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The Regulatory Role of Plasma Membrane Proton-Pumping ATPase in Salt Tolerance of Soybean Plant Growing Under the Salt-Stress Condition

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ABSTRACT: The role of plasma membrane H^+-pumping ATPase in the tolerance to salt stress of soybean plants were studied in the present study. Salt-acclimated plants which had previously acclimated to salt-stress condition accumulated higher concentration of Na^+ in root cells than that of the control and non-acclimated plants. The level of Na^+ accumulation in root cells correlated with the inside-acid proton gradient (ΔpH) across the plasma membrane vesicles. The salt-acclimated plants had higher antiport activity of Na^+ influx /H^+ efflux and H^+-pumping ATPase activity than that of control and non-acclimated plants. It may be concluded that higher H^+-ATPase activity favored salt-acclimated plants growing under the salt-stress environment.

KEY WORDS: Plasma membrane, Proton- pumping ATPase, Proton gradient, Fluorescence, Salt-acclimation, Salt accumulation, Soybean.

INTRODUCTION

Certain plants grow well even under water-stress or salt-stress conditions. These plants usually maintain higher osmotic pressure or lower water potential intracellularly (Greenway and Munns, 1980). Osmolytes for the accumulation and regulation of the intracellular osmotic pressure or water potential are sorbitol (Ahmad *et al.*, 1979), proline (Schobert and Tschesche, 1978), sucrose (Buckhout, 1994), mannitol (Tarczynski *et al.*, 1993), and ions. Intracellular accumulation of Na^+ is considered the characteristics of halophytes and halotolerant algae (Greenway and Munns, 1980; Blumwald and Poole, 1987; Hassidim *et al.*, 1990; Katz *et al.*, 1991, 1992). However, it has been proposed that salt tolerant plant cells usually maintain low level of intracellular Na^+. Schachtman *et al.* (1989) reported that the salt-tolerant species of wheat accumulated lower level of Na^+ than did the salt-sensitive species. Hoffmann and Bisson (1986) reported that aquatic plants, *Chlorophytes,* were able to export intracellular Na^+ across the plasma membrane to reduce the accumulation of Na^+ in the cytoplasm. Kiegle and Bisson (1996) found the amount of Na^+ efflux of saline cultured *Charahat longifolia* had been 31-fold as high as the fresh- water-adapted *C. coralline*. In

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association with the Na^+- influx or efflux, K^+-uptake (Watad *et al.*, 1991) or Ca^{++}-uptake (Perez-Pratet *et al.*, 1992; Wimmers *et al.*, 1992) was enhanced in NaCl-adapted higher plants. However, Flowers *et al.* (1977) showed that the harmful effects of high salinity are resulted from intracellular accumulation of Na^+. In regulating the uptake of Na^+ into halophyte, *Atriplex nummulari* (Braun *et al.*, 1986; Hassidim *et al.*, 1990) halotolerant algae, *Dunaliella salina* (Katz *et al.*, 1991, 1992), and *Heterosigma akashiwo* (Wada *et al.*, 1989) enzyme H^+-ATPase plays an important role. This enzyme also regulates Ca^{++}-uptake (Lynch *et al.*, 1989; Perez-pratet *et al.*, 1992; Wimmers *et al.*, 1992), or K^+-transport into glycophytes (Hassidim *et al.*, 1990) under the salt-stress conditions. However, Rea *et al.* (1992) reported that pyrophosphatase also involves in H^+-pumping and ion uptake. From statements above, it seems not to exist a conclusive salt-tolerance mechanism in plants. In this study, it attempted to figure-out the adaptation of soybean plants to salt-stress conditions is intimately controlled by the accumulation of sodium in root cells, and the Na^+ influx is modulated by plasma membrane H^+-ATPase activity.

MATERIALS AND METHODS

Plant Material

Four-day-old soybean seedlings (*Glycine max* cv. Kaoshiung #10) were transplanted to hydroponic culture system. The roots were submerged in one-fourth strength of Hoagland's nutrient solution and aerated with air at a rate of 30 mL per min. The water potential of nutrient solution was adjusted constantly to –0.8 MPa with saturated NaCl solution. Four days later, plants were divided into three groups: (a) Control group; Plants were further grown in nutrient solution with a consistent water potential of -0.8 MPa for eight days. (b) Salt acclimated group; Plants were transferred from a water potential of -0.8 MPa to -1.2 MPa and were grown for 4 days. Then they were transferred to the solution with -1.6 MPa and grown there. (c) Salt non-acclimated group; Plants were transferred directly from -0.8 MPa to 1.6 MPa after they had grown in a solution of -0.8 MPa for eight days. The water potentials of nutrient solutions were checked daily with digital thermocouple psychrometer, and adjusted to desired values using saturated NaCl solution. Conditions in the growth chamber were: light/ dark period 16:8 hrs; day/night temperature, 28/21°C; light intensity, 734 μEinstein $m^{-2}sec^{-1}$.

Preparation of Plasma Membrane Vesicle

At the end of each treatment as described above, the root systems were removed from plants and were cut into small sections. After washing with ice-cooled distilled water, the root sections were used to isolate and purify the plasma membrane vesicles as the procedures described by Blumwald and Poole (1987) with small modification. One hundred gram of root sections were homogenized in the medium containing 5 mM dithiothreitol, 5 mM EDTA, 5 mM $MgSO_4$, 1 mM PMSF, 30 mM Tris (pH 8.0), 0.5 mM butylated hydroxyltoluene, 0.5 mM dibucaine, 0.25 M sorbitol, and 5% PVP . The homogenate was filtered through four layers of cheesecloth and was centrifuged at 8,000g for 10 min at 4°C. Subsequently, the supernatant was centrifuged at 80,000g for 10 min. The pellets were

suspended in medium containing 1.0 M glycerol, 0.5 mM dibucaine, 0.5 mM butylated hydroxytoluene, 3 mM DTT, 10 mM Tris-Mes (pH 7.5), and then were centrifuged at 80,000g for 30 min. The pellets were further purified using a discontinuous sucrose density gradients (10% to 45%) and collected by centrifugation 80,000g for 30 min. This membrane vesicles which contain 3-5 mg of membrane protein per mL were suspended in 0.25 M sucrose, 0.2 % BSA, 0.5 mM mercaptoethanol, and 1 mM Tris-Mes (pH 6.0). They were stored under liquid nitrogen until needed for assay (Briskin *et al.*, 1995). The whole isolation procedure was performed at 4°C.

Plasma Membrane H^+-ATPase (PM H^+-ATPase) Assay

The enzyme activity was measured as the release of Pi from ATP. One ml reaction medium contained 30 mM Tris-Mes (pHs adjusted to specified value from 5.5 to 8.0 at a 0.5 unit intervals), 3 mM ATP, 2.5 mM mercaptoethanol, 50 mM KCl, 3 mM $MgSO_4$, 25 mM KCl, 5 μM valionmycin, 1 mM NaN_3, 100 mM KNO_3 and 0.1 mM Na-molybdate. NaN_3, KNO_3, and Na-molybdate were used to inhibit mitochondrial ATPase, tonoplast ATPase, and acid phosphatase, respectively (Grandmougin - Ferjani *et al.*, 1997). The reaction was started by the addition of 10 μg membrane protein. After incubation in water bath at 28°C for 30 min, the reaction was terminated by 1 ml of 33% trichloroacetic acid (w/v). Release of Pi was measured colorimetrically according to the procedures described by Staal *et al.* (1991). A control was run without membrane proteins. In all measurements, the ATPase activity was determined with and without vanadate, which is a specific inhibitor of plasma membrane H^+-ATPase. The difference between these two activity was taken as actual H^+-ATPase activity. To measure the effect of NaCl concentration on the PM ATPase activity from salt-acclimated plant, NaCl was added to the assay medium to final concentrations of 30, 60, 90, 120, 150 and 180 mM, respectively.

Measurement of $^{22}Na^+$ Influx and Efflux

The procedures using the measurement of $^{22}Na^+$ influx into and efflux out of roots were followed the method of Kiegle and Bisson (1996) but with modification. For measuring the effect of pH on $^{22}Na^+$ influx, the root sections were pre-incubated in a K^+-free solution containing 20 mM NaCl and $^{22}Na^+$ (9 μCi/mL, 333 kBq) at pH 5.5 (pHi). After the influx of $^{22}Na^+$ into root sections was reached to a steady state, the extracellular pH in incubation medium(pH_o) was then changed to pH 6.0, 6.5, 7.0. 7.5 and 8.0. After 30 min incubation, a half portion of root sections were rinsed twice with a non-radioactive NaCl solution, blotted dry on filter paper, and then weighted. The radioactivity in root sections were then counted as the method described by Cooper (1977). To measure the $^{22}Na^+$ efflux, another half-portion of the root sections were removed from the radioactive solution, rinsed in a non-radioactive NaCl solution, reloaded in a fresh, non-radioactive NaCl solution. After an incubation for 30 min, five mL of extracellular solution was taken for counting of radioactivity (Cooper, 1977).

Proton Transport Assay

The procedures described by Blumwald and Poole (1987) were used to generate (inside-acid) a pH gradient across the plasma membrane vesicles (ΔpH). ATPase-dependent H^+-efflux out of the plasma membrane vesicles was measured by the change in quenching of

acridine orange fluorescence. Twenty μg plasma membrane vesicles was added to 1.0 mL solution containing 250 mM mannitol, 15 mM K-gluconate, 1 mM DTT, 250 mM glycerol, 10 mM Tris-Mes (pH 8.0), 5 mM $MgSO_4$ and 5 μM acridine orange. The reaction was started by addition of ATP to a final concentration of 1 mM. The change in absorbance of acridine orange was monitored using spectrofluorometer (Shimazu, RF 5000) with excitation and emission wavelength at 496 and 540 nm, respectively. The dissipation of ΔpH by Na^+-uptake was measured by recovery of acridine orange fluorescence (Blumwald and Poole, 1987). An aliquot of 0.5 M Na_2SO_4 solution was added to initiate Na^+/H^+ antiport activity. The Na^+ /H^+ antiport activity was monitored by change in recovery of acridine orange fluorescence.

Protein Determination

The protein concentrations were measured using the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

RESULTS

Plasma membrane H^+-pumping ATPase (PM H^+-ATPase) activity was affected by changes of pH(s) in extracellular incubation medium. The optimum pH for this enzyme was about 6.5. The plants which had previously acclimated to salt-stress possessed higher activities of PM H^+-ATPase than those plants without salt-acclimated- and the control-plants. The relative activity of this enzyme in the roots of acclimated- and non-acclimated-plant at optimum pH was about 117 and 39% of the control plants (Fig. 1), respectively. The Na^+-influx into the root cells of salt-acclimated and control plants increased with elevating pH in incubation medium. The rates of Na^+-influx into these two groups of plants increased with increasing inside-acid the proton gradient (ΔpH = pHo - pHi) from 0.5 to 3.0 units. However, changes of ΔpH values had little effect on the Na^+ influx into the non-acclimated plants. The relative amounts of Na^+ influx into the acclimated-, and non-acclimated- plant at 3.0 units were 115%, and 28% of the control plants (Fig. 2). The Na^+ efflux out of the root sections was also affected by the change in ΔpH across the root sections. As shown in Figure 3, rates of Na^+ efflux in the acclimated- and control plants were lower than those measured in non-acclimated plants at ΔpH ranged between 0.5 and 3.0 .units. The relative amount of Na^+-efflux out of acclimated- and control- plants were 3.9- and 2.6% of the non-acclimated plants at the ΔpH 3.0 unit. The specific activities of PM H^+-ATPase were changed with the extracellular Na^+ concentrations. Enzyme activity in acclimated plants increased when Na^+ concentration was increased from 30 to 120 mM (Fig. 4), and reached its maximum at 120 mM. The enzyme activity was also indicated by the levels of generation of ΔpH, and the changes of ΔpH were traced by the quenching of acridine orange fluorescence in the presence of MgATP. There were about 4%, 17% and 21% quenching of the initial fluorescence were observed for the PM vesicles isolated from non-acclimated, control, and acclimated plant, (Fig. 5-A) respectively. These results indicated that PM vesicles were allowed to pump out H^+ as soon as the MgATP was added to reaction mixtures and the PM vesicles derived from the acclimated plants had highest H^+-pumping capability comparing

with the control and non-acclimated plants. And the vesicles from non-acclimated plants had lowest activity. Fig. 5-B indicates the. recovery of fluorescence of the PM vesicles occurred when Na^+ was added to reaction medium. This indicates that Na^+ influx into the vesicles was at the cost of dissipation of ΔpH. The acclimated plants had higher antiport activity of Na^+-influx/H^+-efflux than of control plants. In contrast, the non-acclimated plants had the lowest Na^+/H^+ antiport activity.

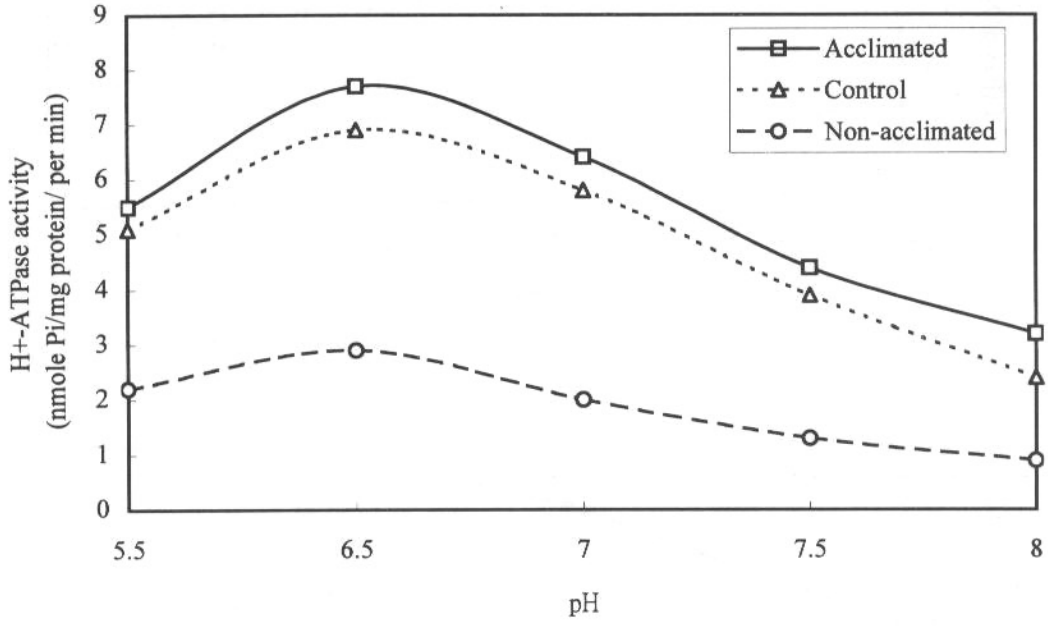

Fig. 1. Effect of pH of assay medium on the plasma membrane H^+-ATPase activity.

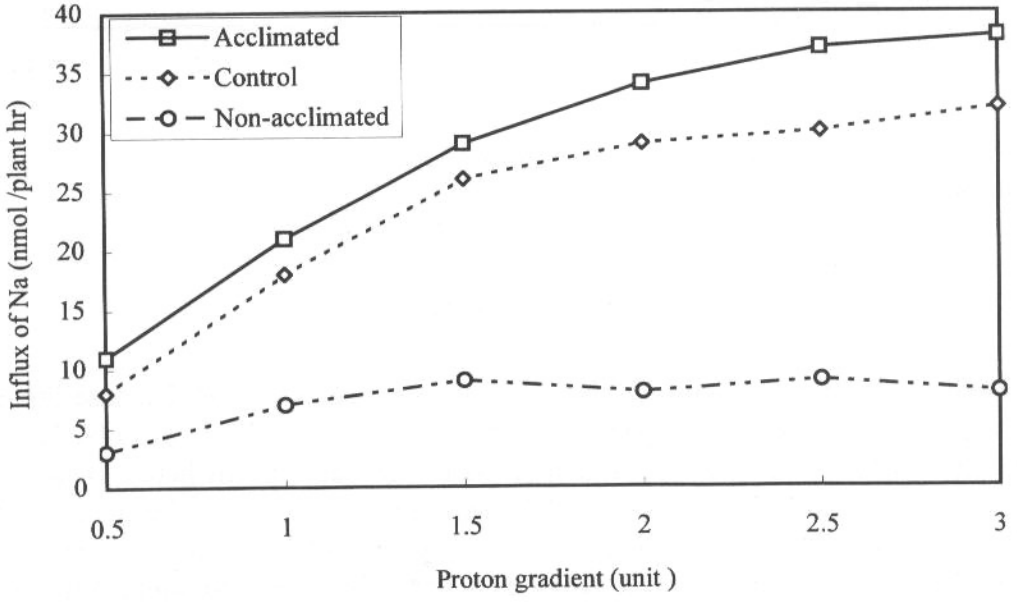

Fig. 2. Effect of inside-acid proton gradient on the influx of Na^+ into the root cells.

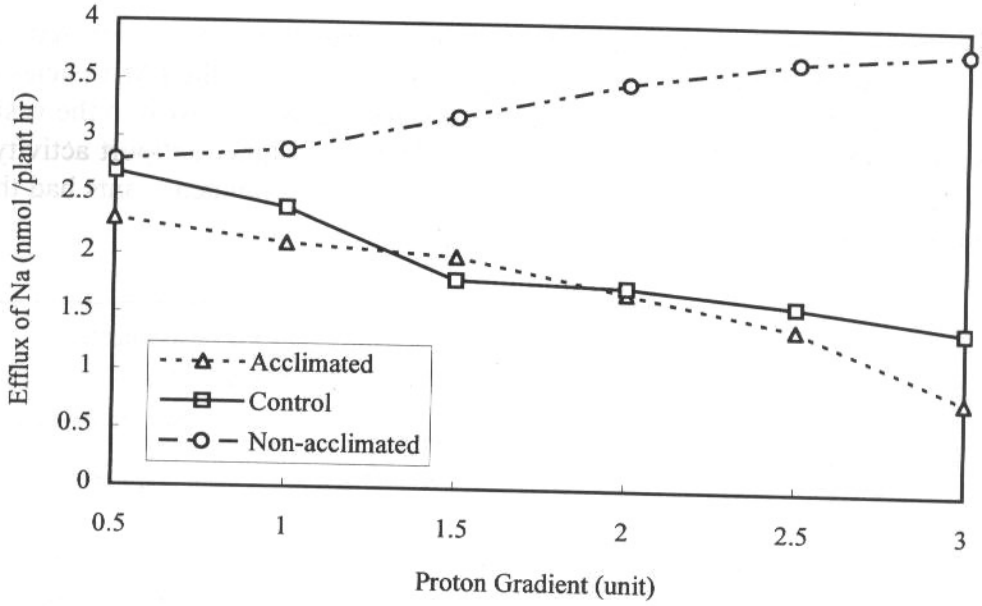

Fig. 3. Effect of inside-acid proton gradient on the efflux of Na^+ from roots.

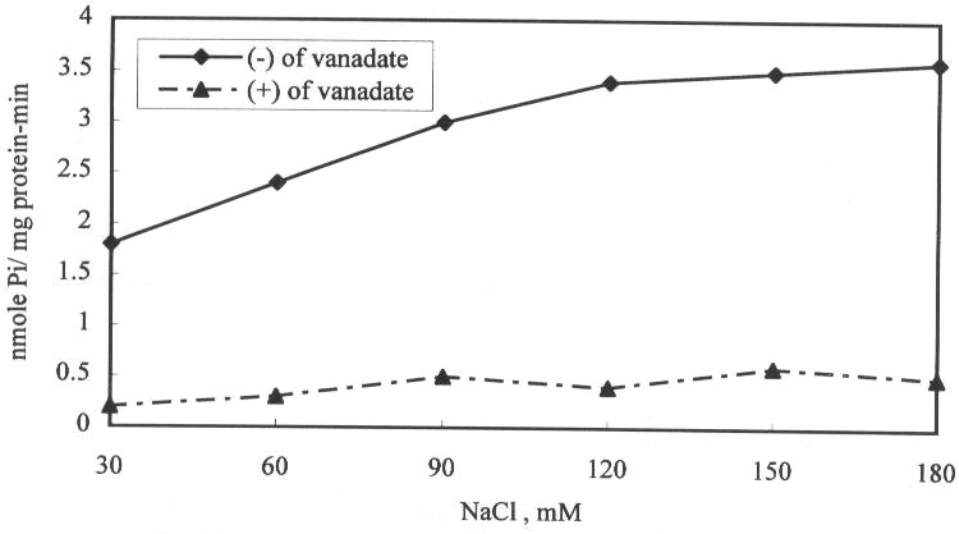

Fig. 4. Effect of NaCl concentration in assay medium on the plasma membrane H^+-ATPase activity of salt-accumulated plants.

DISCUSSION

The preliminary surveys indicated that this enzyme locates on PM, because this enzyme activity was little affected by the specific inhibitors of mitochondiral, vacuolar H^+-ATPase, and acid phosphatase, NaN_3, KNO_3, and molybdate (no data shown here). Besides, the Pi measured was the hydrolytic product of ATP catalyzed by H^+-ATPase, because this enzyme activity was severely inhibited by vanadate but not by Na-molybdate, an specific inhibitor of acid phosphatase (Fig. 4). This result provided a further evidence to support the results of preliminary studies. The occurrence of fluorescence was also inhibited by the vanadate (Fig.

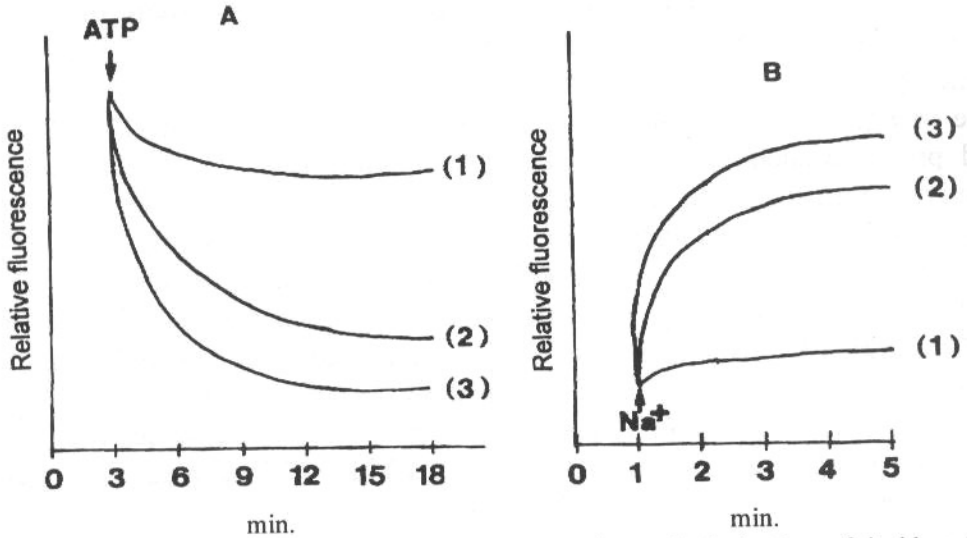

Fig. 5. Changes of acridine orange fluorescence by generation and dissipation of inside-acid ΔpH. A. Generation of inside-acid ΔpH by MgATP monitored by quenching of fluorescence; B. Dissipation of ΔpH by Na^+ monitored by recovery of fluorescence. (1)Non-acclimated plants, (2)Control plants, (3)Acclimated plamts.

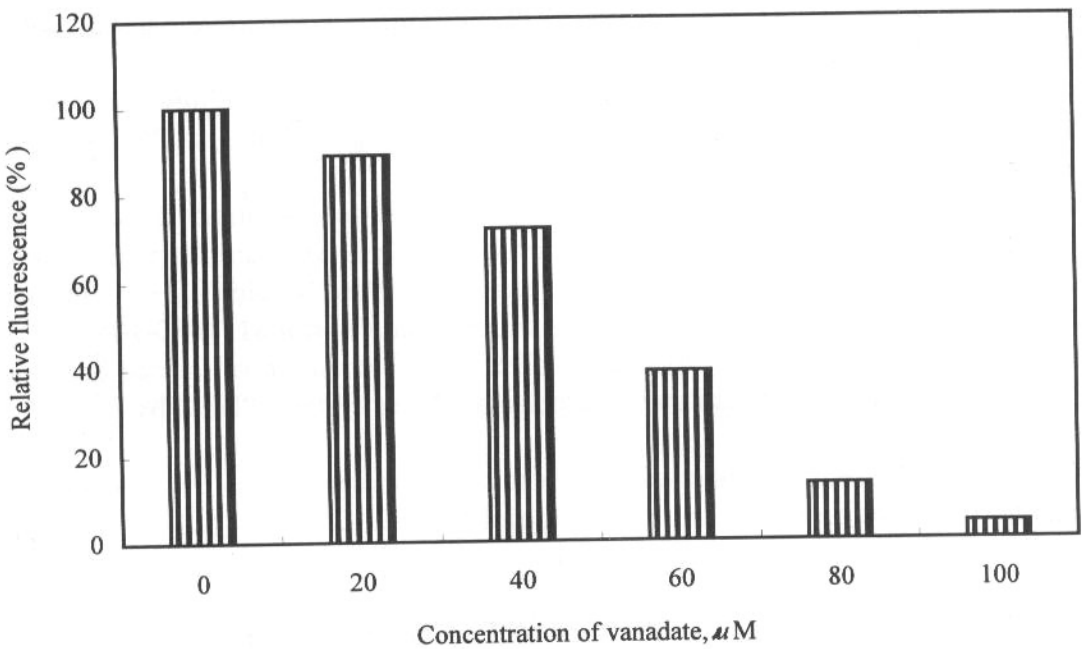

Fig. 6. Effect of vanadate concentrations in assay medium on reduction of fluorescence. The PM vesicles were isolated from acclimated plants.

6). The level of salt accumulation in root cells was regulated by change in inside-acid proton gradient. The ratio of Na^+ influx in the root cells of the acclimated plants to that in the non-acclimated plants increased when ΔpH was elevated. On the contrary, the ratio of Na^+ efflux in the acclimated plants to that in the non-acclimated plants decreased when ΔpH was elevated. This suggests that the generation of inside-acid proton gradient across the plasma membrane much more favors the uptake and accumulation of Na^+ in the salt-acclimated root

cells. Besides, the salt-acclimated plants have higher H^+-ATPase activity than control and non- acclimated plants (Fig. 1). So, there existed an intimately relationship among H^+-ATPase, the ΔpH, and Na^+-uptake into the root cells. The change of fluorescence quenching of acridine orange provides further evidences to support the levels of H^+-ATPase activity modulated proton-gradient across the plasma membrane (Fig. 5). The highest PM H^+-ATPase activity from acclimated plant induced the highest H^+ efflux, and the lowest PM H^+-ATPase activity from non-acclimated plants had the lowest H^+ efflux (Fig. 5-A). It is known that PM H^+-ATPase generates a proton gradient (ΔpH) across the vesicles by hydrolysis of ATP (Michelet and Boutry, 1995). The ΔpH formation provides the driving force for antiport activity of Na^+ influx/H^+ efflux (Fig. 5-B) and these consequently favored plants to uptake and accumulate Na^+. The acridine orange fluorescent quenchings were reduced by vanadate (Fig. 6). These results also support that H^+-ATPase in this study mainly derived from plasma membrane. Therefore, it may be concluded that the PM H^+-ATPase plays an important role in salt-acclimated plants to tolerate the salt-stress environment.

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大豆生長於鹽逆境下細胞膜 H^+-ATPase 活性對其耐鹽性的調節功能

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摘　　要

大豆幼苗分成三個實驗組，即對照組、鹽逆境馴化組、及未馴化組。馴化組植物的根細胞中 Na^+有累積的現象，其細胞中所含 Na^+濃度均比對照組及未馴化組植物高。Na^+的累積受到細胞膜內外的氫離子濃度梯度所影響，因為馴化組植物比其他兩組植物具有較高的氫離子濃度梯度以及較高 Na^+/H^+的互換輸送活性。細胞膜內外的氫離子濃度梯度的形成受到膜 H^+-ATPase 活性所調控，而馴化組植物比其他倆組植物也具有較高的 H^+-ATPase 活性, 因此可歸納而言，高 H^+-ATPase 活性對植物耐監性有重大的影響。

關鍵詞：細胞膜，H^+-ATPase，氫離子梯度，螢光，鹽馴化，鹽累積，大豆。

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