 5.5 to 7.3, although the fluorescence response is relatively poor at pH values greater than 7.4 (Fig. 5). One of the problems encountered in the use of this fluorophore for measurement of cellular pH in plants is the relatively high permeability of the plasma membrane, especially under warm conditions. At 25°C, fluorescein leaks out of cells at a relatively high rate: about 10% of the total fluorophore entrapped in cells leaks out per hour. However, the leakage is extremely slight at low temperature, and no leakage was detected after incubation of loaded cells at 0°C for 6 h (data not shown). The permeation of the fluorophore from cytoplasm into the vacuole was also minimal at low temperatures. For all of these characteristics, the use of FDA for measurement of cytoplasmic pH in mung bean cultured cells under low temperature appears to be quite possible.

In the present study, it was clearly shown that the alteration in cytoplasmic pH in mung bean cultures during cold incubation was quite dependent on the growth stage and the chilling sensitivity of cells as well. In the cells at the early stage of exponential growth, the cytoplasmic pH decreased abruptly after chilling for 6 h (Fig. 9). The rapid reduction in the cytoplasmic pH was mostly reversed upon warming of cells for 2 h. In contrast to the cells at the early stage of exponential growth, no significant acidification of the cytoplasm occurred in the cells at the late stage of exponential growth during cold incubation for 24 h. Upon protracted cold incubation for up to 96 h, the cytoplasmic pH decreased only slightly from 7.3 to 6.8 (data not shown). In the cells at the early stage of exponential growth, the activity of the tonoplast H^+-ATPase declined to 30% of the control level after cold incubation for 6 h and the decline was reversed completely upon warming of cells for 2 h (Yoshida et al., 1993). In contrast to the cells at the early stage of exponential growth, the tonoplast H^+-ATPase in cells at the late stage of exponential growth was relatively cold stable; only slight inactivation occurs after cold incubation for 24 h (Yoshida et al., 1993). Thus, there seems to exist a close correlation between cold-induced inactivation of the enzyme and the cytoplasmic acidosis.

The cold-induced acidification of cytoplasm seems unlikely to be due to an abrupt increase in permeability of the tonoplast to protons and proton equivalents resulting from a phase transition of the lipid bilayers. As reported earlier (Yoshida and Matsuura-Endo, 1991), in tonoplast vesicles prepared from mung bean hypocotyls that were also highly sensitive to chilling, the permeability to protons and proton equivalents decreased linearly with decreasing incubation temperature from 25 to 0°C without any indication of an abrupt change. Instead, both types of proton-translocating enzymes, namely H^+-ATPase and proton-translocating inorganic pyrophosphatase, were markedly suppressed at temperatures below 10°C. It is well known that the sensitivity of the cytoplasmic pH to external pH variations is low, whereas vacuolar pH is more sensitive to the external pH variations (refs. in Kurkdjian and Guern, 1989). The difference in behavior between the two compartments illustrates the tightness of the homeostasis of the cytoplasmic pH and may indicate that exchanges of protons and proton equivalents between cytoplasmic and vacuolar compartments are likely involved in the adaptation of the cell to a large range of the external proton concentrations. In mung bean cultured cells, the cold-induced acidification of cytoplasm was not significantly affected by the variations in the external pH from 4.75 to 7.28 (data not shown), suggesting that the cold-induced acidification of the cytoplasm was not primarily due to an increase in plasma membrane permeability. The observed correlation between the cold-induced inactivation of the tonoplast H^+-ATPase and the cytoplasmic acidification tempts us to presuppose the importance of the tonoplast proton-transport systems for the homeostasis of cytoplasmic pH at low temperatures. It has been demonstrated that hypoxia-induced acidification of cytoplasm in corn and pea root tips occurs independently of lactic acid metabolism (Saint-Ges et al.,

1991) but concomitantly with alkalization of the vacuolar lumen, which likely corresponds to a release of protons from the vacuoles (Roberts et al., 1984).

Cold-induced cytoplasmic acidosis was also observed in mesophyll cells from leaves of *Episcia, Saintpaulia,* and *Cucumis,* which are all extremely sensitive to chilling (S. Yoshida, unpublished data), although the time courses differed among different species. These results suggest that the cold-induced acidification of cytoplasm is common to those extremely chilling-sensitive plants, and the cold-induced acidification of the cytoplasm may be one of the determinants responsible for cell injury. The regulatory system(s) of the pH homeostasis under low temperature might be critically important for cell adaptation to cold environments.

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