Plant Cell Physiol. 33(2): 139–149 (1992)

Stimulation of the Extrusion of Protons and H^+-ATPase Activities with the Decline in Pyrophosphatase Activity of the Tonoplast in Intact Mung Bean Roots under High-NaCl Stress and Its Relation to External Levels of Ca^{2+} Ions

Yoshiyuki Nakamura[1], Kunihiro Kasamo[2], Noriko Shimosato[1], Makoto Sakata[3] and Eiji Ohta[1]

[1] *Department of Instrumentation Engineering, Faculty of Science and Technology, Keio University, Yokohama, 223 Japan*
[2] *Molecular Function Laboratory, National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Kannondai, Tsukuba, Ibaraki, 305 Japan*
[3] *School of Medicine, Kyorin University, Mitaka, Tokyo, 181 Japan*

Extrusion of protons as a response to high-NaCl stress in intact mung bean roots was investigated at different external concentrations of Ca^{2+} ions ($[Ca^{2+}]_{ex}$). The extrusion of protons was gradually enhanced in the roots exposed to 100 mM NaCl, and high $[Ca^{2+}]_{ex}$ diminished this enhancement of the extrusion. Vesicles of plasmalemma and tonoplast were prepared from the roots and the H^+-translocating ATPase (H^+-ATPase) activities associated with the two types of membrane and the H^+-pyrophosphatase (H^+-PPase) activity of the tonoplast were assayed. The plasmalemma ATPase was stimulated in parallel with dramatic increases in the intracellular concentration of Na^+($[Na^+]_{in}$). High $[Ca^{2+}]_{ex}$ prevented the increase in $[Na^+]_{in}$ and diminished the stimulation of ATPase activity. The tonoplast ATPase showed a rapid response to salt stress and was similarly stimulated even at high $[Ca^{2+}]_{ex}$. The activities of both ATPases were, however, insensitive to concentrations of Na^+ ions up to 100 mM. By contrast, H^+-PPase activity of the tonoplast was severely inhibited with increasing $[Na^+]_{in}$ under salt stress and recovered with high $[Ca^{2+}]_{ex}$. These findings suggest that high-NaCl stress increases the intracellular concentration of Na^+ ions in mung bean roots, which inhibits the tonoplast H^+-PPase, and the activity of the plasmalemma H^+-ATPase is thereby stimulated and regulates the cytoplasmic pH.

Key words: Ca^{2+} — Extrusion of protons — H^+-ATPase — H^+-pyrophosphatase — Mung bean (*Vigna mungo*) root — Salt stress.

Many species of higher plant suffer inhibition of growth in saline (generally high-NaCl) environments (salt stress). The salt stress is the result primarily of two factors: a decline in water uptake caused by the increase in external osmotic pressure (osmotic stress); and physiological disturbances in plant cells caused by an ionic imbalance in the cytoplasm of the plant cells (ionic stress) (Greenway and Munns 1980, Flowers et al. 1977). The plant cells can become adapted to osmotic stress via increases in the internal osmotic pressure of the cells, a process that is facilitated by the accumulation of solutes in their vacuoles. Osmotic adjustment is also a fundamental response of plant cells to salt stress.

Many important metabolic processes in living plant cells take place in the cytoplasm of the cells and are affected by levels of various cations, such as, Na^+, K^+, H^+, Mg^{2+} and Ca^{2+} ions. Intracellular compartmentalization of ions is, hence, an important component of salt adaptation of plant cells. It is widely assumed at present that the death of plant cells exposed to saline conditions is caused by a high ratio of Na^+ to K^+ ions in the cytoplasm, which is due

Abbreviations: ATPase, adenosine triphosphatase; BSA, bovine serum albumin; $[Ca^{2+}]_{ex}$, external concentration of Ca^{2+} ions; DCCD, *N,N'*-dicyclohexylcarbodiimide; DTT, dithiothreitol; EGTA, ethyleneglycol-*bis*-*N, N, N', N'*, tetraacetic acid; $[Na^+]_{in}$, intracellular concentration of Na^+ ions; PPase, pyrophosphatase.

to drastic increases in the influx of Na^+ ions into the cells and in the efflux of K^+ ions from the cells (Katsuhara and Tazawa 1986, 1988). The ability of plant cells to survive under saline conditions can be summarized as the ability to maintain normal ionic conditions (high K^+, low Na^+, neutral pH, and so on) in the cytoplasm of the cells. Plants have various mechanisms for controlling the cytoplasmic concentration of Na^+ ions, such as sequestration of the Na^+ ions into the vacuole via the action of a Na^+/H^+ antiporter at the tonoplast (Blumwald and Poole 1987, Garbarino and DuPont 1988) and pumping out Na^+ ions across the plasmalemma through Na^+/H^+ exchange mechanisms (Katz et al. 1986, Watad et al. 1986). The control of ion transport across the plasmalemma and the tonoplast is a key factor in salt tolerance of plant cells. In recent years, intensive studies of mechanisms of salt tolerance in plants have been conducted at the cellular level, and the importance of the control of ion transport across the plasmalemma and tonoplast in salt adaptation of plants has been demonstrated (Lerner 1985, Cramer et al. 1985, 1986, 1987, Katsuhara and Tazawa 1988, 1990, Katsuhara et al. 1989).

Numerous studies in recent years have proved evidence for the widespread existence of an electrogenic H^+-translocating ATPase in the plasmalemma and the tonoplast of higher plant cells (Sze 1985, Marrè and Denti 1985, Kasamo 1986a). The enzyme generates an electrochemical potential gradient of protons, which is believed to serve as the driving force for the transport of other solutes across both types of membrane (Poole 1978, Spanswick 1981). The H^+-ATPase plays a central role in the control of ion transport in plant cells. The enzyme also plays an important role in the homeostatic regulation of the conditions inside plant cells in response to environmental changes, for example, regulation of intracellular pH (Raven and Smith 1976, Smith and Raven 1979). It is to be expected that H^+-ATPases associated with the plasmalemma and tonoplast also make a significant contribution to salt adaptation of plant cells. There have been some investigations of the effects of external salinity on H^+-ATPase activity in salt-tolerant halophytes (Lerner et al. 1983, Braun et al. 1986, Matsumoto and Chung 1988, Brüggemann and Janiesch 1988): However, only studies have been performed using salt-sensitive plants (Chung and Matsumoto 1989).

The essential role of Ca^{2+} ions has been extensively documented with respect to various cellular functions that are associated with the growth and development of plants (Bangerth 1979, Roux and Slocum 1982, Hepler and Wayne 1985). In particular, regulation of membrane functions is thought to be one of the most important roles of Ca^{2+} ions in plant cells (Tazawa et al. 1987). Ca^{2+} ions have a significant effect on osmotic adjustment and on ion-selective transport, influencing the regulation of ion transporters such as K^+-channels and H^+-ATPase (Yapa et al. 1986, Tazawa et al. 1987, Katsuhara et al. 1989, Okazaki and Tazawa 1990). Ca^{2+} ions facilitate the salt tolerance of plant cells by controlling membrane permeability to Na^+ and K^+ ions (Cramer et al. 1986, 1987, Katsuhara and Tazawa 1986). We described in a previous report the protective effect of high $[Ca^{2+}]_{ex}$ on the elongation and maintenance of $[K^+]_{in}$ in intact mung bean roots (Nakamura et al. 1990).

In this study, we investigated the effect of salinity on the extrusion of protons from intact mung bean roots and the activities of H^+-ATPase and H^+-PPase associated with the plasmalemma and the tonoplast of the root cells at different $[Ca^{2+}]_{ex}$. A discussion is presented of the role of H^+-ATPase in salt tolerance of mung bean roots in relation to the mitigating effects of Ca^{2+} ions on salt stress.

Materials and Methods

Plant materials—Intact roots of mung bean [*Vigna mungo* (L.) Hepper] were used for experiments. Seedlings were cultivated for 48 h at 30°C in the dark, as described by Itoh et al. (1986). The cultivation medium consisted of a solution of 0.1 mM KCl and 0.5 mM $CaCl_2$. Two-day-old seedlings were then subjected to osmotic stress or to salt stress by transfer to the experimental cultivation medium which contained 200 mM mannitol or 100 mM NaCl. The seedlings were further incubated for 1 to 12 h under stress or under control conditions at 30°C in the dark.

Extrusion of protons from intact roots—Two-day-old seedlings were divided into three groups and transferred to three kinds of cultivation medium to serve a control, osmotic-stressed and salt-stressed seedlings. At 1, 3, 6 and 12 h after the transfer, 100 seedlings in each group were transferred to 300 ml of one of three kinds of experimental medium which had the same composition as the medium used for cultivation. The pH of the experimental medium was initially adjusted at 7.0 with 1 mM MES-Tris. The decrease in the pH of the medium after the transfer was measured with a glass combination pH-sensitive electrode and an estimation of the protons extruded was made by titration of the medium to pH 7.0 with 0.1 N KOH.

Preparation of membranes—About 4 g fresh weight of apical segments of 5 mm in length were collected from about 1,000 intact roots kept respectively under the control (0.1 mM KCl and 0.5 mM $CaCl_2$), osmotic-stress (plus 200 mM mannitol) and salt-stress (plus 100 mM NaCl) conditions and used for preparation of membranes. Plasmalemma and tonoplast vesicles were prepared by a modified version of the method of Kasamo (1988a). The segments of roots were homogenized in a grinding medium which consisted of 0.25 M mannitol, 25 mM HEPES-Tris (pH 7.5), 2 mM EGTA, 1 mM DTT and 1% (w/v) BSA. The homogenate was centrifuged at $1,500 \times g$ for 10 min and the supernatant was recentrifuged at $10,000 \times g$ for 30

min to generate a tonoplast-enriched pellet. The resulting supernatant was centrifuged at 80,000 × *g* for 30 min for preparation of a microsomal pellet. The two different pellets, for preparation of tonoplast and plasmalemma, respectively, were suspended in a gradient mixture which contained 0.25 M mannitol, 2.5 mM HEPES-Tris (pH 7.3), 1 mM EDTA and 1 mM DTT and subjected to centrifugation on a step gradient of dextran T70 (1%/8%) in the gradient mixture at 105,000 × *g* for 90 min. The plasmalemma-enriched and tonoplast-enriched membrane fractions were localized in the 8% dextran pellet and at the interface between 1% and 8% dextran, respectively (Mandala and Taiz 1985, Kasamo 1988a). The interface was collected and this fraction was further centrifuged at 140,000 × *g* for 40 min for preparation of the tonoplast fraction. The membrane fractions were stored at −20°C for 12 h before the assays of ATPase and PPase activity.

Assay of ATPase activity—The activity of H^+-ATPases was measured by the method described by Kasamo (1986). The reaction medium consisted of 3 mM Tris-ATP, 3 mM $MgSO_4$, 50 mM KCl, 30 mM MES and 2 μM gramicidin D, and the pH of the medium was adjusted to 6.5 and 7.0 for assays of the plasmalemma and tonoplast, respectively. Activities of ATPases associated with the plasmalemma and the tonoplast were assayed in the presence of 100 mM KNO_3 and 50 μM Na_3VO_4, respectively, to inhibit the other ATPase activity.

Assay of PPase activity—The activity of H^+-PPase associated with the tonoplast was assayed in a reaction medium that contained 1 mM $MgSO_4$, 50 mM KCl, 40 mM Tris-MES (pH 8.0) and 2 μM gramicidin D. The reaction was incubated for 30 min at 38°C, as in the case of the assay for ATPase activities.

Protein determination—Protein in the membrane vesicles was quantitated by the method of Bradford (1976) with BSA as the standard.

Measurement of the intracellular concentration of Na^+ ions—One to two hundred two-day-old seedlings cultivated for 24 h with 100 mM NaCl and various concentrations of $CaCl_2$ were transferred to a cold solution of 0.1 mM KCl in order to wash away Na^+ ions in the apoplast of the roots. After this solution had been blotted from the root surface, two kinds of segment were cut off with a razor blade: one from 0–5 mm (elongating region) and one from 10–15 mm (mature region) from the root tip. Cell sap from the segments was sampled and the concentration of Na^+ ions in the cell sap was measured by flame photometry as previously described (Nakamura et al. 1990).

Results

Extrusion of protons from intact mung bean roots—The extrusion of protons from intact mung bean roots was investigated after 1, 3, 6 and 12 h of incubation under various conditions. Figures 1-A and 1-B show the acidification of the experimental medium by the roots after 3-h and 12-h incubations, respectively. The roots incubated with 100 mM NaCl for 12 h markedly promoted the acidification of the medium, whereas the roots incubated with 200 mM mannitol had only a slight effect on the acidification.

Figure 2 shows the time course of changes in the rate of extrusion of protons, which was calculated on the basis of the fresh weight of the roots for $[Ca^{2+}]_{ex}$ of 0.5 and 5 mM. The rate of extrusion of protons from the control roots was almost constant, about 0.3 μmol (g fr wt)$^{-1}$ min^{-1}, except after an 1-h incubation with a high $[Ca^{2+}]_{ex}$ (5 mM). In the NaCl-stressed roots, the rate of extrusion of protons gradually increased to twice that in the control roots during the course of a 12-h incubation. Increasing $[Ca^{2+}]_{ex}$ from 0.5 mM to 5 mM suppressed the enhancement of the extrusion of protons under the high-NaCl stress. A similar increase in the external osmotic pressure, achieved by addition of 200 mM mannitol to the cultivation medium, had no significant effect on the rate of extrusion of protons, regardless of $[Ca^{2+}]_{ex}$.

The rate of extrusion of protons from the root cells under non-saline conditions was estimated for a range of $[Ca^{2+}]_{ex}$ from 0 mM to 10 mM. The extrusion of protons was slightly enhanced with increases in $[Ca^{2+}]_{ex}$ from 0.5 mM to 5 mM and was suppressed when $[Ca^{2+}]_{ex}$ was higher than 8 mM. The absence of Ca^{2+} ions from the medium repressed the extrusion of protons to a considerable extent, via injury to membranes that was induced by depletion of Ca^{2+} ions (Fig. 3).

The effects of inhibitors of H^+-ATPase on the extrusion of protons from intact mung bean roots were investigated (Fig. 4). The addition of DCCD at 50 μM to the

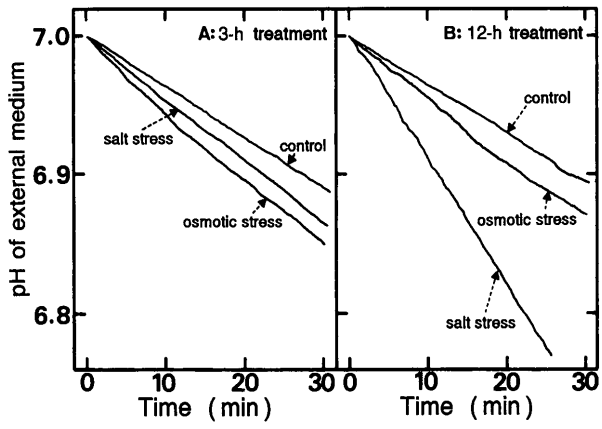

Fig. 1 Typical time course of decreases in the pH of the external medium when intact mung bean roots were incubated for 3 or 12 h under control (0.1 mM KCl, 0.5 mM $CaCl_2$), osmotic-stress (plus 200 mM mannitol) and salt-stress (plus 100 mM NaCl) conditions. The experiments were conducted six times and similar results were obtained.

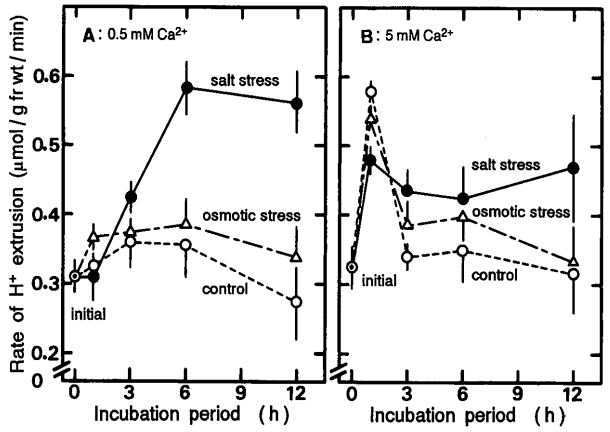

Fig. 2 Time course of changes in rates of extrusion of protons from intact mung bean roots incubated under various conditions in the presence of 0.5 mM (A) and 5 mM (B) Ca^{2+} ions. Values are the means of results from 6 experiments and the vertical bars represent standard deviations (SD).

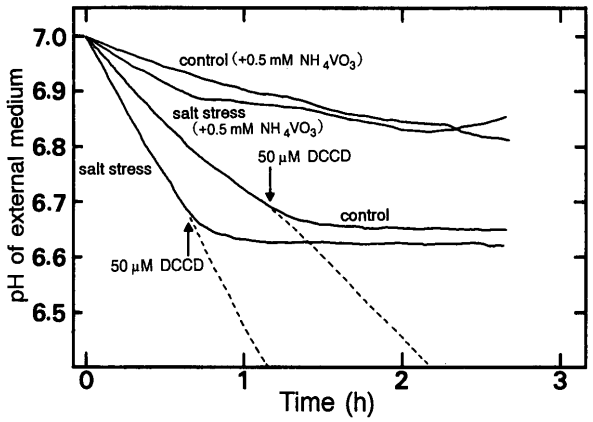

Fig. 4 Effect of inhibitors of H^+-ATPase activity on extrusion of protons from intact mung bean roots under control (0.1 mM KCl, 0.5 mM $CaCl_2$) and salt-stress (plus 100 mM NaCl for 12 h) conditions. Data show the kinetics typical of those obtained in three separate individual experiments.

external medium completely suppressed the decrease in pH of the medium in the case of both control and salt-stressed roots. Moreover, vanadate (NH_4VO_3) at 0.5 mM also suppressed the acidification of the external medium by the roots.

H^+-ATPase activities associated with the plasmalemma and tonoplast—Vesicles of plasmalemma and tonoplast were prepared from the control and stressed roots after 1, 3, 6 and 12 h of incubation in medium that contained 0.5 mM Ca^{2+} ions. The ATPase activities associated with the plasmalemma increased substantially during the high-NaCl stress, although the activity gradually increased even under the non-saline conditions (Fig. 5). The tonoplast ATPase showed a more rapid increase in activity just after the start of exposure to high-NaCl stress (after a 1- to 3-h incubation), and then it decreased to that in the tonoplast of control roots after 12 h of NaCl stress. Increased external osmotic pressure, achieved by addition of 200 mM mannitol to the medium, had no significant effect on the ATPase activities of either type of membrane throughout the incubation.

The stimulatory effect of external salinity on the H^+-ATPase activity of mung bean roots incubated for 3 or 12 h with 100 mM NaCl was compared for external levels of Ca^{2+} ions between 0.5 mM and 5 mM (Table 1). At 0.5 mM Ca^{2+}, the activity of the plasmalemma ATPase increased by 21% and 49% after incubation for 3 h and 12 h, respec-

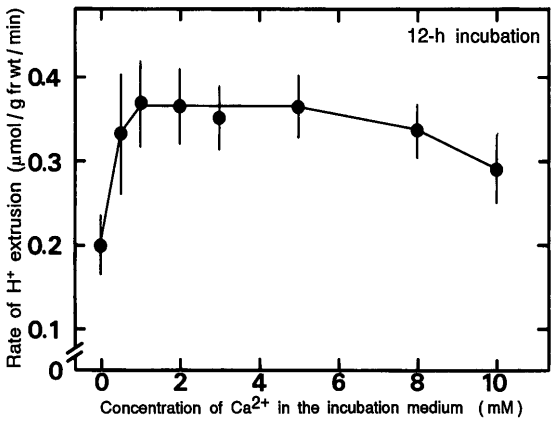

Fig. 3 Effects of the external concentration of Ca^{2+} ions on the rate of extrusion of protons from two-day-old mung bean roots incubated for 12 h under non-saline conditions. Values are the means of results from 5 individual experiments and the vertical bars represent SD.

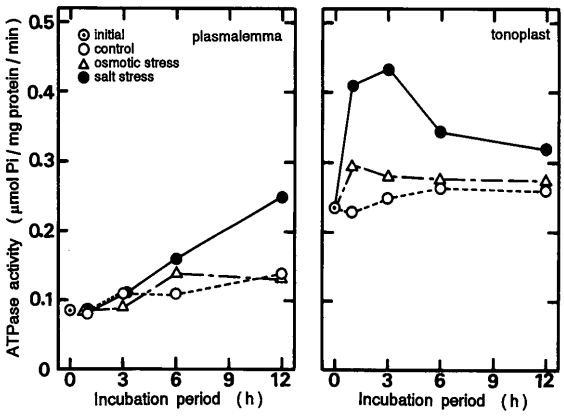

Fig. 5 Time course of changes in H^+-ATPase activity associated with the plasmalemma and the tonoplast of mung bean roots under various conditions with an external concentration of 0.5 mM Ca^{2+} ions.

Table 1 Effects of salinity on H^+-ATPase activities associated with the plasmalemma and tonoplast of intact mung bean roots incubated at low and high external concentrations of Ca^{2+} ions

	$[Ca^{2+}]_{ex}$	ATPase activity (μmol P_i (mg protein)$^{-1}$ min^{-1})			
		plasmalemma		tonoplast	
		0 mM NaCl (control)	100 mM NaCl (salt stress)	0 mM NaCl (control)	100 mM NaCl (salt stress)
3-h treatment	0.5 mM	0.156±0.017 (5)	0.188±0.016 (5)	0.243±0.021 (5)	0.343±0.023 (5)
	5 mM	—	0.147±0.012 (5)	—	0.155±0.014 (5)
12-h treatment	0.5 mM	0.193±0.017 (11)	0.287±0.037 (7)	0.295±0.024 (11)	0.387±0.030 (7)
	5 mM	0.183±0.024 (5)	0.212±0.018 (7)	0.251±0.046 (5)	0.362±0.061 (7)

ATPase activity was assayed in membrane fractions prepared from two-day-old roots incubated for 3 or 12 h with 0 or 100 mM NaCl. Data are shown as the mean±SD with the number of experiments given in parentheses.

tively. The ATPase activity of the tonoplast increased by more than 40% and 30% after 3 h and 12 h of incubation, respectively. By contrast, at 5 mM Ca^{2+}, the plasmalemma ATPase was slightly inhibited after a 3-h incubation and it was stimulated by only 10% after the 12-h incubation. Furthermore, the ATPase activity of the tonoplast was significantly depressed after a 3-h incubation and increased by about 20% after 12 h of the salt stress.

Figure 6 shows the effects of $[Ca^{2+}]_{ex}$ on the H^+-ATPase activity associated with the plasmalemma and tonoplast of the root cells under non-saline conditions. The activity of the H^+-ATPases associated with the plasmalemma and the tonoplast remained almost constant at $[Ca^{2+}]_{ex}$ from 0.5 mM to 8 mM and activities were inhibited at $[Ca^{2+}]_{ex}$ of 0 and 10 mM.

Addition of NaCl to the assay medium had less effect on the activities of the H^+-ATPases associated with the plasmalemma and the tonoplast of control and salt-stressed roots (Fig. 7).

Activity of H^+-PPase associated with tonoplast—The effects of high-NaCl stress on the activity of H^+-PPase associated with the tonoplast were examined at low (0.5 mM) and high (5 mM) external levels of Ca^{2+} (Table 2). At $[Ca^{2+}]_{ex}$ of 0.5 mM, the activity of the tonoplast H^+-PPase decreased to 78% and to 49% of that of the tonoplast of control roots (incubated with 0 mM NaCl) after 3 h and 12

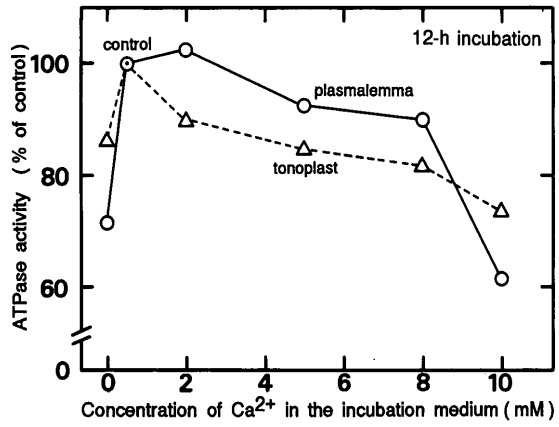

Fig. 6 Effects of the external concentration of Ca^{2+} ions on ATPase activities associated with the plasmalemma and the tonoplast in mung bean roots incubated with 0.1 mM KCl. The control values for ATPase activities of the plasmalemma and tonoplast were 0.199 and 0.260 μmol P_i (mg protein)$^{-1}$ min^{-1}, respectively. The results are typical of those obtained in two identical experiments.

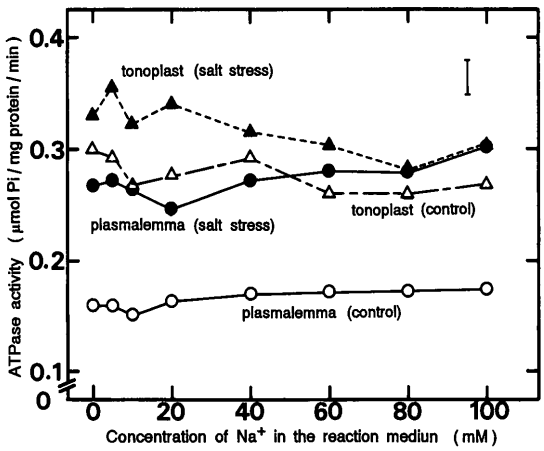

Fig. 7 Effects of the concentration of Na^+ ions in the reaction medium on the ATPase activity in mung bean roots incubated under saline and non-saline conditions at 0.5 mM Ca^{2+}. Activity at 0 mM NaCl is the control (100%). Values are the means of results of 3 individual experiments and the vertical bar represents the maximum standard deviation in all experiments.

Table 2 Effects of salinity on H^+-PPase activity of the tonoplast of intact mung bean roots at low and high external concentrations of Ca^{2+} ions

$[Ca^{2+}]_{ex}$	PPase activity (μmol P_i (mg protein)$^{-1}$ min^{-1})			
	3-h treatment		12-h treatment	
	0 mM NaCl (control)	100 mM NaCl (salt stress)	0 mM NaCl (control)	100 mM NaCl (salt stress)
0.5 mM	0.233±0.027 (3)	0.182±0.021 (3)	0.241±0.023 (3)	0.117±0.009 (3)
5 mM	—	0.230±0.025 (3)	—	0.282±0.039 (3)

PPase activity was assayed using tonoplast-enriched fractions prepared from roots conditioned as described in Table 1. Data are shown as the mean±SD with the number of experiments given in parentheses.

h of salt stress, respectively. At $[Ca^{2+}]_{ex}$ of 5 mM, the H^+-PPase was completely protected from the high-NaCl stress.

Figure 8 shows the effects of Na^+ ions on the activity of the H^+-PPase associated with the tonoplast of the roots under control conditions. In contrast to the results for H^+-ATPase activity, increasing concentrations of NaCl in the assay medium seriously inhibited the PPase activity of the tonoplast in the presence of 50 mM KCl.

Effects of high $[Ca^{2+}]_{ex}$ on the intracellular concentration of Na^+ ions in mung bean roots under high-NaCl stress—Figure 9 shows the time course of changes in intracellular concentrations of Na^+ ions in apical elongating and mature regions of intact mung bean roots exposed to salt stress of 100 mM NaCl. The intracellular concentration of Na^+ ions increased dramatically after the roots had been exposed to salt stress at $[Ca^{2+}]_{ex}$ of 0.5 mM (closed symbols). High levels of external Ca^{2+} ions (5 mM) were effective in preventing an increase in $[Na^+]_{in}$ during at least 18 h of salt stress (open symbols). The effects of $[Ca^{2+}]_{ex}$ on the intracellular concentration of Na^+ in mung bean roots subjected to salt stress at 100 mM NaCl are represented in Figure 10. Ca^{2+} ions at 5 mM in the medium were able fully to protect the root cells from increases in the intracellular concentration of Na^+ ions.

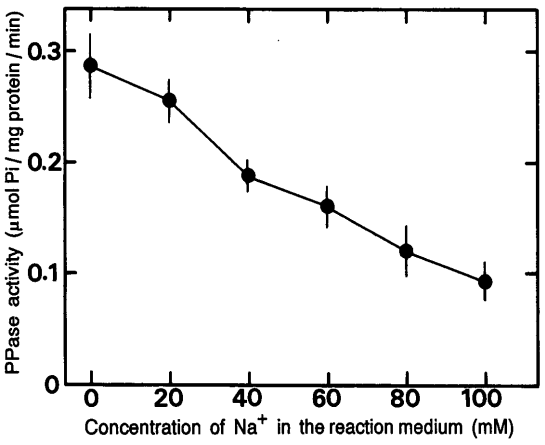

Fig. 8 Effects of the concentration of Na^+ ions in the assay medium on the PPase activity of the tonoplast of mung bean roots incubated under non-saline conditions. Values are the means of results from duplicate samples and the vertical bars represent SD.

Discussion

Stimulation of H^+-ATPases associated with the plas-

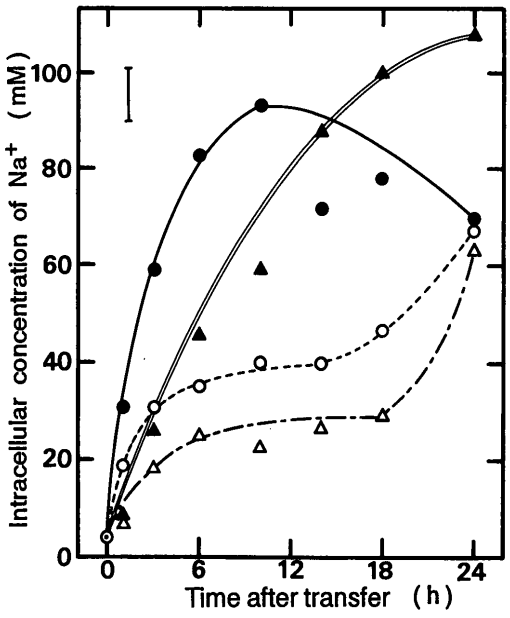

Fig. 9 Changes in intracellular concentrations of Na^+ ions in the elongating region of mung bean roots incubated with 0.5 mM (●) and 5 mM (○) Ca^{2+} ions, and in the mature region of the roots incubated with 0.5 mM (▲) and 5 mM (△) Ca^{2+} ions under high-NaCl conditions (100 mM NaCl). The symbol ⊙ represents the concentration of Na^+ ions in elongating and mature regions of roots before the treatment with 100 mM NaCl. Values are the means of results of 3 individual experiments and the vertical bar represents the maximal SD in all experiments.

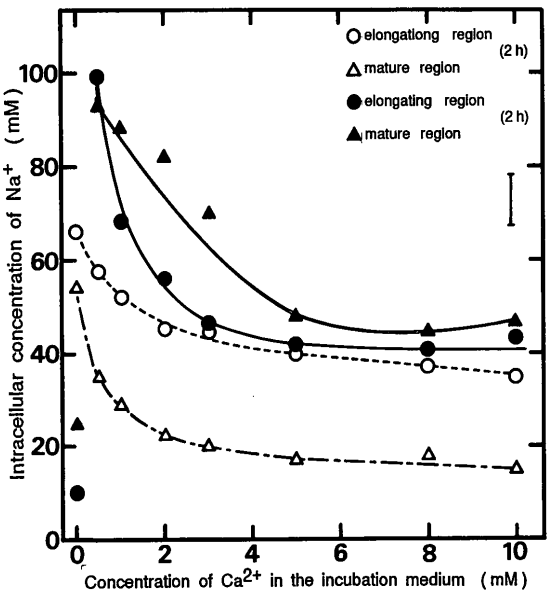

Fig. 10 Effects of the external concentration of Ca^{2+} ions on intracellular concentrations of Na^+ ions in mung bean roots incubated for 2 or 12 h under salt stress (100 mM NaCl). Values are the means of results of 4 individual experiments and the vertical bar represents the maximal SD.

malemma and tonoplast in intact roots under high-NaCl stress—The proton-extrusion activity of mung bean root cells was markedly stimulated, in a time-dependent manner, under saline conditions (Figs. 1, 2). Salt stress includes an osmotic effect, and some evidence for osmotically induced extrusion of protons from plant cells has been presented (Reinhold et al. 1984, Reuveni et al. 1987). In the present study, however, no obvious effects of increases in external osmotic pressure on the extrusion of protons were observed in the case of intact mung bean roots incubated with 200 mM mannitol, namely, in a solution that was isotonic to a solution of 100 mM NaCl. Moreover, 50 mM Na_2SO_4 had a similar effect on the extrusion of protons to that of 100 mM NaCl (data not shown). Therefore, the enhancement of the extrusion of protons from root cells that had been conditioned with 100 mM NaCl is due to the ion-specific effect of Na^+ ions. Nunes et al. (1983) and Watad et al. (1986) also reported the Na^+-induced stimulation of the efflux of protons from higher plant cells.

It has been suggested that the extrusion of protons involves the action of a proton-translocating ATPase (H^+-ATPase) that is associated with the plasmalemma (Mengel and Schubert 1985, Matsumoto 1988, Chen et al. 1990). In mung bean roots, specific inhibitors of H^+-ATPases, namely, DCCD and vanadate, completely suppressed the extrusion of protons from the control and the salt-stressed roots (Fig. 4). This observation suggests a close relationship between the extrusion of protons from the intact mung bean roots and the H^+-ATPase activity in the root cells. However, Watad et al. (1986) reported the vanadate-insensitive extrusion of protons, which they suggested was driven by a Na^+/H^+ antiport at the plasmalemma, in tobacco cultured cells incubated with 100 mM NaCl.

Figure 5 shows the substantial stimulation of the H^+-ATPase activity associated with the plasmalemma and the tonoplast under the high-NaCl stress. The stimulation of ATPase activity associated with plasmalemma was correlated with the enhancement with time of the extrusion of protons from the roots under saline conditions (Fig. 2A). The tonoplast ATPase showed an even more rapid response to the high-NaCl stress. Kasamo (1988b) reported that the H^+-ATPase associated with the tonoplast responded to low temperature prior to the response of the ATPase in the plasma membrane of rice cultured cells. The tonoplast H^+-ATPase seems to be more sensitive to changes in external conditions than the plasmalemma H^+-ATPase. The rapid stimulation of the tonoplast H^+-ATPase under salt stress might be advantageous for operation of the Na^+/H^+ antiport across the tonoplast.

Substitution of 100 mM NaCl by 50 mM Na_2SO_4 had a similar stimulatory effect on the ATPase activities of both the plasmalemma and tonoplast (data not shown), even though it has been reported by others that the tonoplast ATPase is stimulated by Cl^- ions (Churchill and Sze 1984, Griffith et al. 1986). The stimulation of ATPase activities associated with both types of membrane appears to be a Na^+-induced phenomenon.

Diminishing effect of high levels of external Ca^{2+} ions on the stimulation of H^+-ATPase activities under saline conditions in intact mung bean roots—The increase in $[Ca^{2+}]_{ex}$ to 5 mM diminished the extent of stimulation of H^+-ATPase activity under high-NaCl conditions (Table 1). The enhancement of the extrusion of protons from the roots under the high-NaCl stress was also suppressed by high $[Ca^{2+}]_{ex}$ (Fig. 2). The initial transitory activation of the extrusion of protons observed in the control and the stressed roots, has been suggested to be attributable to an influx of Ca^{2+} ions through the Ca^{2+}/H^+ antiport at the plasmalemma (Zocchi and Hanson 1983, Rasi-Caldogno et al. 1982, Sze 1985). Ca^{2+} ions were reported to have an inhibitory effect on the H^+-ATPase activity associated with the plasmalemma (DuPont et al. 1982, Bennett et al. 1985, Torimitsu et al. 1985), and it is possible that high $[Ca^{2+}]_{ex}$ may increase intracellular levels of Ca^{2+} ions and thereby suppress the ATPase activity in mung bean roots under saline conditions. However, the cytoplasmic level of Ca^{2+} ions in plant cells is said to be tightly regulated by various mechanisms (Williamson 1981): Indeed, no significant effects of $[Ca^{2+}]_{ex}$ on the extrusion of protons and the H^+-ATPase activity were observed in the mung bean roots over a range of concentrations from 0.5 mM to 8 mM (Figs. 3, 6). High $[Ca^{2+}]_{ex}$ indirectly affects the ATPase activities in

mung bean roots under NaCl-stress, with the effects being mediated by some other factors.

The interaction between Na^+ and Ca^{2+} ions at the plasmalemma of plant cells is considered to be an important factor in the effects of salt stress on plant: high external Na^+ ions displace Ca^{2+} ions from the plasmalemma and, thus, disrupts the membrane integrity, disturbing the mechanisms for the selective transport of ions across the membrane; and high $[Ca^{2+}]_{ex}$ protect the plant membrane from the adverse effects of Na^+ ions and regulates the permeability of the membrane (Cramer et al. 1985, 1986, Cramer and Läuchli 1986). High $[Ca^{2+}]_{ex}$ is known to control the influx of Na^+ ions into salt-stressed plant cells (Jacoby and Hanson 1985, Katsuhara and Tazawa 1986, Cramer et al. 1987). The treatment of mung bean roots with high $[Ca^{2+}]_{ex}$ under the salt stress of 100 mM NaCl brought about the control of intracellular levels of Na^+ ions in the roots (Figs. 9, 10). Unfortunately, we were unable to obtain any information about the ionic environment in the cytoplasm and vacuoles of the root cells separately. However, since less of the volume of the cells in the elongating regions was occupied with vacuoles (Torimitsu et al. 1985), we can speculate that the measurements from the elongating regions (0–5 mm from the root tip) and those from the mature regions (10–15 mm from the root tip) reflect, to a considerable extent, the concentration of Na^+ ions in the cytoplasm and the vacuole, respectively. It is suggested that the diminished stimulation of H^+-ATPase activity under high-NaCl stress with high $[Ca^{2+}]_{ex}$ is correlated with the control of the intracellular concentration of Na^+ ions that is induced by high $[Ca^{2+}]_{ex}$.

Mechanisms for activation of H^+-ATPase activities associated with the plasmalemma and tonoplast in mung bean roots under high-NaCl stress—As mentioned above, the activity of H^+-ATPases is expected to be affected by Na^+ ions. However, the ATPases associated with the plasmalemma and the tonoplast in mung bean root cells are rather insensitive to Na^+ ions at concentrations up to 100 mM (Fig. 7). Some factors other than a direct effect of Na^+ ions must stimulate the H^+-ATPase activities in salt-stressed mung bean roots.

Katsuhara et al. (1989) reported a decrease in cytoplasmic pH in salt-sensitive *Nitella* cells treated with 100 mM NaCl. They ascribed this cytoplasmic acidification to the inhibition of H^+-translocating pyrophosphatase (H^+-PPase) in the tonoplast that was induced by increases in the concentration of Na^+ ions with concomitant decreases in the concentration of K^+ ions in the cytoplasm of the salt-stressed cells. Takeshige and Hager (1988) showed that the PP_i-dependent transport of protons was seriously inhibited by a high concentration of Na^+ ions, whereas the ATP-dependent transport was relatively insensitive to high levels of Na^+ ions. Shimmen and MacRobbie (1987) also pointed out the essential role of K^+ ions in the PP_i-dependent transport of protons across the tonoplast of *Chara*. In mung bean roots, the tonoplast H^+-PPase was also inhibited by Na^+ ions, even in the presence of adequate levels of K^+ ions (Fig. 8), and high-NaCl stress induced dramatic increases in the concentration of Na^+ ions (Figs. 9, 10), which were accompanied by a remarkable decrease in the concentration of K^+ ions (Nakamura et al. 1990) in the root cells. The severe inhibition of H^+-PPase activity in the tonoplast of mung bean roots under the salt stress of 100 mM NaCl (Table 2) can, therefore, be putatively ascribed to the increase in levels of Na^+ ions in the root cells. High $[Ca^{2+}]_{ex}$ prevented the increase in the intracellular concentration of Na^+ ions (Figs. 9, 10) and, consequently, the H^+-PPase activity was maintained under the salt stress (Table 2). It is suggested that the inhibition of H^+-PPase activity leads to the suppression of the transport of protons across the tonoplast and results in cytoplasmic acidification in mung bean roots under high-NaCl stress. Moreover, operation of a Na^+/H^+ antiporter at the tonoplast, for sequestration of Na^+ ions in the vacuole in order to avoid what from salt stress, is also partly responsible for cytoplasmic acidification. Further investigations of proton-pumping activity across the tonoplast and changes in intracellular pH in mung bean roots under saline conditions are necessary. The cytoplasmic pH in cells of mung bean roots was estimated to be about 7.2 under normal conditions (Torimitsu et al. 1984). The optimal pH for activity of the H^+-ATPase associated with the plasmalemma in higher plants has been recognized to be about 6.5 (Anthon and Spanswick 1986, Kasamo 1986b). Thus, the decrease in cytoplasmic pH can be ascribed to stimulation of the H^+-ATPase associated with the plasmalemma in mung bean roots at $[Ca^{2+}]_{ex}$ of 0.5 mM under the high-NaCl stress due to the presence of 100 mM NaCl. Stimulation of proton-pumping activity at the plasmalemma, induced by cytoplasmic acidification, has been reported in other plant materials (Sanders et al. 1981, Felle and Bertl 1986). Brüggemann and Janiesch (1987) also suggested that an optimal pH for activity of the plasmalemma ATPase on the acidic side would be advantageous for the regulation of cytoplasmic pH in salt-tolerant plants.

The stimulation of the tonoplast ATPase cannot, however, be explained in terms of cytoplasmic acidification since the optimal pH for the tonoplast ATPase is around 7.2, being alkaline rather than acidic (Bennett et al. 1984, Poole et al. 1984). Furthermore, the activity of the tonoplast ATPase increased rapidly prior to any significant increase in $[Na^+]_{in}$, and high $[Ca^{2+}]_{ex}$ is less effective for the suppression of stimulation of ATPase activity in the tonoplast than in the plasmalemma (Table 1). These results suggest that the ATPase activity of the tonoplast is stimulated via some mechanisms different from those that stimulate the ATPase activity of the plasmalemma. The stimulation of the ATPase activity of the tonoplast might be related to

the operation of a Na^+/H^+ antiport system in the tonoplast for the control of the cytoplasmic concentration of Na^+ ions that is regulated for salt adaptation of plants (Matsumoto and Chung 1988, Reuveni et al. 1990).

The increase in activities of H^+-ATPases associated with the plasmalemma and tonoplast and the decline in activity of the tonoplast H^+-PPase were detected in reaction media specific for each enzyme, in the absence of Na^+ ions (Table 2), suggesting the modulation of certain properties of these enzymes, such as the affinity for their respective substrates and the rate of turnover. Further investigations are needed of the mechanisms of the catalytic activities of H^+-ATPase and H^+-PPase in mung bean roots under the salt stress.

In summary, stimulation of the H^+-ATPase activities associated with the plasmalemma and tonoplast was observed concomitant with dramatic increases in intracellular concentrations of Na^+ ions in intact mung bean roots under salt stress due to the presence of 100 mM NaCl. High $[Ca^{2+}]_{ex}$ prevented the influx of Na^+ ions into the root cells and diminished the stimulation of the ATPase activity associated with the plasmalemma. H^+-ATPases in both types of membrane were insensitive to the concentration of Na^+ ions, whereas the tonoplast H^+-PPase was severely inhibited by increases in the intracellular concentration of Na^+ ions under salt stress. The stimulation of H^+-ATPase activity of the plasmalemma in mung bean root cells under high-NaCl stress appears to be correlated with cytoplasmic acidification, via the inhibition of the H^+-PPase activity of the tonoplast by a dramatic increase in the intracellular concentration of Na^+ ions under high-NaCl stress.

The authors express their gratitude to Prof. T. Kishi of Keio University for allowing them to use an atomic absorption spectrophotometer and to Drs. K. Kuchitsu and K. Sakano of the National Institute of Agrobiological Resources for their helpful suggestions. They also wish to thank Dr. T. Sato of Keio University and Mr. Y. Irie for helping to prepare the manuscript, and Mr. N. Shimura, Mr. R. Morichi and Mr. K. Maki for their technical support. The authors are grateful to Dr. K. Itoh of Nippon Steel Corporation and Dr. K. Oka of Fujitsu Laboratories Ltd. and Dr. Y. Yazaki of the National Institute of Agrobiological Resources for their encouragement of our study. This work was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and by a grant from Ito Ham Food Incorporated.

References

Anthon, G. E. and Spanswick, R. M. (1986) Purification and properties of H^+-translocating ATPase from the plasma membrane of tomato roots. *Plant Physiol.* 81: 1080–1085.

Bangerth, F. (1979) Calcium-related physiological disorders of plants. *Annu. Rev. Phytopathol.* 17: 97–122.

Bennett, A. B., O'Neill, S. D., Eilmann, M. and Spanswick, R. M. (1985) H^+-ATPase activity from storage tissue of *Beta vulgaris*; modulation of ATPase activity by reaction substrates and products. *Plant Physiol.* 78: 495–499.

Bennett, A. B., O'Neill, S. D. and Spanswick, R. M. (1984) H^+-ATPase activity from storage tissue of *Beta vulgaris*. *Plant Physiol.* 74: 538–544.

Blumwald, E. and Poole, R. J. (1987) Salt tolerance in suspension cultures of sugar beet; induction of Na^+/H^+ antiport activity at the tonoplast by growth in salt. *Plant Physiol.* 83: 884–887.

Bradford, M. M. (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.

Braun, Y., Hassidim, M., Lerner, H. R. and Reinhold, L. (1986) Studies on H^+-ATPases in plants of varying resistance to salinity. *Plant Physiol.* 81: 1050–1056.

Brüggemann, W. and Janiesch, P. (1987) Characterization of plasma membrane H^+-ATPase from salt-tolerant and salt-sensitive *Plantago* species. *J. Plant Physiol.* 130: 395–411.

Brüggemann, W. and Janiesch, P. (1988) Properties of native and solubilized plasma membrane ATPase from the halophyte *Plantago crassifolia*, grown under saline and non-saline conditions. *Physiol. Plant.* 74: 615–622.

Chen, S. L., Chen, C. T. and Kao, C. H. (1990) Acidification of deionized water by roots of intactrice seedlings. *Plant Cell Physiol.* 31: 569–573.

Chung, G. C. and Matsumoto, H. (1989) Localization of the NaCl-sensitive membrane fraction in cucumber roots by centrifugation on sucrose density gradients. *Plant Cell Physiol.* 30: 1133–1138.

Churchill, K. A. and Sze, H. (1984) Anion-sensitive, H^+-pumping ATPase of oat roots; direct effect of Cl^-, NO_3^- and disulfonic stilbene. *Plant Physiol.* 76: 490–497.

Cramer, G. R. and Läuchli, A. (1986) Ion activities in solution in relation to Na^+–Ca^{2+} interactions at the plasmalemma. *J. Exp. Bot.* 37: 321–330.

Cramer, G. R., Läuchli, A. and Epstein, E. (1986) Effects of NaCl and $CaCl_2$ on ion activities in complex nutrient solutions and root growth of cotton. *Plant Physiol.* 81: 792–797.

Cramer, G. R., Läuchli, A. and Polito, V. (1985) Displacement of Ca^{2+} by Na^+ from the plasmalemma of root cells; a primary response to salt stress? *Plant Physiol.* 79: 207–211.

Cramer, G. R., Lynch, J., Läuchli, A. and Epstein, E. (1987) Influx of Na^+,K^+, and Ca^{2+} into roots of salt-stressed cotton seedlings; effects of supplemental Ca^{2+}. *Plant Physiol.* 83: 510–516.

DuPont, F. M., Giorgi, D. L. and Spanswick, R. M. (1982) Characterization of proton-translocating ATPase in microsomal vesicles from corn roots. *Plant Physiol.* 70: 1694–1699.

Felle, H. and Bertl, A. (1986) Light-induced cytoplasmic pH changes and their interrelation to the activity of the electrogenic proton pump in *Riiccia fluitans*. *Biochem. Biophys. Acta* 848:

176–182.

Flower, T. J., Troke, P. F. and Yeo, A. R. (1977) The mechanism of salt tolerance in halophytes. *Annu. Rev. Plant Physiol.* 28: 89–121.

Garbarino, J. and DuPont, F. M. (1988) NaCl induces a Na^+/H^+ antiport in tonoplast vesicles from barley roots. *Plant Physiol.* 86: 231–236.

Greenway, H. and Munns, R. (1980) Mechanisms of salt tolerance in nonhalophytes. *Annu. Rev. Plant Physiol.* 31: 149–190.

Griffith, C. J., Rea, P. A., Blumwald, E. and Poole, R. J. (1986) Mechanism of stimulation and inhibition of tonoplast H^+-ATPase of *Beta vulgaris* by chloride and nitrate. *Plant Physiol.* 81: 120–125.

Hepler, P. and Wayne, R. O. (1985) Calcium and plant development. *Annu. Rev. Plant Physiol.* 36: 397–439.

Itoh, K., Yamada, T., Ishikawa, H., Ohta, E. and Sakata, M. (1986) Role of K^+ and Cl^- in osmotic adjustment in roots and hypocotyls of intact mung bean seedlings. *Plant Cell Physiol.* 27: 1445–1450.

Jacoby, B. and Hanson, J. B. (1985) Controls on $^{22}Na^+$ influx in corn roots. *Plant Physiol.* 77: 930–934.

Kasamo, K. (1986a) Comparison of plasma membrane and tonoplast H^+-translocating ATPases in *Phaseolus mungo* L. roots. *Plant Cell Physiol.* 27: 49–59.

Kasamo, K. (1986b) Purification and properties of the plasma membrane H^+-translocating adenosine triphosphatase of *Phaseolus mungo* L. roots. *Plant Physiol.* 80: 818–824.

Kasamo, K. (1988a) Inhibition of tonoplast and plasma membrane H^+-ATPase activity in rice (*Oryza sativa* L.) culture cells by local anesthetics. *Plant Cell Physiol.*29: 215–221.

Kasamo, K. (1988b) Response of tonoplast and plasma membrane ATPases in chilling-sensitive and -insensitive rice (*Oryza sativa* L.) culture cells to low temperature. *Plant Cell Physiol.* 29: 1085–1094.

Katsuhara, M., Kuchitsu, K., Takeshige, K. and Tazawa, M. (1989) Salt stress-induced cytoplasmic acidification and vacuolar alkalization in *Nitellopsis obtusa* cells. *Plant Physiol.* 90: 1102–1107.

Katsuhara, M., Mimura, T. and Tazawa, M. (1989) Patch-clamp study on a Ca^{2+}-regulated K^+ channel in the tonoplast of the brackish characeae *Lamprothamnium succinctum*. *Plant Cell Physiol.* 30: 549–555.

Katsuhara, M. and Tazawa, M. (1986) Salt tolerance in *Nitellopsis obtusa*. *Protoplasma* 135: 155–161.

Katsuhara, M. and Tazawa, M. (1988) Changes in sodium and potassium in *Nitellopsis* cells treated with transient salt stress. *Plant Cell Environ.* 11: 71–74.

Katsuhara, M. and Tazawa, M. (1990) Mechanism of calcium-dependent salt tolerance in cells of *Nitellopsis obtusa*: role of intracellular adenine nucleotides. *Plant Cell Environ.* 13: 179–184.

Katz, A., Kaback, R. and Avron, M. (1986) Na^+/H^+ antiport in isolated plasma membrane vesicles from the halotolerant alga *Dunaliella salina*. *FEBS Lett.* 202: 141–144.

Lerner, H. R. (1985) Adaptation to salinity at plant cell level. *Plant and Soil.* 89: 3–14.

Lerner, H. R., Reinhold, L., Guy, R., Braun, Y., Hassidim, M. and Poljakoff-Mayber, A. (1983) Salt activation and inhibition of membrane ATPase from roots of the halophyte *Atriplex nummularia*. *Plant Cell Environ.* 6: 501–506.

Mandala, S. and Taiz, L. (1985) Partial purification of a tonoplast ATPase from corn coleoptiles. *Plant Physiol.* 78: 327–333.

Marrè, E. and Ballarin-Denti, A. (1985) The proton pumps of the plasmalemma and the tonoplast of higher plants. *J. Bioenerg. Biomembr.* 17: 1–21.

Matsumoto, H. (1988) Repression of proton extrusion from intact cucumber roots and the proton transport rate of microsomal membrane vesicles of the roots due to Ca^{2+} starvation. *Plant Cell Physiol.* 29: 79–84.

Matsumoto, H. and Chung, G. P. (1988) Increase in proton-transport activity of tonoplast vesicles as an adaptive response of barley roots to NaCl stress. *Plant Cell Physiol.* 29: 1133–1140.

Mengel, K. and Schubert, S. (1985) Active extrusion of protons into deionized water by roots of intact maize plants. *Plant Physiol.* 79: 344–348.

Nakamura, Y., Tanaka, K., Ohta, E. and Sakata, M. (1990) Protective effect of external Ca^{2+} on elongation and the intracellular concentration of K^+ in intact mung bean roots under high NaCl stress. *Plant Cell Physiol.* 31: 815–821.

Nunes, M. A., Correia, M. M. and Lucas, M. D. (1983) NaCl-stimulated proton efflux and cell expansion in sugar-beet leaf discs. *Planta* 158: 103–107.

Okazaki, Y. and Tazawa, M. (1990) Calcium ion and turgor regulation in plant cells. *J. Membr. Biol.* 114: 189–194.

Poole, R. J. (1978) Energy coupling for membrane transport. *Annu. Rev. Plant Physiol.* 29: 437–460.

Poole, R. J., Briskin, D. P., Kratky, Z. and Johnstone, R. M. (1984) Density gradient localization of plasma membrane and tonoplast from storage tissue of growing and dormant red beet. *Plant Physiol.* 74: 549–556.

Rasi-Caldogno, F., de Michelis, M. I. and Pugliarello, M. C. (1982) Active transport of Ca^{2+} in membrane vesicles from pea: Evidence for a H^+/Ca^{2+} antiport. *Biochim Biophys. Acta* 693: 287–295.

Raven, J. A. and Smith, F. A. (1976) Cytoplasmic pH regulation and electrogenic H^+ extrusion. *Curr. Adv. Plant Sci.* 8: 649–660.

Reinhold, L., Seiden, A. and Volokita, M. (1984) Is modulation of the rate of proton pumping a key event in osmoregulation? *Plant Physiol.* 75: 846–849.

Reuveni, M., Colombo, R., Lerner, H. R., Pradet, A. and Poljakoff-Mayber, A. (1987) Osmotically induced proton extrusion from carrot cells in suspension culture. *Plant Physiol.* 85: 383–388.

Reuveni, M., Bennett, A. B., Bressan, R. A. and Hasegawa, P. M. (1990) Enhanced H^+ transport capacity and ATP hydrolysis activityof the tonoplast H^+-ATPase after NaCl adaptation.

Plant Physiol. 94: 524–530.

Roux, S. J. and Slocum, R. D. (1982) Role of calcium in mediating cellular functions important for growth and development in higher plants. *Calcium and Cell Function*, vol. 3 Academic Press, Inc.

Sanders, D., Hansen, U.-P. and Slayman, C. (1981) Role of the plasma membrane proton pump in pH regulation in non-animal cells. *Proc. Natl. Acad. Sci. USA* 78: 5903–5907.

Shimmen, T. and MacRobbie, E. A. (1987) Characterization of two proton transport systems in the tonoplast of plasmalemma-permeabilized *Nitella* cells. *Plant Cell Physiol.* 28: 1023–1031.

Smith, F. A. and Raven, J. A. (1979) Cytoplasmic pH and its regulation. *Annu. Rev. Plant Physiol.* 30: 289–311.

Spanswick, R. M. (1981) Electrogenic ion pumps. *Annu. Rev. Plant Physiol.* 32: 267–289.

Sze, H. (1985) H^+-translocating ATPases; advances using membrane vesicles. *Annu. Rev. Plant Physiol.* 36: 175–208.

Takeshige, K. and Hager, A. (1988) Ion effects on H^+-translocating adenosine triphosphatase and pyrophosphatase associated with the tonoplast of *Chara corallina*. *Plant Cell Physiol.* 29: 649–657.

Tazawa, M., Shimmen, T. and Mimura, T. (1987) Membrane control in the characeae. *Annu. Rev. Plant Physiol.* 38: 95–117.

Torimitsu, K., Hayashi, M., Ohta, E. and Sakata, M. (1985) Effect of K^+ and H^+ stress and role of Ca^{2+} in the regulation of intracellular K^+ concentration in mung bean roots. *Physiol. Plant.* 63: 247–252.

Torimitsu, K., Yazaki, Y., Nagasuka, K., Ohta, E. and Sakata, M. (1984) Effect of external pH on the cytoplasmic and vacuolar pHs in mung bean root-tip cells; a ^{31}P nuclear magnetic resonance study. *Plant Cell Physiol.* 25: 1403–1409.

Watad, A. E. A., Pesci, P. A., Reinhold, L. and Lerner, H. R. (1986) Proton fluxes as a response to external salinityin wild type and NaCl-adapted *Nicotiana* cell lines. *Plant Physiol.* 81: 454–459.

Williamson, R. E. (1981) Free Ca^{2+} concentration in the cytoplasm: a regulator of plant cell function. *What's New Plant Physiol.* 12: 45–48.

Yapa, P. A. J., Kawasaki, T. and Matsumoto, H. (1986) Changes of some membrane-associated enzyme activities and degradation of membrane phospholipids in cucumber roots due to Ca^{2+} starvation. *Plant Cell Physiol.* 27: 223–232.

Zocchi, G. and Hanson, J. B. (1983) Calcium transport and ATPase activity in microsomal vesicle fraction from corn roots. *Plant Cell Environ.* 6: 203–209.

(Received April 26, 1991; Accepted December 13, 1991)