

Impairment of Tonoplast H⁺-ATPase as an Initial Physiological Response of Cells to Chilling in Mung Bean (*Vigna radiata* [L.] Wilczek)¹

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ABSTRACT

Biochemical alterations of cellular membranes in chilling-sensitive mung bean (*Vigna radiata* [L.] Wilczek) hypocotyls were investigated with reference to chilling injury. Reversible decreases in activities of tonoplast H⁺-ATPase and *in vivo* respiration became manifest within 24 hours of chilling when tissues suffered no permanent injury as assessed by electrolyte leakage and regrowth capacity. These changes were found to be the earliest cellular responses to chilling. A density-shift on a sucrose density gradient was observed in Golgi membranes early in the chilling treatment, suggesting that Golgi function and/or membrane biogenesis via the Golgi may have been altered upon chilling. After chilling more than 2 days, irreversible changes were generally produced in cellular membranes including the plasma membrane, endoplasmic reticulum, and mitochondria. Respiratory functions remained intact in mitochondria isolated from tissues prechilled for 24 hours, but were impaired after prechilling for 3 days. Given the important role of the tonoplast H⁺-ATPase in the active transport of ions and metabolites, the early decline in the tonoplast H⁺-ATPase activity may give rise to an alteration of the cytoplasmic environment and, consequently, trigger a series of degenerative reactions in the cells.

Although a number of studies have been carried out to elucidate the mechanisms involved in chilling injury in plants, many problems remain to be explained. For a better understanding of the mechanisms, it is important to identify the cellular site(s) and the physical features of the primary reaction(s) sensing low temperatures and to determine the physiological transducers transmitted from the low temperature sensing reaction. The temperature sensing site(s) has been suggested to reside on cellular membranes and either lipids (10, 12, 16) or proteins (1) may be involved. Lyons (9) and Raison (10) proposed the thermotropic lipid phase-transition hypothesis, which is the most widely accepted hypothesis explaining the molecular mechanisms of chilling injury in plants. A number of studies have been carried out to substantiate this hypothesis using mostly mitochondrial and plastid membranes as model systems. Nevertheless, there remains

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uncertainty as to how the chill-induced physical changes in those membranes can be sequentially transduced into cell injury. Furthermore, there seems to be no specific reason to assume that those organelles are exclusive cellular sites for sensing low temperatures.

In our earlier studies, using extremely chill-sensitive cultured cells (27), degeneration of cell structures occurred within a short period of chilling, *i.e.*, 6 to 12 h at 0°C. Partial dilation and microvesiculation of RER and other deteriorative changes were followed by marked morphological changes in the intramembranous particles on the tonoplast fracture faces. Furthermore, the chilled cells retained the capacity to grow after transfer to a warm temperature until the onset of the structural alteration in the tonoplast. It is assumed that chill-induced structural and functional deterioration in the tonoplasts is also involved in the earliest cellular events triggered by chilling. In the present study, we have attempted to analyze the biochemical changes in various cellular membranes, which take place during chilling in the cells in chill-sensitive mung bean seedlings. Special attention was paid to determining the earliest changes manifested in intact cells immediately upon chilling and to distinguish them from the secondarily transduced ones.

MATERIALS AND METHODS

Plant Materials

Seeds of mung bean (*Vigna radiata* [L.] Wilczek) were imbibed and germinated at 26°C in the dark as reported elsewhere (28, 29).

Chilling Treatment

Intact 3.5-d-old seedlings were chilled at 0°C in the dark for various periods. During chilling, the relative humidity was kept at 100% to avoid an additive effect of dehydration stress. After various periods of chilling, hypocotyls were excised and tested for electrolyte leakage and capacity for regrowth.

Electrolyte Leakage Test

Two g of hypocotyls were excised from nonchilled or chilled seedlings and cut into small segments, then immersed in 10 mL of distilled water. After incubation at 26°C in the dark with constant shaking at 60 cycles/min, the amount of electrolytes in the leached solution was measured with a conduc-

tivity meter. The extent of electrolyte leakage from tissues was expressed as a percentage of the total amount of electrolytes leached from freeze-thawed (-80°C) tissues. The conductivity of nonchilled tissues was taken as 0%.

Preparation of Membrane Fractions

Hypocotyls excised from nonchilled or chilled seedlings were washed once with distilled water and prechilled at 4°C before use. The prechilled tissues were sliced into a homogenizing medium chilled at 0°C and immediately homogenized with a Polytron PT 30 at the medium speed setting for 30 s. The homogenization medium contained 0.25 M sucrose, 75 mM Mops/KOH buffer (pH 7.6), 5 mM EDTA, 5 mM EGTA, 10 mM KF, 2 mM PMSF, 2 mM SHAM,² 2.5 mM potassium metabisulfite, 1.5% (w/v) PVP (mol wt 24,000), 0.5% defatted BSA, and 10 $\mu\text{g}/\text{mL}$ butylated hydroxytoluene. The slurry was passed through four layers of gauze and subjected to differential centrifugation at 3,600g for 10 min followed by 156,000g for 20 min. The 3,600 to 156,000g pellet was designated the crude total membrane fraction. The crude total membrane fraction was resuspended in 8 mL of sucrose suspension medium which contained 0.25 M sucrose, 5 mM Mops-KOH buffer (pH 7.3), 1 mM EDTA, 10 mM KCl, 10 $\mu\text{g}/\text{mL}$ butylated hydroxytoluene, and 1 mM DTT, then loaded on a discontinuous sucrose density gradient made up in the same buffer (10 mL of 32% and 7 mL of 43% sucrose, w/w). After centrifugation at 189,000g for 2.5 h in a Hitachi RP 50.2 rotor at 2°C , a membrane fraction enriched in tonoplast, ER, and Golgi, and a membrane fraction enriched in plasma membrane and mitochondria were recovered at the interfaces of sample/32% (w/w) and the 32/43% (w/w) sucrose layers, respectively (28). For preparation of a mitochondria-enriched fraction, tissues were homogenized as described above and the homogenate centrifuged at 1,500g for 7 min. The resulting supernatant was further centrifuged at 10,000g for 15 min. The 1,500 to 10,000g pellet was resuspended in the sucrose suspension medium (8 mL) and loaded on a discontinuous sucrose density gradient which consisted of 32% (w/w, 16 mL) and 43% (w/w, 12 mL) sucrose solutions. After centrifugation at 89,000g for 2 h in a Hitachi SW-27 rotor at 2°C , the mitochondria-enriched fraction was recovered from the 32/43% sucrose interface. Membrane fractions thus obtained were diluted with the sucrose suspending medium and pelleted by centrifugation at 156,000g for 20 min. The membrane pellets were resuspended in a sorbitol-buffer system containing 0.25 M sorbitol, 1 mM DTT, and 5 mM Mops-KOH (pH 7.3), and used for enzyme analysis and measurements of *in vitro* respiration.

Linear Sucrose Density Gradient

The crude total membrane fractions (3,600–156,000g pellet) prepared from nonchilled or chilled hypocotyl tissues were resuspended in the sucrose suspending buffer (6 mL) and loaded on a linear sucrose density gradient (30 mL, 15–50%, w/w) made up in the sucrose suspending medium. After

centrifugation at 86,000g for 16 h at 2°C in a Hitachi SW 27 rotor, 1.2-mL aliquots were collected from the top of the gradient.

Measurements of Respiratory Activities *In Vitro* and *In Vivo*

Mitochondrial respiration *in vitro* was measured polarographically with a Beckman Fieldlab oxygen analyzer using a Clark electrode and a thermostatted 4.3-mL reaction cell. O_2 saturated distilled water was used to calibrate the instrument. The reaction mixture contained 0.1 mL of mitochondria (200 μg protein) and 4.2 mL of buffer solution containing 0.25 M sorbitol, 20 mM K-phosphate buffer (pH 7.2), 1 mM MgCl_2 , 0.1% defatted BSA, 20 mM sodium succinate (substrate for oxidation, state 4 respiration), and 116 μM ADP (state 3 respiration), when added. *In vivo* assays of respiration were performed polarographically at 25°C with the same apparatus as described above using 200 mg of nonchilled or chilled hypocotyl segments in 1 mM CaSO_4 solution.

Enzyme Assays

ATPase activities were assayed in a reaction mixture containing 3 mM Na-ATP, 3 mM MgSO_4 , 30 mM Tris-Mes (pH 7.0), 50 mM KCl, 1 mM Na-molybdate, 5 mM NaN_3 , 0.03% (w/v) Triton X-100 in the presence or absence of 100 μM orthovanadate or 100 mM KNO_3 . For mitochondrial ATPase assay, the pH of the reaction mixture was adjusted to 9.0, and activity was measured in the presence or absence of 5 mM NaN_3 . The inhibitor-sensitive ATPase activity was determined by subtracting the activity with inhibitor from the control activity without inhibitor. PPase activity (23) was assayed in a reaction mixture containing 3 mM MgSO_4 , 3 mM Na-PPi, 30 mM Tris-Mes (pH 8.0), 50 mM KCl, 1 mM Na-molybdate in the presence or absence of 0.03% (w/v) Triton X-100. UDPase activity was measured according to the method of Nagahashi and Kane (13). All enzyme assays were performed at 30°C . NADH Cyt *c* reductase and Cyt *c* oxidase activities were measured as reported elsewhere (28). Protein content was determined according to the method of Bradford (4) using BSA as the standard.

Assay of Adenine Nucleotides

Levels of adenine nucleotides were determined by the luciferase bioluminescence assay described by Ball and Atkinson (2). This assay allows determinations of all three adenine nucleotides by using pyruvate kinase and adenylate kinase for the stepwise conversions of ADP and AMP to ATP. Ten g (fresh weight) of nonchilled or chilled hypocotyl were homogenized in 10 mL of 1 N HClO_4 chilled at 0°C with a Polytron PT 10 at the maximum speed setting. The acid extracts were neutralized with solid KH_2CO_3 and were buffered with 0.2 M Tris-HCl (pH 7.3), then centrifuged at 10,000g for 10 min. The neutralized samples were frozen at -80°C until use.

Proton Pumping Assays

The formation of an inside-acid pH gradient across membrane vesicles was measured as the rate of fluorescent quench-

² Abbreviations: SHAM, salicylhydroxamic acid; PPase, pyrophosphatase; ACC, 1-aminocyclopropane-1-carboxylic acid.

ing of quinacrine (18). An aliquot of the tonoplast-enriched fraction (the sample/32% sucrose interfaces, 150–200 μg protein) was added to a reaction mixture containing 250 mM sorbitol, 30 mM Hepes-bis-tris-propane, 1 mM (PPi-dependent H^+ -pump) or 3 mM (ATP-dependent H^+ -pump) MgSO_4 , 50 mM KCl, and 10 μM quinacrine (pH 7.5), in a final volume of 2.0 mL. After temperature equilibration at 20°C, proton translocation was initiated by the addition either of Na-PPi or bis-tris-propane-ATP at a final concentration of 1 or 3 mM, respectively, and the fluorescence decrease with time measured with a Shimadzu spectrofluorimeter model RF-540 at excitation and emission wavelengths of 423 (slit width, 3 nm) and 500 nm (slit width, 5 nm), respectively. The initial rate of quenching (relative fluorescent change per min, Q_v) was followed as a measure of the rate of proton pumping. The total extent of quenching at the steady state (where proton pumping and proton leaks are equilibrated), which was reversed to nearly 80% level of original fluorescent intensity by addition of 3 μM carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone, was used as a measure of the pH gradient (ΔQ) across the membrane vesicles. The PPi and ATP-dependent proton-translocating activities observed with the tonoplast-enriched membrane fraction were insensitive to vanadate, but the latter activity was highly sensitive to KNO_3 , suggesting all the proton-pump activities were of tonoplast origin.

RESULTS

Development of Chilling

The development of chilling injury in mung bean hypocotyls was assessed by measurement of electrolyte leakage after exposing seedlings to 0°C for various periods, then returning them to a warm condition. Table I shows changes in the electrolyte leakage as a function of the chilling period. Electrolyte leakage was negligible after 1-d chilling, but appeared after 2-d chilling and increased thereafter in proportion to the chilling period. After 4-d chilling, nearly 50% of the cellular electrolytes leaked out, indicating severe damage to the plasma membrane and, presumably, to other cellular membranes,

Table I. Effect of Chilling on Electrolyte Leakage from Mung Bean Hypocotyls

The 3.5-d old etiolated seedlings were chilled at 0°C for various periods. After the chilling, hypocotyls were excised and submitted to an electrolyte-leakage test as described in "Materials and Methods." The degree of electrolyte leakage was expressed as the percentage of the total amount of electrolytes leached from freeze-thawed (–80°C) tissues. The results are mean values \pm SE of three determinations.

Chilling Period	Electrolyte Leakage
<i>d</i>	%
0 (nonchilled)	0
1	1.9 \pm 0.6
2	24.5 \pm 1.2
3	32.1 \pm 1.6
4	47.2 \pm 0.9
5	62.2 \pm 1.1
6	79.2 \pm 2.1
7	81.1 \pm 2.0

including the tonoplast. When the electrolyte-leakage measurement was followed at 0°C before rewarming, no detectable change was observed in the tissues for up to 5 d (data not shown), suggesting that the membrane damage allowing electrolyte-leakage was a secondary cellular event. The degree of electrolyte leakage after warming to 26°C was correlated with the capacity for regrowth of hypocotyls after transfer to a warm condition (6). When seedlings were chilled for 1 d and returned to 26°C, hypocotyl growth was only slightly inhibited. However, after chilling for 2 d the capacity for regrowth was severely reduced, and chilling more than 3 d resulted in a complete loss of the regrowth capacity.

Changes in Enzyme Activities Associated with Various Cellular Membranes

Figure 1 shows the effects of chilling on enzyme activities associated with various cellular membranes. Enzyme assays were carried out with a total crude membrane fraction (3,600–156,000g pellets) isolated from nonchilled or chilled hypocotyls. Activity of KNO_3 -sensitive ATPase (tonoplast) was found to be the most sensitive to chilling and began to decline during the first day of chilling. The activity of the vanadate-sensitive ATPase (mostly plasma membrane) also showed a slight decrease after 1-d chilling. Activities of other membrane-bound enzymes such as the antimycin A-resistant NADH Cyt *c* reductase (endoplasmic reticulum), UDPase (Golgi), NaN_3 -sensitive ATPase and Cyt *c* oxidase (mitochondria) showed essentially no change after 1-d chilling. However, they declined after 2-d chilling, suggesting a secondary physiological event. PPase is known to be specifically bound to the tonoplast (5) and constitutes another type of proton-translocating system in plants (17, 23). Almost all the particulate PPase was associated with the tonoplast in mung bean hypocotyl cells (data not shown). When the PPase activity was assayed in the presence of 0.03% Triton X-100, the activity increased by about 7-fold, suggesting a latent enzyme (M Maeshima, S Yoshida, in preparation). In contrast with the KNO_3 -sensitive ATPase (tonoplast H^+ -ATPase), the activity of the Triton-stimulated PPase showed no change after 1-d chilling. However, it declined upon prolonged chilling. The control PPase activity, which was assayed in the absence of detergent, increased after prolonged chilling.

Enzyme assays were performed with partially purified membrane fractions after a discontinuous sucrose density gradient (32/43%, w/w) to determine more precisely the changes in activities of membrane-bound enzymes during chilling (Fig. 2). Activity changes in antimycin A-insensitive NADH Cyt *c* reductase, Cyt *c* oxidase, and KNO_3 -sensitive ATPase were essentially the same as observed in Figure 1. However, the vanadate-sensitive ATPase activity in the plasma membrane-enriched fraction (32/43% interface) did not show any change after 1-d chilling, but did so after 2-d chilling. The initial decline in the vanadate-sensitive ATPase activity in Figure 1, therefore, was presumably due to a specific loss of enzyme activity associated with other cellular membranes. The early decline in the tonoplast H^+ -ATPase activity (*i.e.*, 1-d chilling) was completely reversed to the control level within 6 h upon rewarming of the prechilled seedlings at 26°C (data not shown). On the other hand, the

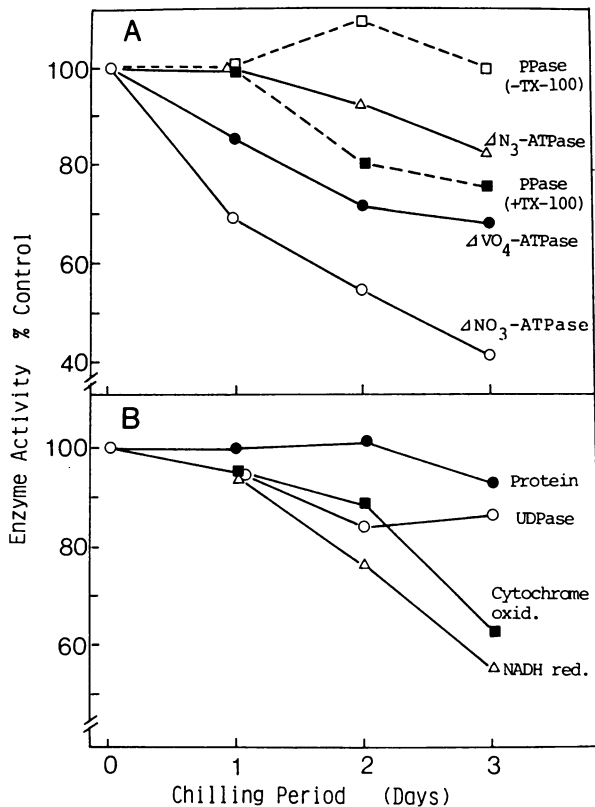


Figure 1. Effects of chilling on enzyme activities associated with various cellular membranes. Enzyme assays were carried out with the total crude membrane fraction (3,600–156,000g pellets) prepared from mung bean hypocotyls previously chilled at 0°C for various periods. Experimental details are described in "Materials and Methods." Data are averages of triplicate determinations and the variations for the data were about 5%. Specific enzyme activities in the membrane fraction prepared from nonchilled hypocotyls were taken as 100% and are as follows: KNO₃-sensitive ATPase, 5.5 μmol·mg⁻¹·h⁻¹; vanadate-sensitive ATPase, 11.0 μmol·mg⁻¹·h⁻¹; NaN₃-sensitive ATPase, 14.0 μmol·mg⁻¹·h⁻¹; PPase (-TX-100), 5.1 μmol·mg⁻¹·h⁻¹; PPase (+TX-100), 21.6 μmol·mg⁻¹·h⁻¹; UDPase, 89 μmol·mg⁻¹·h⁻¹; NADH Cyt c reductase, 1.3 μmol·mg⁻¹·min⁻¹; Cyt c oxidase, 61 nmol·mg⁻¹·min⁻¹. A, (■) and (□) PPase in the presence or absence of the detergent, respectively, (●) vanadate-sensitive ATPase, and (○) KNO₃-sensitive ATPase. B, (●) protein content, (○) UDPase, (■) Cyt c oxidase, and (Δ) NADH Cyt c reductase (antrimycin A-insensitive).

decline became irreversible after 2-d chilling, as did enzymes associated with the plasma membrane, endoplasmic reticulum, Golgi, and mitochondria (data not shown). When experiments were performed with highly enriched plasma membrane (28) and tonoplast (29) fractions, the vanadate-sensitive and the KNO₃-sensitive ATPases showed nearly the same changes upon chilling as observed in Figure 2 (data not shown).

Proton-Translocating Activity

Figure 3 shows changes in the ATP-dependent and PPI-dependent proton-pump activities of the tonoplast-enriched membrane fractions as a function of the chilling period. Both the initial rate of fluorescent quenching of quinacrine (Q_0 ,

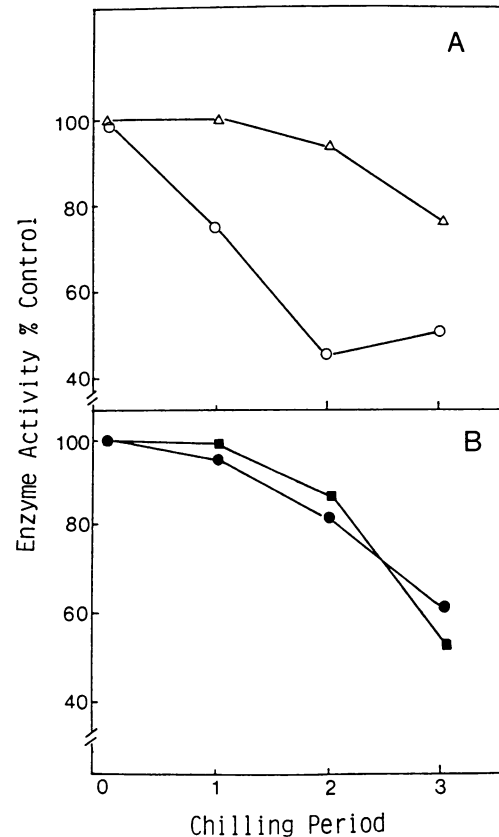


Figure 2. Effects of chilling on enzyme activities associated with various cellular membranes. Enzyme assays were carried out with partially purified membrane fractions after a discontinuous sucrose density gradient centrifugation (32/43%, w/w) of the total crude membrane fraction (3,600–156,000g pellet). Enzyme activities were measured as described in "Materials and Methods." Data are averages of triplicate determinations and the variations for the data were about 5%. Specific enzyme activities in the membrane fractions prepared from nonchilled hypocotyls were taken as 100% and are as follows: KNO₃-sensitive ATPase, 14.2 μmol·mg⁻¹·h⁻¹; vanadate-sensitive ATPase, 30.8 μmol·mg⁻¹·h⁻¹; NADH Cyt c reductase, 2.9 μmol·mg⁻¹·min⁻¹; Cyt c oxidase, 234 nmol·mg⁻¹·min⁻¹. A, Sample/32% interface fraction. (Δ) NADH cytochrome c reductase, (○) KNO₃-sensitive ATPase. B, The 32/43% interface fraction; (●) Vanadate-sensitive ATPase and (■) Cyt c oxidase.

initial rate of proton translocation) and the total extent of fluorescent quenching in the steady state (ΔQ , formation of ΔpH) showed an abrupt decline after 1-d chilling, analogous to the reduction in the KNO₃-sensitive ATPase activity (Figs. 1 and 2). Upon prolonged chilling, the proton-translocating function declined further. On the contrary, PPI-dependent proton-pump showed no change throughout the chilling period.

Respiratory Changes during Chilling

Figure 4 shows the changes in respiratory activity of hypocotyl tissue during chilling. After chilling for 1 d, the respiration decreased by about 36%, though it was completely reversed to the control level after transfer to 26°C for 3 h in the dark (inset, Fig. 4). Upon prolonged chilling, the decline in

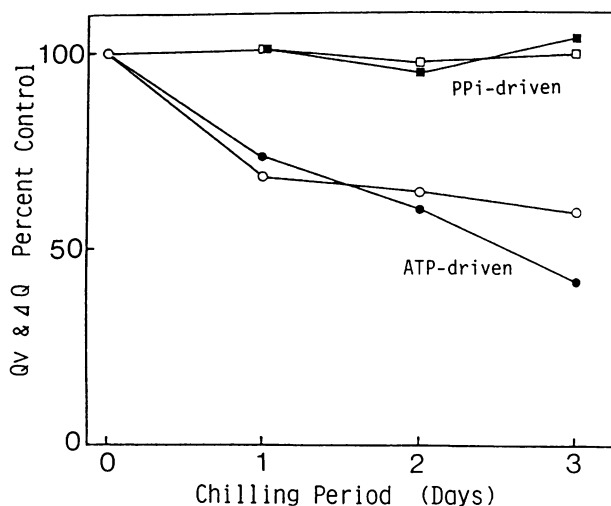


Figure 3. Effects of chilling on ATP-dependent and PPI-dependent proton-pumping activities in the tonoplast-enriched membrane fraction. The tonoplast-enriched fraction (sample/32% interface) was obtained by a discontinuous sucrose density gradient as described in Figure 2. The initial rate of H^+ -translocation (Q_v) and formation of a pH gradient across the membrane vesicles (ΔQ) were measured as the rate of fluorescent quenching of quinacrine and as the total extent of quenching at the steady state, respectively. Data are averages of triplicate determinations and variations for the data were about 5%. Experimental details are described in "Materials and Methods." (□) and (■) PPI-dependent ΔQ and Q_v , respectively; (○) and (●) ATP-dependent ΔQ and Q_v , respectively.

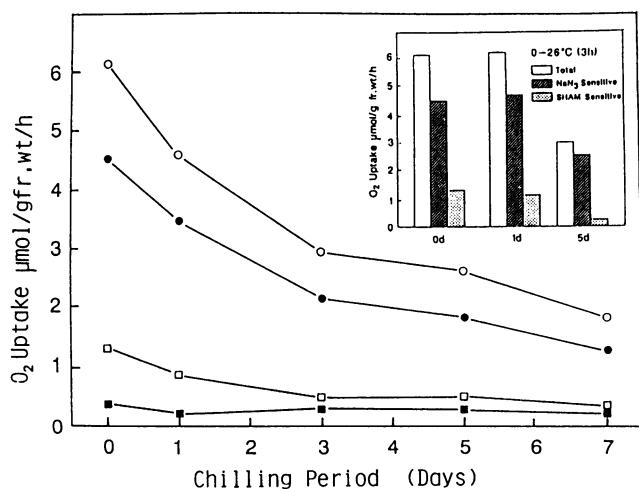


Figure 4. Effects of chilling on *in vivo* respiration of mung bean hypocotyls. Seedlings were previously chilled at 0°C for the indicated period and excised hypocotyl segments were submitted to measurements of O_2 -uptake. Inset: After chilling for 1 or 5 d, seedlings were transferred to 26°C for 3 h in the dark prior to O_2 -uptake measurements. O_2 -uptake by the tissue segments were measured polarographically at 25°C . Data are averages of triplicate determinations and the variations of the data were about 5%. (○) Total O_2 -uptake (without addition of inhibitors), (●) NaN_3 -sensitive, (□) SHAM-sensitive, and (■) residual O_2 -uptake in the presence of 5 mM NaN_3 and 5 mM SHAM.

respiration continued and became irreversible. The SHAM-sensitive, NaN_3 -resistant respiration (alternative pathway) was estimated to be around 20% of the total respiration in control tissues and it declined concurrently with the NaN_3 -sensitive rate during chilling. Table II shows the changes in respiratory activity of mitochondria isolated from nonchilled and chilled hypocotyl tissues. In contrast with the respiratory activity *in vivo*, the isolated mitochondrial respiration was not affected by 1 d of chilling, but it decreased after chilling more than 3 d. The decrease was greater in state 3 than in state 4, and thus resulted in a decline in the respiratory control ratio. The P/O ratio also decreased after chilling for 5 d, indicating an impairment of respiratory function of the mitochondria. This result correlates with activity changes in mitochondrial Cyt *c* oxidase and the NaN_3 -sensitive ATPase (Figs. 1 and 2).

Changes in Adenine Nucleotide Levels

Figure 5 shows changes in adenine nucleotide levels in hypocotyl tissues as a function of chilling period. The total amount of adenine nucleotides decreased remarkably during chilling (inset). After the first day of chilling the total level fell to about 60% of the control value. The decrease was mostly due to the declines in the levels of ATP and ADP. The decrease, however, returned to around 80% of the control level after warming to 26°C for 4 h (data not shown). Prolonged chilling up to 5 d resulted in a further decline of the nucleotide levels to about 20% of the control value. Adenylate energy charge decreased slightly from 0.76 to 0.60 after chilling for 5 d (inset, Fig. 4).

Changes in Density Gradient Profiles of Cellular Membranes

To get insight into the changes in buoyant densities of cellular membranes during chilling, the total crude membrane fraction (3,600–156,000g pellets) was fractionated on a linear sucrose density gradient (15–50%, w/w). Figure 6 shows the density gradient profiles of the KNO_3 -sensitive ATPase and PPase activities. The ATPase activity showed a broad peak at fractions 11 to 14 with a shoulder at fractions 9 to 10 (Fig. 6A). This may indicate that the tonoplast consisted of two populations which differed in density, *i.e.*, a lighter and a heavier one, and presumably, originated from different cell populations in the hypocotyls, *i.e.*, from the top and the base.

Table II. Alteration in Mitochondrial Respiration *In Vitro* by Chilling

Mitochondrial fractions were isolated from nonchilled or chilled mung bean hypocotyls and respiration was measured as described in "Materials and Methods." The results are mean values \pm SE of three determinations.

Chilling Period	Respiration		Respiratory Control Ratio	P/O Ratio
	State 4	State 3		
<i>d</i>	O_2 nmol \cdot mg $^{-1}$ \cdot min $^{-1}$			
0 (nonchilled)	185 \pm 11	346 \pm 9	1.87	2.67
1	191 \pm 10	364 \pm 13	1.90	2.63
3	155 \pm 8	243 \pm 10	1.56	2.38
5	114 \pm 9	164 \pm 8	1.43	1.92

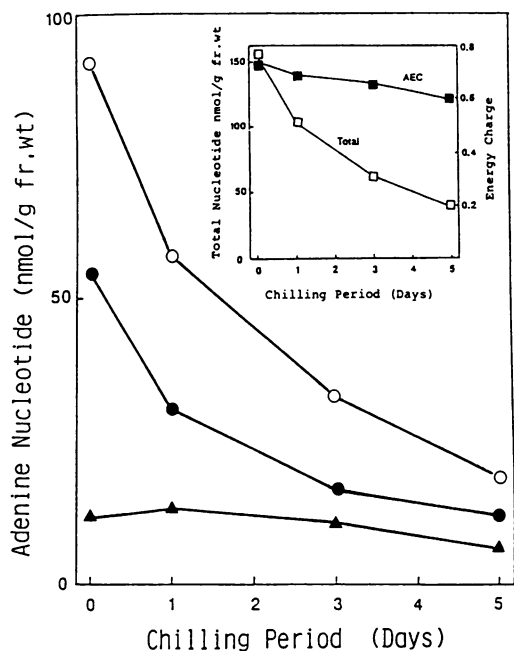


Figure 5. Changes in adenine nucleotide levels of mung bean hypocotyls during chilling. Data are averages of triplicate measurements and variations for data were about 5%. Experimental details are described in "Materials and Methods." (○) ATP, (●) ADP, and (▲) AMP. The inset shows changes in adenylate energy charge (AEC) and total amount of adenine nucleotides (total).

The decrease in activity during chilling was more apparent in the heavier tonoplast fraction; the activity in the lighter one was relatively constant until 2 d of chilling. There was a good correspondence between density gradient profiles of the KNO_3 -sensitive ATPase and PPase activities. When the PPase activity was assayed in the presence of Triton X-100 (Fig. 6C), the activity did not show any significant change until 2 d of chilling, although a moderate reduction occurred after chilling for 3 d. The situation was much different when the activity was assayed without addition of the detergent (Fig. 6B). The activity increased after chilling, suggesting either that the sidedness of the isolated tonoplast vesicles changed as a result of chilling or the chilling altered a topological feature of the enzyme.

Figure 7 shows changes in the density gradient profiles of UDPase and total ATPase activities during chilling. As assessed by the distribution profile of UDPase activity (Fig. 7A), Golgi membranes from non-chilled control tissues were banded at fractions 15 to 17 with a symmetric peak. Upon chilling, the activity peak of UDPase shifted to a much denser portion of the sucrose density gradient (fractions 21 to 22), suggesting a change in the average buoyant density or a change in the density of the *trans*-face of the Golgi cisternae with which the enzyme is supposed to be specifically associated (19). It should be stressed that the density shift can be detected even during the early, reversible phase of chilling, *i.e.*, 1-d chilling. Total ATPase activity (Fig. 7B) was assayed in a reaction mixture containing 1 mM Na-molybdate and 5 mM NaN_3 to eliminate activities of nonspecific phosphatase and mitochondrial ATPase, respectively, and 0.03% Triton X-100

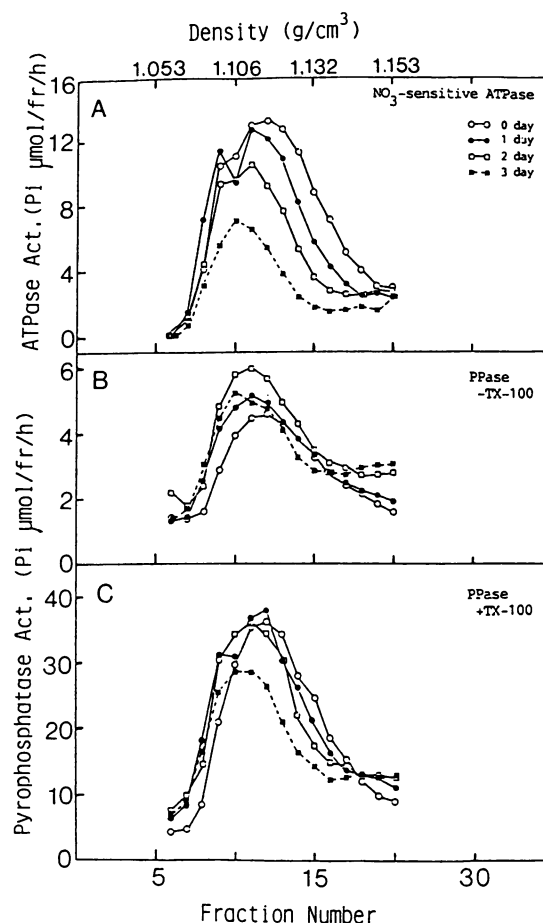


Figure 6. Effect of chilling on the density gradient profiles of the KNO_3 -sensitive ATPase and pyrophosphatase activities. Fifty g (fresh weight) of hypocotyl tissues excised from nonchilled or chilled seedlings were used for each experiment. After sucrose density gradient centrifugation described in "Materials and Methods," aliquots of 1.2 ml were fractionated from the top of the gradient. Pyrophosphatase activities were assayed in the presence or absence of 0.03% (w/v) Triton X-100. Each sucrose gradient was identical with respect to sucrose concentration of the fractions. A, KNO_3 -sensitive ATPase; B, PPase (-TX-100), C, PPase (+TX-100), (○) nonchilled, (●) 1-d chilled, (□) 2-d chilled, and (■) 3-d chilled.

to evaluate activity of any latent ATPases, although ATPase associated with endoplasmic reticulum is severely inhibited by the detergent (7, 26). The activity peak at the denser portion of the gradient, *i.e.*, fractions 23 to 24, corresponded to plasma membrane ATPase as assessed by vanadate-sensitivity. Although mitochondria cofractionated with plasma membrane in these fractions, addition of 5 mM NaN_3 completely inhibited the mitochondrial ATPase activity (data not shown). No detectable change was seen in the density of the plasma membranes during chilling. The activity peak at a lighter portion of the gradient, *i.e.*, fractions 9 to 13, corresponded to tonoplast ATPase (see Fig. 6, A-C). A significant level of ATPase activity was detected in the region between the activity peaks of the tonoplast and the plasma membrane ATPases, *i.e.*, fractions 15 to 18, where the Golgi membranes cofractionated. The activity in these fractions was significantly lowered upon chilling for 1 d and further declined with

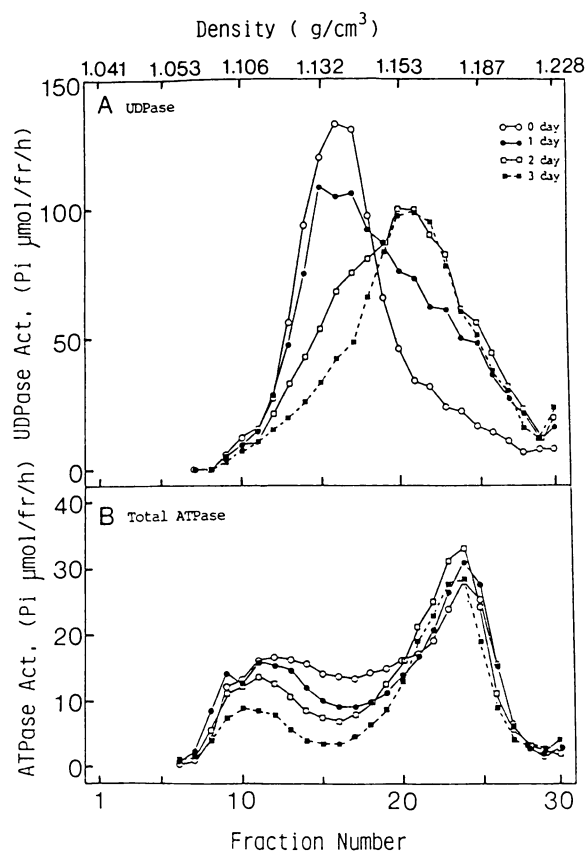


Figure 7. Effects of chilling on density gradient profiles of UDPase and total ATPase activities. Fifty g of hypocotyl tissues excised from nonchilled or chilled seedlings were used for each experiment. After sucrose density gradient centrifugation, aliquots of 1.2 mL were fractionated from the top of the gradient. Total ATPase activity was measured in the presence of Na-molybdate (1 mM) and NaN_3 (5 mM). Experimental details are described in "Materials and Methods." Each sucrose gradient was identical with respect to sucrose concentration of the fractions. A, UDPase; B, total ATPase. (○) Nonchilled, (●) 1-d chilled, (□) 2-day chilled, and (■) 3-d chilled.

increased chilling periods, similar to the tonoplast ATPase. Activity peaks of vanadate-sensitive ATPases were distributed at fractions 15 to 18 and 23 to 24 in accord with the distribution profiles of Golgi membranes and plasma membranes, respectively (data not shown). This supports our earlier results (7) that a vanadate-sensitive ATPase is associated with Golgi membranes. The observed changes in the Golgi membranes were reversible in the early process of chilling, within 1 d, but became irreversible after that period (data not shown).

The activity peak of antimycin A-resistant NADH Cyt *c* reductase appeared at fractions 10 to 11 and did not show any significant shift in the density after chilling, although the activity level fell significantly after chilling more than 2 d (data not shown).

DISCUSSION

When the cellular manifestation of injury in plants is approached from a biochemical point of view, special care must be paid to distinguish the initial cellular events transduced by chilling from secondary cellular events which are subsequently

propagated as a result of degenerative cell injury. Impairments of photosynthetic and respiratory functions in chloroplasts and mitochondria isolated from chilled tissues have been suggested (21, 25), although it is sometimes difficult to determine whether such impairments are the result or the cause of cell injury. In mung bean hypocotyls, electrolyte leakage from cells after rewarming cannot be the cause of injury, but is probably the result of a secondary-induced cellular event. In the present study, special emphasis has been placed on determination of the earliest, reversible alterations in cellular membranes, which could become manifest in intact cells immediately or time-dependently upon chilling. Etiolated mung bean hypocotyls were highly sensitive to chilling and irreversibly injured after exposure to 0°C more than 2 to 3 d. They were completely normal within the first 24 h of chilling as assessed by electrolyte leakage and the regrowth capacity. Therefore, this plant seems to be an ideal material to investigate the earliest biochemical processes transduced by chilling, before subsequent deteriorative changes are irreversibly triggered in the cells.

One of the most important findings in the present study was the reversible decline of tonoplast H^+ -ATPase activity and its proton-translocating function in the first day of chilling, when the tissues suffered no permanent injury. The early decline of the tonoplast ATPase activity may suggest that the enzyme, or the host membrane, has been altered in a reversible way. The tonoplast proton translocating ATPase is felt to function in the regulation of cellular pH levels and the proton-antiports of Ca^{2+} (3), basic amino acids (14) and sugars (22). The chill-induced decline of the H^+ -ATPase activity and the impairment of the proton-translocating function, whether reversible or irreversible, may therefore result in a perturbation of the cellular compartmentations of solutes and ions, especially protons and Ca^{2+} . In contrast to the ATPase, the tonoplast PPase and the proton-pump activities were rather stable for chilling periods up to 3 d. As reported earlier (29), mung bean tonoplast H^+ -ATPase activity was not stimulated by the addition of 0.016% Triton X-100 into the reaction mixture, whereas the plasma membrane ATPase was stimulated severalfold by the addition of the detergent (28), suggesting that these isolated membrane vesicles were mostly in a normal sidedness. In purified tonoplast vesicles, on the other hand, the PPase activity was observed to be latent and stimulated dramatically by the addition of 0.015% detergent into the reaction mixture (data not shown), suggesting an inside-out orientation to the vesicles. However, an effect of detergent on a conformational change or an alteration of some regulatory factor of the enzyme cannot be excluded. According to Shimmen and MacRobbie (20), however, the tonoplast H^+ -ATPase and the PPase in *Nittela* cells are reported to be exposed to the cytoplasmic surface. To resolve this discrepancy, further detailed studies on the topological feature of the PPase are needed.

According to Woods *et al.* (24), the cessation of protoplasmic streaming in chilling sensitive plant cells at lower temperatures is probably due to increases in the cytoplasmic Ca^{2+} concentration as a result of an immediate change in intracellular compartmentation. An immediate efflux of protons from vacuoles into cytoplasm as a result of a temperature-

dependent alteration of the physical state of the tonoplast membrane, if occurring, may also directly affect cellular metabolism and structures as a result of an abrupt lowering of the cytoplasmic pH. The capacity to generate a pH gradient across the isolated tonoplast vesicles became *nil* below 5°C (S Matsuura, C Yoshida, M Maeshima, in preparation). With this background, it is hypothesized that early responses of tonoplast activities to chilling might represent a primary or, at the least, initial cellular event, which subsequently would be transduced into several types of secondary cellular events, finally leading to cell death. The decline in membrane-bound enzyme activities including the plasma membrane ATPase, NADH Cyt *c* reductase, mitochondrial Cyt *c* oxidase and even the tonoplast H⁺-ATPase in the latter effects of chilling, more than 2 d, are all considered as secondary cellular events.

In the present study, *in vivo* respiration by hypocotyl segments was reversibly depressed after 1 d of chilling whereas no change occurred in the *in vitro* respiration of the isolated mitochondria from prechilled tissues. The ATP level in the hypocotyl cells decreased appreciably even after the first day of chilling. This rapid decline in the ATP level may be caused by the depression of respiration as mentioned above, although the direct effect of a temperature-dependent alteration of physical state in the mitochondrial membranes cannot be ruled out (15). The fall of ATP level may in turn affect not only the primary and secondary energy-dependent transport of ions and metabolites into their cellular compartments, but also general cellular metabolic reactions require that ATP. Our recent studies (8) demonstrate that *in vivo* incorporation of [³⁵S]methionine into proteins in mung bean hypocotyls was markedly reduced (*i.e.*, by 60%) by the prechilling at 0°C for 1 d, but it was completely reversed after rewarming to 26°C within 6 h. ACC-dependent ethylene formation by hypocotyl tissues was also demonstrated to decline reversibly after a 1-d chilling (6). Although the exact reasons are not fully understood, alterations of cytoplasmic pH and the cellular localization of ions, especially Ca²⁺, may also be responsible for the early and reversible declines in protein synthesis, ACC-dependent ethylene formation and respiration.

It should be also noted that Golgi membranes underwent a marked shift of buoyant density upon chilling, when assessed by the UDPase activity profile. UDPase is known to be specifically located in the *trans*-face cisternae of the Golgi apparatus (19), and, therefore, it is possible that the density profile represented the *trans*-Golgi cisternae. Although the reason is not understood, it is envisaged that the physiological functions of the Golgi apparatus might have been altered even in the early, reversible phase of chilling. The early decline of the membrane-associated ATPase activity, which was highly sensitive to vanadate (7), may partially support this idea. It is generally acknowledged that the Golgi apparatus plays a fundamental role in membrane biogenesis (11). Therefore, in future studies, close attention should be addressed to the effects of low temperatures upon general pathways of membrane biogenesis in chilling-sensitive plants, with special reference to the decline of tonoplast H⁺-ATPase activity during the early phase of chilling treatment.

LITERATURE CITED

1. Asahi T, Maeshima M, Uritani I (1982) Effect of temperature on the activity and stability of higher plant cytochrome *c* oxidase. In A Sakai, PH Li, eds, Plant Hardiness and Freezing Stress II. Academic Press, New York, pp 671–682
2. Ball WJ, Atkinson, DE (1975) Adenylate energy charge in *Saccharomyces cerevisiae* during starvation. J Bacteriol 121: 975–980
3. Blumwald E, Poole RJ (1986) Kinetics of Ca²⁺/H⁺ antiport in isolated tonoplast vesicles from storage tissues of *Beta vulgaris* L. Plant Physiol 80: 727–731
4. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
5. Chanson A, Pilet P-E (1987) Localization in sucrose density gradients of the pyrophosphatase-dependent proton transport of maize root membranes. Plant Physiol 84: 1431–1436
6. Etani S, Yoshida S (1987) Reversible and irreversible reduction of ACC-dependent ethylene formation in mung bean (*Vigna radiata* [L.] Wilczek) hypocotyls caused by chilling. Plant Cell Physiol 28: 83–91
7. Kawata T, Yoshida S (1988) Characterization of ATPases associated with various cellular membranes isolated from etiolated hypocotyls of *Vigna radiata* [L.] Wilczek. Plant Cell Physiol 29:1399–1410
8. Kawata T, Yoshida S (1988) Alterations *in vivo* protein synthesis in chilling sensitive mung bean hypocotyls caused by chilling stress. Plant Cell Physiol 29:1423–1427
9. Lyons JM (1973) Chilling injury in plants. Annu Rev Plant Physiol 24: 445–466
10. Lyons JM, Raison JK (1970) Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling. Plant Physiol 45: 386–389
11. Morre DJ (1979) Membrane biogenesis. Annu Rev Plant Physiol 26: 441–481
12. Murata N, Yamaya J (1984) Temperature-dependent phase behavior of phosphatidylglycerols from chilling-sensitive and chilling-resistant plants. Plant Physiol 74: 740–746
13. Nagahashi J, Kane AD (1982) Triton-stimulated nucleotide diphosphatase activity: Subcellular localization in corn root homogenates. Protoplasma 112: 167–173
14. Ohsumi Y, Anraku Y (1981) Properties of H⁺-translocating adenosine triphosphatase in vacuolar membranes of *Saccharomyces cerevisiae*. J Biol Chem 256: 10859–10863
15. Raison JK, Chapman EA (1976) Membrane phase changes in chilling-sensitive *Vigna radiata* and their significance to growth. Aust J Plant Physiol 3: 291–299
16. Raison JK, Lyons JM, Mehlhorn RT, Keith AD (1971) Temperature-induced phase changes in mitochondrial membranes detected by spin-labeling. J Biol Chem 246: 4036–4040
17. Rea PA, Poole RJ (1985) Proton-translocating inorganic pyrophosphatase in red beet (*Beta vulgaris* L.) tonoplast vesicles. Plant Physiol 77: 46–52
18. Roger RL, Spanswick RM (1985) Characterization of anion effects on the nitrate-sensitive ATP-dependent proton pumping activity of soybean (*Glycine max* L.) seedling root microsomes. Plant Physiol 77: 352–357
19. Roth J, Berger EG (1982) Immunochemical localization of galactosyl-transferase in HeLa cells: codistribution with thiamine pyrophosphatase in *trans*-Golgi cisternae. J Cell Biol 93: 223–229
20. Shimmen T, MacRobbie EAC (1987) Characterization of two proton transport systems in the tonoplast of plasmalemma-permeabilized *Nitella* cells. Plant Cell Physiol 28: 1023–1031
21. Taylor AO, Slack CR, McPherson HG (1974) Plants under climatic stress. VI. Chilling and light effects on photosynthetic enzymes of sorghum and maize. Plant Physiol 54: 696–701
22. Thom M, Komor E (1984) H⁺-sugar antiport as the mechanism of sugar uptake by sugar cane vacuoles. FEBS Lett 173: 1–4
23. Wang, Y, Leigh RA, Kaestner KH, Sze H (1986) Electrogenic

- H⁺-pumping pyrophosphatase in tonoplast vesicles of oat roots. *Plant Physiol* **81**: 497-502
24. **Woods CM, Polito VS, Reid MS** (1984) Response to chilling stress in plant cells. II. Redistribution of intracellular calcium. *Protoplasma* **121**: 17-24
 25. **Yamaki S, Uritani I** (1973) Mechanism of chilling injury in sweet potato. Part VIII. Morphological changes in chilling injured sweet potato root. *Agric Biol Chem* **37**: 183-186
 26. **Yoshida S, Kawata T** (1988) Isolation of smooth endoplasmic reticulum and tonoplast from mung bean hypocotyls (*Vigna radiata* [L.] Wilczek) by a Ficoll gradient and two-polymer phase partition. *Plant Cell Physiol* **29**: 1391-1397
 27. **Yoshida S, Niki T, Sakai A** (1979) Possible involvement of the tonoplast lesion in chilling injury of cultured cells. In JM Lyons, D Graham, JK Raison, eds, *Low Temperature Stress in Crop Plants*. Academic Press, New York, pp 275-290
 28. **Yoshida S, Kawata T, Uemura M, Niki T** (1986) Properties of plasma membrane isolated from chilling-sensitive etiolated seedlings of *Vigna radiata* L. *Plant Physiol* **80**: 152-160
 29. **Yoshida S, Kawata T, Uemura M, Niki T** (1986) Isolation and characterization of tonoplast from chilling-sensitive etiolated seedlings of *Vigna radiata* L. *Plant Physiol* **80**: 161-166