



Increased vacuolar Na^+/H^+ exchange activity in *Salicornia bigelovii* Torr. in response to NaCl

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Abstract

Shoots of the halophyte *Salicornia bigelovii* are larger and more succulent when grown in highly saline environments. This increased growth and water uptake has been correlated with a large and specific cellular accumulation of sodium. In glycophytes, sensitivity to salt has been associated with an inability to remove sodium ions effectively from the cytoplasm in order to protect salt-sensitive metabolic processes. Therefore, in *Salicornia bigelovii* efficient vacuolar sequestration of sodium may be part of the mechanism underlying salt tolerance. The ability to compartmentalize sodium may result from a stimulation of the proton pumps that provide the driving force for increased sodium transport into the vacuole via a Na^+/H^+ exchanger. In current studies, increased vacuolar pyrophosphatase activity (hydrolysis of inorganic pyrophosphate and proton translocation) and protein accumulation were observed in *Salicornia bigelovii* grown in high concentrations of NaCl. Based on sodium-induced dissipation of a pyrophosphate-dependent pH gradient in vacuolar membrane vesicles, a Na^+/H^+ exchange activity was identified and characterized. This activity is sodium concentration-dependent, specific for sodium and lithium, sensitive to methyl-isobutyl amiloride, and independent of an electrical potential. Vacuolar Na^+/H^+ exchange activity varied as a function of plant growth in salt. The affinity of the transporter for Na^+ is almost three times higher in plants grown in high levels of salt ($K_m = 3.8$ and 11.5 mM for plants grown in high and low salt, respectively) suggesting a role for exchange activity in the salt adaptation of *Salicornia bigelovii*.

Key words: Euhalophyte, *Salicornia bigelovii*, salt tolerance, vacuolar H^+ -pyrophosphatase, vacuolar Na^+/H^+ exchange.

Introduction

Traditionally, studies directed toward understanding salt tolerance have investigated the response of glycophytes (plants that are sodium (Na^+)-sensitive) to salt stress. For example, these studies have focused on comparisons of ion-transporting activities of closely related species grown in the presence of high NaCl concentrations (Schachtman *et al.*, 1991; Staal *et al.*, 1991; Wilson and Shannon, 1995) and physiological parameters (water potential, turgor pressure, photosynthesis, respiration, or ion content) in plants grown with and without salt stress (Gale *et al.*, 1967; Hoffman *et al.*, 1980; Termaat *et al.*, 1985; Erdei and Teleisnik, 1993). While these studies provide evidence that genetic variability for salt tolerance exists, the actual mechanisms underlying salt tolerance are not well understood. An alternative approach is to make similar comparative measurements with halophytes which grow optimally in elevated levels of Na^+ (Flowers *et al.*, 1977). Such species can be grown in high or low Na^+ concentrations, making it possible to compare growth and physiological parameters at different salinity levels without the confounding effects of Na^+ toxicity.

Halophytes utilize numerous strategies that have evolved for adaptation to growth in high Na^+ concentrations: (1) exclusion of Na^+ at the soil–root boundary and, therefore, from all tissues, (2) exclusion of Na^+ from the xylem and, therefore, from leaf tissue, thus preventing disruption of photosynthesis, (3) inclusion of Na^+ and synthesis of compatible solutes to maintain osmotic adjustment, (4) inclusion of Na^+ and its subsequent sequestration in vacuoles (Braun *et al.*, 1986; O’Leary, 1995; Blumwald, 2000; Blumwald *et al.*, 2000), and (5) inclusion of Na^+ and its eventual elimination through secretion by leaves (Flowers *et al.*, 1977). Evolutionarily, inclusion and sequestration have been favoured in the most extreme saline environments (O’Leary, 1995).

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Therefore, it is important to focus plant improvement methods on this latter strategy.

Salicornia bigelovii is a succulent marine halophyte belonging to the Chenopodiaceae. It grows optimally in a broad range of Na⁺ concentrations between 100 and 400 mM NaCl. *S. bigelovii* is a 'true' halophyte or euhalophyte; it not only tolerates high Na⁺ concentrations, but requires them for optimal growth (Ayala and O'Leary, 1995). Because this species does not exclude Na⁺ or sequester it within specialized structures and does not differentially produce osmotic solutes in response to salt (Weeks, 1986), an understanding of the mechanisms underlying its tolerance to salt may be useful for the improvement of salt tolerance in glycophytic crop species (Ayala et al., 1996).

Comparisons of *S. bigelovii* grown in 200 and 5 mM NaCl demonstrate that exposure to salt alters plant growth; plants grown in 200 mM NaCl are twice as tall and 2.5 times more succulent (Ayala and O'Leary, 1995). Previous investigation of a number of physiological parameters showed that changes in ion concentrations are highly correlated with the observed growth differences. In 200 mM NaCl-grown plants, potassium, magnesium and calcium levels were reduced, but Na⁺ levels were almost three times higher than in 5 mM NaCl-grown plants (Pfister, 1999).

It has been proposed that the additional Na⁺ in *S. bigelovii* grown in 200 mM NaCl accumulates in the vacuole. Metabolic processes in the cytoplasm of a number of halophytes, including members of the Chenopodiaceae, have been shown to be highly salt-sensitive (Flowers, 1972; Greenway and Osmond, 1972). Sequestration in the vacuole would protect salt-sensitive enzymes in the cytoplasm. Furthermore, vacuolar accumulation of Na⁺ would provide an osmotic driving force for the uptake of water in highly saline environments which would explain the increased size and succulence of *S. bigelovii* grown in 200 mM NaCl.

Vacuolar Na⁺ accumulation could be accomplished by the combined activities of proton (H⁺) pumps and a Na⁺/H⁺ exchanger on the vacuolar membranes. H⁺ pumps use metabolic energy in the form of inorganic pyrophosphate (PP_i) or adenosine triphosphate (ATP) to transport H⁺ against a concentration gradient. Increased activity of one or both of these vacuolar H⁺ pumps could create an energy gradient in the form of a transmembrane pH gradient (Δ pH, Sze, 1985; Rea and Poole, 1986; Rea and Sanders, 1987). A vacuolar Na⁺/H⁺ exchanger could couple the use (dissipation) of the Δ pH to the uptake of Na⁺ into the vacuole (Blumwald and Poole, 1987; Barkla et al., 1995; Apse et al., 1999; Blumwald, 2000; Blumwald et al., 2000). Previous studies demonstrated a positive correlation between increased plant size, Na⁺ accumulation, and increased activity of the vacuolar H⁺-ATPase (V-H⁺ATPase) in *S. bigelovii* as a function of Na⁺

concentration during growth (Ayala et al., 1996). Current studies in this laboratory demonstrate higher vacuolar pyrophosphatase activity (PP_i hydrolysis and Δ pH formation) in plants grown in high concentrations of NaCl. Therefore, mechanisms appear to be in place that may provide an increased driving force (Δ pH) to support the vacuolar Na⁺ accumulation in *S. bigelovii* grown in high levels of salt.

By monitoring Na⁺-induced dissipation of a pyrophosphate-dependent Δ pH in vacuolar membrane vesicles, a Na⁺/H⁺ exchange activity has been identified and characterized. Specificity for Na⁺ and initial rates of Na⁺-induced dissipation of Δ pH as a function of Na⁺ concentration were determined, and effects of specific inhibitors and membrane potential on exchange activity were investigated. Finally, Na⁺/H⁺ exchange was compared in vacuolar-enriched membrane fractions from 200 and 5 mM NaCl-grown plants in order to determine if Na⁺-dependent regulation of activity is correlated with salt tolerance in *S. bigelovii*.

Materials and methods

Plant material

S. bigelovii seeds were collected in the autumn of 1996 from Estero Morúa, a coastal estuary near Puerto Peñasco, Mexico. All plants were grown in the greenhouse at the University of Arizona. Relative humidity, light, and temperature conditions over the period in which plants were grown were as previously described (Ayala and O'Leary, 1995; Ayala et al., 1996). Seeds were germinated on vermiculite in flats lacking drainage and misted daily with tap water. Cation concentrations in tap water were determined by atomic absorption spectroscopy to be in mM: 1.1 Na⁺, 0.04 K⁺, 0.8 Ca²⁺, and 0.08 Mg²⁺ (Pfister, 1999). On the third day after sowing, seedlings were irrigated with nutrient solution (composition in mM: 3 Ca(NO₃)₂, 2 KNO₃, 2 KH₂PO₄, 2 MgSO₄; in μ M: 7.6 MnCl₂, 40 H₃BO₃, 0.3 CuCl₂, 1.3 ZnSO₄, 3 MoO₃, and 4.2 mg l⁻¹ Fe-EDTA) supplemented with either 5 or 200 mM NaCl (Pfister, 1999). On subsequent days, seedlings were irrigated with tap water every first and second day and with the appropriate salt-supplemented nutrient solution every third day. After each irrigation, extra tap water or nutrient solution was decanted so that no standing solution remained. After 21 d of growth in vermiculite, seedlings typically had one internode and were approximately 1–2 cm long from cotyledons to shoot tip (seedlings grown in 200 mM NaCl appeared slightly larger than those grown in 5 mM NaCl). Seedling roots were gently freed of vermiculite by immersing in tap water and transferred to hydroponic solutions (nutrient solution supplemented with salt as described above) with constant aeration. The hydroponic solutions were brought to volume daily and changed once per week. Analysis of ion levels in discard solutions showed that this schedule maintained the desired salt concentrations (Pfister, 1999). After 21 d growth in appropriate nutrient solutions, whole shoots were harvested and used for the preparation of vacuolar membrane vesicles.

Reagents

Tetrasodium pyrophosphate was purchased from Boehringer Mannheim (Indianapolis, IN) and converted to PP_i-bis-tris-propane (PP_i-BTP) by cation exchange with Dowex 50W-W8

resin (H⁺ form) and titration with BTP. Antiserum to the V-H⁺PPase was the gift of Dr Philip A Rea (University of Pennsylvania). Aminomethylenebisphosphonate (AMBP) was the gift of Dr Alexander A Baykov (AN Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia). All general reagents, including the Diagnostic Protein Assay Kit, were purchased from Sigma-Aldrich.

Isolation of vacuolar membrane vesicles

Because most salt-induced increases in dry matter production observed in halophytes are the result of enhanced Na⁺ accumulation in the shoot (O'Leary, 1995), all studies of V-H⁺PPase activity focused on shoot tissue. All procedures for preparation of vacuolar membrane vesicles were conducted at 4 °C. Entire shoots were harvested and tissue was homogenized in a precooled mortar and pestle in a buffer containing either 250 mM mannitol (for 5 mM NaCl-grown plants) or 500 mM mannitol (for 200 mM NaCl-grown plants), 0.2% fatty acid-free BSA, 25 mM HEPES (buffered with BTP to pH 7.5), 3 mM EGTA, 1 mM DTT, 1 mM iodoacetamide, 0.1 mM phenylmethyl sulfonyl fluoride, 0.01 mM pepstatin A, and 0.083 g ml⁻¹ polyvinylpyrrolidone. Three ml of homogenization buffer were used g⁻¹ tissue. To remove cell debris, the homogenate was filtered through three layers of cheesecloth and then centrifuged for 15 min at 13 000 g. The supernatant was collected and centrifuged for 30 min at 60 000 g to pellet the microsomal membranes. The pellet was resuspended gently without aeration in resuspension buffer containing either 250 mM or 500 mM mannitol, 2.5 mM HEPES (buffered with BTP to pH 7.5), 10% glycerol, and 1 mM DTT. The resuspended microsomes were layered on a two-step dextran gradient (6% and 12%); dextran (average molecular weight 73 000) was prepared in the appropriate resuspension buffer (w/w). Because microsomal protein yields were higher for 200 mM NaCl-grown plants, 10 g fresh weight equivalent (200 mM NaCl-grown plants) and 30 g fresh weight equivalent (5 mM NaCl-grown plants) of microsomal resuspension were loaded per gradient. Gradients were centrifuged at 70 000 g for 2 h. Vacuolar-enriched membrane vesicles were harvested from the 0–6% interface; this fraction typically contained membrane protein at a concentration of 0.6–1.2 µg µl⁻¹. Vesicles were stored at –80 °C; aliquots were thawed once and the unused portions were discarded.

V-H⁺PPase assays

Hydrolysis of PP_i was determined by measuring the release of inorganic phosphate (P_i). Reaction mixes (final volume 0.5 ml) contained in mM: 0.3 BTP-PP_i, 1.3 MgSO₄, 50 KCl, 0.005 gramicidin-D, 30 BTP (buffered to pH 8.5 with HEPES) and 5–10 µg of vacuolar membrane protein. Ammonium molybdate (final concentration of 100 µM) was added to all reactions to inhibit activity from non-specific phosphatases. Reactions were initiated by the addition of vacuolar membrane protein, and P_i release was assayed at 35 °C for 60 min (Fiske and Subbarow, 1925; Hodges and Leonard, 1974; Schumaker and Sze, 1986); P_i release was linear for at least 60 min (data not shown). All activities are reported on the basis of mol PP_i hydrolysed (P_i release divided by 2). Data shown in Fig. 2 represent aminomethylenebisphosphonate (AMBP)-sensitive V-H⁺PPase activity which was calculated as the difference in activity in the absence and presence of 0.075 mM AMBP. AMBP, a structural analog of PP_i has been shown to inhibit the plant V-H⁺PPase (Zhen *et al.*, 1994; Gordon-Weeks *et al.*, 1999).

SDS-PAGE and immunoblot analysis

After TCA precipitation (12.5% final concentration), vacuolar membrane proteins were resuspended in a buffer containing 62 mM TRIS-HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol. Protein was denatured at 50 °C for 10 min prior to loading on 7.5% polyacrylamide gels (Laemmli, 1970). After separation by SDS-PAGE, gels were either silver-stained to visualize proteins (Oakley *et al.*, 1980) or the proteins were transferred electrophoretically to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was subsequently incubated with diluted antiserum (1:1000) directed against the peptide TKAADVGADLVGKIE (the putative hydrophilic loop IV of the substrate site of the V-H⁺PPase in *Arabidopsis thaliana*; Zhen *et al.*, 1997). To visualize reacting proteins, the membrane was incubated with alkaline phosphatase-conjugated goat antiserum directed against rabbit IgG and then stained for alkaline phosphatase activity (Burnette, 1981; Ward *et al.*, 1992, respectively).

Proton transport assays

Formation of an inside-acid pH gradient (ΔpH) in vacuolar membrane vesicles (pH of vacuolar compartment < pH of external solution) by the V-H⁺PPase was monitored by measurement of a decrease (quench) in quinacrine fluorescence. H⁺ transport reactions (1 ml total volume) were prepared in 1.5 ml cuvettes designed for use with a mini-stir bar. Except where noted, reactions contained in mM: 30 HEPES-BTP pH 8.5, 50 KCl, 0.002 quinacrine, 0.3 mM PP_i-BTP, and either 50 µg ml⁻¹ (for reactions with vesicles isolated from 200 mM NaCl-grown plants) or 100 µg ml⁻¹ (for reactions with vesicles isolated from 5 mM NaCl-grown plants) vacuolar membrane protein in the appropriate resuspension buffer. Reactions were mixed by inversion several times and then placed in a dark chamber with constant stirring in a luminescence spectrophotometer (Perkin Elmer model LS-5B). Reactions were equilibrated in the dark with stirring for 5 min before beginning fluorescence readings. Quinacrine fluorescence was monitored at 495 nm after illumination at 427 nm. Initial levels of fluorescence were set to approximately 85%, and fluorescence was measured for 2 min to establish baseline levels before starting the reaction. All additions to reactions were made in a darkened room. Reactions were initiated by the addition of MgSO₄ to a final concentration of 1.3 mM, and quenching of fluorescence was allowed to proceed until a constant level of fluorescence was achieved (steady-state). All subsequent additions were made once steady-state had been reached.

To determine initial rates of ΔpH formation (change in fluorescence min⁻¹; Δ%F min⁻¹), changes in relative fluorescence were measured between 1 and 2 min after the start of the reaction. Total quench was calculated from the difference in fluorescence immediately before the addition of MgSO₄ and at steady-state.

Na⁺/H⁺ exchange assays

Na⁺/H⁺ exchange activity in vacuolar membrane vesicles isolated from shoot tissue was assayed as follows: (1) ΔpH formation was allowed to proceed until steady-state was achieved (measured as a constant level of fluorescence quench), (2) Na⁺ was added to dissipate the ΔpH (measured as recovery of fluorescence), (3) 15 s after addition of Na⁺, the change in relative fluorescence (Δ%F) was recorded, (4) initial rates of Na⁺-induced dissipation were converted to units of Δ%F min⁻¹, (5) specific activity was calculated by dividing the initial rate by the mass of vacuolar membrane protein in the reaction (Δ%F min⁻¹ mg⁻¹ protein). Preliminary analyses

determined that initial rates are non-linear beyond 15 s after the addition of Na^+ .

Control experiments were conducted to monitor potential effects of organic solvents used in transport assays; no solvent effects were observed (data not shown).

Results

V-H⁺PPase hydrolytic activity and protein accumulation

The V-H⁺PPase possesses two distinct, but coupled, activities: hydrolysis of inorganic pyrophosphate and proton translocation. To characterize the V-H⁺PPase in *S. bigelovii*, the biochemical properties of the hydrolytic activity were determined and levels of the protein were examined. Analysis of these parameters demonstrated that both increased in plants grown in 200 mM NaCl relative to those in plants grown in 5 mM NaCl (Table 1; Fig. 1). Hydrolysis of pyrophosphate was 1.5 and 2 times greater in 200 mM NaCl-grown plants (without and with 50 mM K⁺, respectively; Table 1).

V-H⁺PPase hydrolytic activity in the presence of Na⁺

Inhibition of V-H⁺PPase hydrolytic activity in *S. bigelovii* was observed in the presence of 50 mM NaCl (20% and 16% inhibition for vesicles isolated from 200 and 5 mM NaCl-grown plants, respectively; Table 1) and 50 mM LiCl (60% and 50% inhibition for vesicles isolated from 200 and 5 mM NaCl-grown plants, respectively; Table 1). This inhibition of activity *in vitro* contrasts with the observed stimulation of V-H⁺PPase activity and protein levels in plants grown in 200 mM NaCl. Therefore, to investigate the effects of NaCl on the activity of the V-H⁺PPase further, hydrolytic activity was measured in the presence of a range of concentrations of NaCl added *in vitro* (Fig. 2). It was determined that low NaCl concentrations had minimal effect on V-H⁺PPase activity (0–20 mM NaCl, $\leq 10\%$ inhibition), but higher concentrations (100 mM NaCl) led to approximately 50% inhibition.

PP_i-dependent pH gradient formation

In order to test the hypothesis that the V-H⁺PPase is involved in vacuolar Na⁺ sequestration in *S. bigelovii*, the H⁺ translocating activity of the enzyme was characterized. Experiments were conducted to compare the magnitude of the PP_i-dependent ΔpH in vesicles isolated from 200 and 5 mM NaCl-grown plants (Fig. 3). Proton transport was monitored using a quinacrine fluorescence assay in which a decrease (quench) in fluorescence accompanies inside-acid ΔpH formation (Bennett and Spanswick, 1983). The rate and magnitude of ΔpH formation were always higher in reactions with vesicles isolated from 200 mM NaCl-grown plants (Fig. 3).

Table 1. Effect of cations on V-H⁺PPase hydrolytic activity in vacuolar membrane vesicles isolated from *S. bigelovii* grown in 200 and 5 mM NaCl

Reactions were performed as described in 'Materials and methods' with the addition of various cation chloride salts to a final concentration of 50 mM. Values represent means \pm SE, $n = 3$, except for NaCl treatments, which represent means, $n = 2$.

C ⁺ Cl (50 mM)	V-H ⁺ PPase hydrolytic activity ($\mu\text{mol mg}^{-1} \text{h}^{-1}$ (% Control))	
	200 mM NaCl	5 mM NaCl
None	10.9 \pm 2.4 (100)	7.4 \pm 0.4 (100)
KCl	23.9 \pm 9.0 (219)	11.7 \pm 0.3 (158)
RbCl	24.1 \pm 3.8 (221)	11.3 \pm 0.8 (153)
NH ₄ Cl	22.7 \pm 4.0 (208)	10.0 \pm 0.6 (135)
CsCl	18.1 \pm 4.7 (160)	9.6 \pm 0.7 (130)
NaCl	8.7 (80)	6.2 (84)
LiCl	4.4 \pm 1.2 (40)	3.7 \pm 0.2 (50)

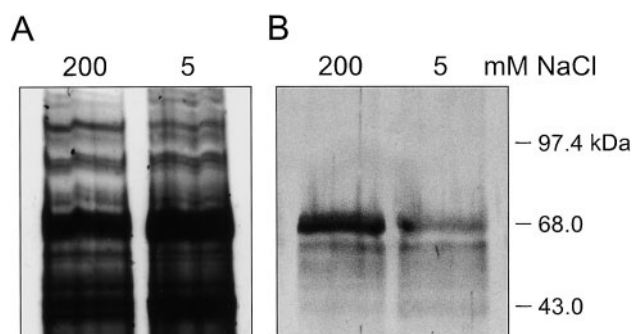


Fig. 1. V-H⁺PPase protein levels in *S. bigelovii* grown in 200 and 5 mM NaCl. Vacuolar membrane proteins (5 μg per lane) were separated in 7.5% polyacrylamide gels and visualized by silver-staining (A) or transferred to Immobilon-P membranes and allowed to react with antiserum directed against the putative hydrophilic loop IV of the substrate-binding subunit of the V-H⁺PPase in *Arabidopsis thaliana* (B). Results shown are representative of three independent experiments.

Kinetics of Na⁺/H⁺ exchange

The ΔpH required for identification and characterization of Na⁺/H⁺ exchange was generated by the V-H⁺PPase. Unless noted, experiments described were conducted using vesicles isolated from plants grown in 200 mM NaCl.

If present, a vacuolar Na⁺/H⁺ exchanger should dissipate ΔpH in vacuolar membrane vesicles upon the addition of NaCl. Consistent with this hypothesis, the addition of increasing concentrations of NaCl induced a greater dissipation of ΔpH (Fig. 4A). The specific activity of this dissipation was saturable (Fig. 4B), and kinetic transformations of these data using a Hanes-Woolf plot revealed a K_m of 3.8 mM and V_{max} of 3214 $\Delta\%F \text{min}^{-1} \text{mg}^{-1}$ protein (Fig. 4C).

Cation specificity of H⁺-coupled exchange

In order to investigate the ion specificity of the exchange activity, the ability of different cations to dissipate ΔpH

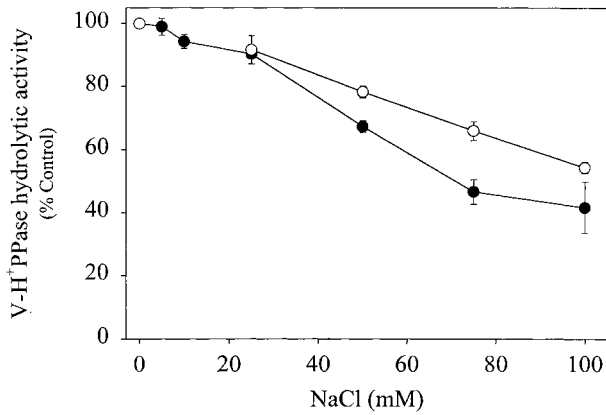


Fig. 2. Effect of NaCl added *in vitro* on V-H⁺ PPase hydrolytic activity in vacuolar membrane vesicles isolated from *S. bigelovii* grown in 200 and 5 mM NaCl. Reactions were performed as described in 'Materials and methods' with the final NaCl concentration varied from 0 to 100 mM. Data shown represent AMBP-sensitive activity. Specific activities were normalized by setting the activity observed without NaCl to 100%. For reactions containing vacuolar membrane vesicles isolated from plants grown in 200 mM NaCl (●), 100% activity was equal to 19.1 ± 2.6 μmol mg⁻¹ h⁻¹. For reactions containing vacuolar membrane vesicles isolated from plants grown in 5 mM NaCl (○), 100% activity was equal to 13.9 ± 4.4 μmol mg⁻¹ h⁻¹. Values represent means ± SE, n = 3.

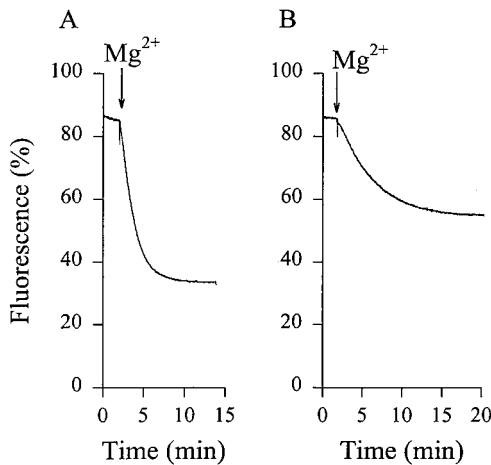


Fig. 3. PP_i-dependent pH gradient formation in vacuolar membrane vesicles isolated from *S. bigelovii* grown in 200 and 5 mM NaCl. Reactions were performed as described in 'Materials and methods'. An inside-acid ΔpH was formed in vacuolar membrane vesicles isolated from plants grown in 200 mM NaCl (A) and 5 mM NaCl (B). Vesicles used in reactions A and B were isolated from plants grown during the same time period. The reactions consisted of 30 mM HEPES-BTP (pH 8.5), 50 mM KCl, 2 μM quinacrine, 0.3 mM PP_i-BTP, and either 50 μg ml⁻¹ (for reactions with vesicles isolated from 200 mM NaCl-grown plants) or 100 μg ml⁻¹ (for reactions with vesicles isolated from 5 mM NaCl-grown plants) vacuolar membrane protein in the appropriate resuspension buffer. Reactions were illuminated at 427 nm and quinacrine fluorescence was monitored at 495 nm. ΔpH formation was initiated by the addition of MgSO₄ to a final concentration of 1.3 mM. Results shown are representative of four independent experiments.

was determined (Table 2). After ΔpH reached steady-state, chloride salts of Li⁺, Na⁺, K⁺, Rb⁺, Cs⁺, and BTP were added at 1 mM and 5 mM final concentration. Under the assay conditions used (in the presence

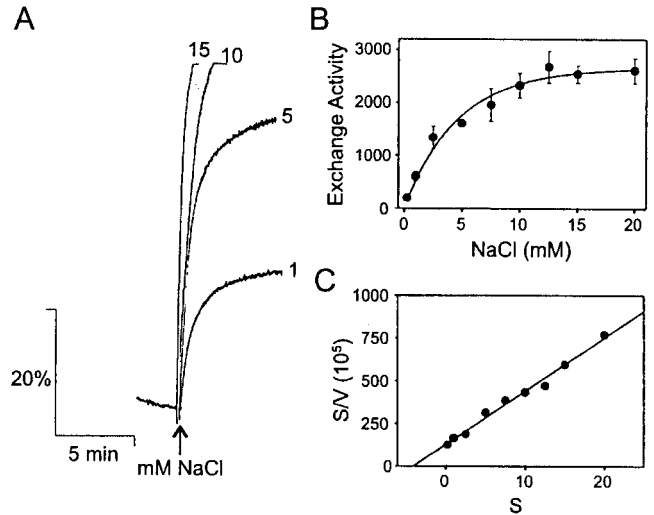


Fig. 4. Dissipation of the PP_i-dependent pH gradient as a function of NaCl concentration in vacuolar membrane vesicles isolated from *S. bigelovii* grown in 200 mM NaCl. ΔpH was formed in vesicles isolated from 200 mM NaCl-grown plants as described in Fig. 3. (A) ΔpH was dissipated by the addition of 1, 5, 10, and 15 mM NaCl as indicated. ΔpH formation is not shown, but was similar for all treatments. Traces were aligned at the point of addition of NaCl. One experiment representative of three or four is shown. (B) ΔpH was dissipated by a range of NaCl concentrations from 0.25–20 mM NaCl and the initial rates of dissipation were determined as described in 'Materials and methods'. Values represent means ± SE, n = 3–4. (C) Data shown in (B) were transformed using a Hanes-Woolf plot in order to determine kinetic parameters (–X intercept = K_m = 3.8 mM NaCl, K_m/Y intercept = V_{max} = 3214 Δ% F min⁻¹ mg⁻¹ protein).

Table 2. Cation specificity of the dissipation of PP_i-dependent ΔpH in vacuolar membrane vesicles isolated from *S. bigelovii* grown in 200 mM NaCl

ΔpH was formed in vesicles isolated from 200 mM NaCl-grown plants as described in Fig. 3 and the effects of various chloride salts on ΔpH were determined. Values represent means ± SE, n = 3.

C ⁺ Cl	Concentration (mM)	Exchange activity Initial rate of dissipation (Δ% F min ⁻¹)
LiCl	1	14.7 ± 1.3
	5	19.0 ± 5.0
NaCl	1	30.0
	5	80.0 ± 0.0
KCl	1 or 5	No effect
RbCl	1 or 5	No effect
CsCl	1 or 5	No effect
BTPCl	1 or 5	No effect

of 50 mM K⁺), dissipation of ΔpH is specific for Na⁺ and ions of similar charge and ionic radius. Both Na⁺ and Li⁺ dissipate ΔpH; however Li⁺ dissipates ΔpH less effectively than Na⁺.

Inhibitor sensitivity of Na⁺/H⁺ exchange

The sensitivity of Na⁺-induced dissipation to specific inhibitors of Na⁺ transport activities was investigated. No effect on dissipation was observed with amiloride

concentrations up to 750 μM (data not shown). However, some degree of sensitivity to a more potent form of amiloride was observed. Methyl-isobutyl amiloride (MIA) inhibited the initial rate of dissipation of ΔpH by 2.5 mM NaCl approximately 40% when added before ΔpH was formed (Fig. 5).

A steady-state ΔpH was required in order to measure the effect of MIA on the Na^+ -induced dissipation of ΔpH . Addition of MIA after ΔpH formation reached steady-state resulted in disruption of the ΔpH (data not shown). However, ΔpH formed in the presence of MIA was stable but reduced. To eliminate any effects of the magnitude of the ΔpH on initial rates of Na^+/H^+ exchange, the rate of Na^+ -induced dissipation in the presence of inhibitor was always compared to the rate in reactions with similar levels of ΔpH but without inhibitor. K^+ concentrations were reduced to limit the activity of the $\text{V-H}^+\text{PPase}$ resulting in a reduced ΔpH .

Driving force for Na^+/H^+ exchange

To demonstrate further that vacuolar Na^+/H^+ exchange was responsible for Na^+ -induced dissipation of ΔpH , the contribution of the electrical potential ($\Delta\Psi$) of the membrane was investigated. If another Na^+ transporter, distinct from the Na^+/H^+ exchanger, could drive the uptake of Na^+ into vacuolar membrane vesicles, a driving force for the efflux of H^+ via a Na^+ diffusion potential might be created. Na^+ -induced dissipation would be observed, but would not be due to the vacuolar Na^+/H^+ exchanger. To address this possibility, Na^+ -induced

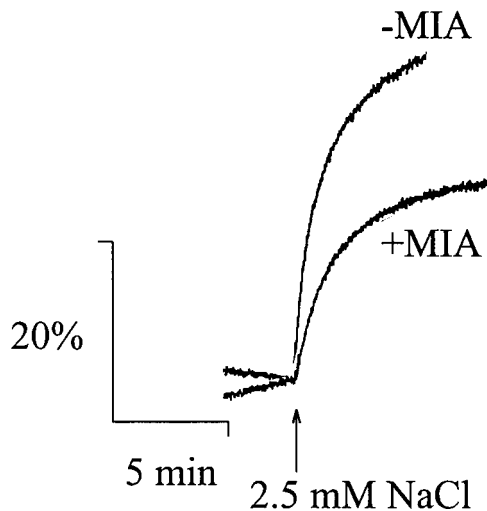


Fig. 5. Effect of MIA on NaCl-induced dissipation of PP_i -dependent ΔpH in vacuolar membrane vesicles isolated from *S. bigelovii* grown in 200 mM NaCl. ΔpH was formed as described in Fig. 3 in the presence and absence of 20 μM MIA in vesicles isolated from 200 mM NaCl-grown plants. ΔpH formation is not shown, but was similar for both treatments. Traces are aligned at the point of addition of 2.5 mM NaCl. Initial rates of dissipation of ΔpH were reduced in the presence of MIA in three separate experiments (average inhibition, relative to control, $44 \pm 9.3\%$). One experiment representative of three is shown.

dissipation of ΔpH was performed in the presence of a K^+ ionophore, valinomycin (Barkla *et al.*, 1995). Valinomycin eliminates $\Delta\Psi$ and H^+ efflux due specifically to $\Delta\Psi$. Any difference in Na^+ -induced dissipation of ΔpH with and without valinomycin would indicate the degree of contribution of $\Delta\Psi$. The ΔpH was formed in the presence of KIDA (potassium iminodiacetic acid, rather than KCl in order to limit the concentration of permeant anion while maintaining K^+ concentrations for the stimulation of the $\text{V-H}^+\text{PPase}$) without and with valinomycin (Fig. 6A, B). ΔpH formation was increased in the presence of valinomycin indicating that the ionophore was effectively dissipating $\Delta\Psi$. The initial rate of dissipation of ΔpH by 2.5 mM NaCl was identical with or without valinomycin, demonstrating that $\Delta\Psi$ does not play a significant role in Na^+ -induced dissipation of ΔpH . Furthermore, similar rates of dissipation in the presence or absence of valinomycin suggest an electroneutral exchange of Na^+ for H^+ .

Na^+/H^+ exchange activity in plants grown in 200 and 5 mM NaCl

In order to determine whether Na^+/H^+ exchange was regulated in a manner consistent with a role in salt tolerance, activity was compared in vesicles isolated from *S. bigelovii* grown at the two salinities. When comparisons were made under conditions of maximal ΔpH formation (Fig. 7A, C), Na^+/H^+ exchange activity was apparently higher in reactions containing vesicles isolated from 200 mM NaCl-grown plants. However, direct comparison of activity is complicated by the fact that ΔpH formation is greater in vesicles isolated from 200 mM NaCl-grown plants relative to that in vesicles

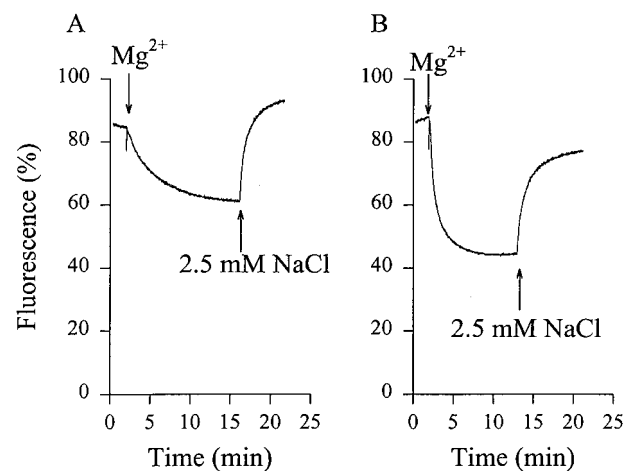


Fig. 6. Influence of $\Delta\Psi$ on initial rate of NaCl-induced dissipation of PP_i -dependent ΔpH in vacuolar membrane vesicles isolated from *S. bigelovii* grown in 200 mM NaCl. ΔpH was formed as described in Fig. 3 in the presence of 50 mM KIDA and in the absence (A) or presence (B) of 1 μM valinomycin in vesicles isolated from 200 mM NaCl-grown plants. One experiment representative of two is shown.

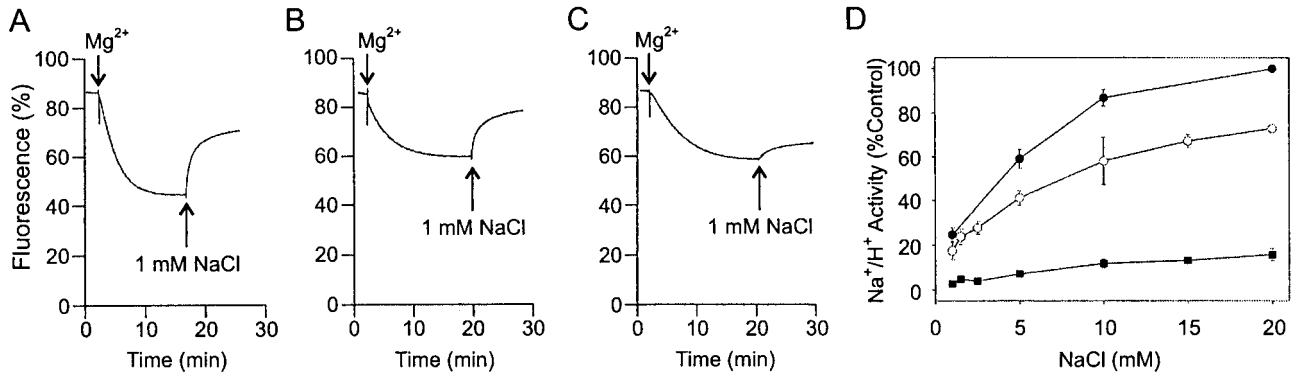


Fig. 7. NaCl-induced dissipation of ΔpH in vacuolar membrane vesicles isolated from *S. bigelovii* grown in 200 and 5 mM NaCl. Reactions were performed as described in Fig. 3 and contained either $50 \mu\text{g ml}^{-1}$ (for reactions containing vesicles isolated from 200 mM NaCl-grown plants) or $100 \mu\text{g ml}^{-1}$ (for reactions containing vesicles isolated from 5 mM NaCl-grown plants) vacuolar membrane protein in the appropriate resuspension buffer. Shown are three representative traces for reactions with: (A) vesicles isolated from 200 mM NaCl-grown plants and 50 mM KCl, (B) vesicles isolated from 200 mM NaCl-grown plants and 4 mM KCl, (C) vesicles isolated from 5 mM NaCl-grown plants and 50 mM KCl. The KCl concentration was reduced in the reaction shown in (B) in order to achieve ΔpH formation equivalent to that in reactions containing vesicles isolated from 5 mM NaCl-grown plants. One experiment representative of three is shown. (D) Membrane vesicles were isolated from plants grown during the same time period for each of the three separate experiments. ΔpH was formed as described in Fig. 3 in the presence of 50 mM KCl with vesicles isolated from 200 mM NaCl-grown plants (●), in the presence of 2, 2.5, or 4 mM KCl with vesicles isolated from 200 mM NaCl-grown plants (○), and in the presence of 50 mM KCl with vesicles isolated from 5 mM NaCl-grown plants (■). After ΔpH was formed, a range of NaCl concentrations was added, and initial rates of dissipation were determined. Within each experiment, values were normalized to the specific activity observed in the presence of 50 mM KCl with vesicles isolated from 200 mM NaCl-grown plants and dissipated with 20 mM NaCl ($2440 \pm 330 \Delta\%F \text{ min}^{-1} \text{ mg}^{-1}$ protein). Values represent means \pm SE, $n = 3$.

isolated from plants grown in 5 mM NaCl. Increased activity of the V-H⁺PPase in plants grown at the higher salinity accounts for this difference (Table 1; Fig. 3). Furthermore, experiments demonstrated that the magnitude of ΔpH affected the initial rates of Na⁺/H⁺ exchange activity (data not shown). Therefore, the difference in activity could have been due to: (1) actual stimulation of exchange activity due to growth of the plants in 200 mM NaCl or (2) apparent stimulation of exchange activity due to the increased driving force (ΔpH) in 200 mM NaCl-grown plants.

To eliminate the second possibility and directly compare Na⁺/H⁺ exchange activity in plants grown at the two salinities, ΔpH formation was reduced in reactions containing vesicles isolated from 200 mM NaCl-grown plants. As with experiments investigating the effect of MIA on exchange activity, this was accomplished by lowering the concentration of KCl in the reaction. Na⁺/H⁺ exchange activity (Fig. 7) was compared in reactions performed with: (A) optimal ΔpH formation in vesicles isolated from 200 mM NaCl-grown plants, (B) ΔpH formation in vesicles isolated from 200 mM NaCl-grown plants that was less than or equivalent to optimal ΔpH formation in vesicles isolated from 5 mM NaCl-grown plants, and (C) optimal ΔpH formation in vesicles isolated from 5 mM NaCl-grown plants. In each experiment, a slightly different KCl concentration was required to produce a ΔpH in vesicles isolated from 200 mM NaCl-grown plants that was equivalent to the ΔpH in vesicles isolated from 5 mM NaCl-grown plants; this may be due to slight variations in

V-H⁺PPase activity or vesicle integrity from preparation to preparation. After the formation of equivalent ΔpH in vesicles isolated from 200 and 5 mM NaCl-grown plants, the initial rates of Na⁺-induced dissipation of ΔpH were measured over a range of NaCl concentrations from 1 to 20 mM (Fig. 7D). Even with reduced ΔpH , Na⁺/H⁺ exchange activity (in the presence of 20 mM NaCl) was approximately 9-fold greater for 200 mM NaCl-grown plants (1740 ± 270 and $190 \pm 44 \Delta\%F \text{ min}^{-1} \text{ mg}^{-1}$ protein for 200 and 5 mM NaCl-grown plants, respectively, with equivalent magnitudes of ΔpH). Kinetic transformation of the data indicated that the affinity of the transporter for Na⁺ was almost three times higher in plants grown in 200 mM NaCl ($K_m = 3.8$ and 11.5 mM for plants grown in 200 and 5 mM NaCl, respectively).

Discussion

In S. bigelovii, multiple mechanisms exist for establishing the vacuolar pH gradient in response to plant growth in salt

The increased accumulation of the 68 kDa V-H⁺PPase catalytic subunit in vacuolar membrane vesicles isolated from *S. bigelovii* grown in 200 mM NaCl (Fig. 1) suggests that increased protein levels may be at least partly responsible for the stimulation of V-H⁺PPase activity. This effect on protein accumulation is in contrast to the effect of salt on V-H⁺ATPase protein levels. Previous studies with *S. bigelovii* showed that the hydrolytic activity of the V-H⁺ATPase was also stimulated in

200 mM NaCl-grown plants (Ayala *et al.*, 1996); however, V-H⁺ATPase protein levels did not increase in these plants. These results suggest that, while these two NaCl-stimulated H⁺ translocating activities may both contribute to a ΔpH that is necessary for Na⁺ sequestration, they may be regulated by different cellular mechanisms.

Additional evidence for multiple cellular pathways underlying salt adaptation in *S. bigelovii* comes from measurements of the effects of Na⁺, added *in vitro*, on H⁺-pump activity. Absence of *in vitro* Na⁺-effects on V-H⁺PPase activity indicates that the stimulation of activity observed in plants grown in 200 mM NaCl may not be due to a direct effect of Na⁺ on the enzyme, but rather may reflect the action of Na⁺ operating through a pathway requiring cytoplasmic factors. By contrast, stimulation of the V-H⁺ATPase by Na⁺ was observed both *in vivo* and *in vitro* (Ayala *et al.*, 1996).

The accumulation of V-H⁺PPase protein and its activities in response to plant growth in salt vary widely depending upon the species. As was found in this study for *S. bigelovii*, increased V-H⁺PPase activities were measured in vacuolar membranes isolated from sunflower roots (although protein levels were unchanged; Ballesteros *et al.*, 1996) and carrot culture cells (Colombo and Cerana, 1993) in response to NaCl. Conversely, activities decreased in vacuolar membranes isolated from wheat roots (Wang *et al.*, 2000), mung bean roots (Nakamura *et al.*, 1992), and cowpea hypocotyls (although proteins levels were unchanged; Otoch *et al.*, 2001). A clear model for the role and regulation of the V-H⁺PPase in the response of the plant to salt stress awaits functional studies with both glycophytes and halophytes. Progress toward this goal has been made in recent studies in which the salt tolerance of *Arabidopsis thaliana* (Gaxiola *et al.*, 2001) and *Saccharomyces cerevisiae* (Gaxiola *et al.*, 1999) was improved with the overexpression of the V-H⁺PPase from *Arabidopsis thaliana*.

Na⁺/H⁺ exchange activity is present in *S. bigelovii*

The affinity of the exchanger in *S. bigelovii* for Na⁺ (3.8 mM) is similar to that observed for Na⁺/H⁺ exchange in vacuolar vesicles isolated from some glycophytes, including rice (10 mM; Fukuda *et al.*, 1998), barley (9 mM; Garbarino and DuPont, 1988), and sunflower (8 mM; Ballesteros *et al.*, 1997). The affinity is higher than that determined for the halophyte *Mesembryanthemum crystallinum* (51 mM; Barkla *et al.*, 1995). The V_{\max} observed for Na⁺/H⁺ exchange in *S. bigelovii* (3214 Δ%F min⁻¹ mg⁻¹ protein) is higher than reported values for rice vacuolar exchange (2160 Δ%F min⁻¹ mg⁻¹ protein) and that from *M. crystallinum* (1007 Δ%F min⁻¹ mg⁻¹ protein) (Fukuda *et al.*, 1998 and Barkla *et al.*, 1995, respectively).

The K_m and V_{\max} values calculated for Na⁺/H⁺ exchange in *S. bigelovii* are consistent with the idea that this activity plays a vital role in the accumulation of Na⁺ within the vacuole, thus protecting salt-sensitive cytoplasmic metabolism and providing an osmotic driving force for the uptake of water and continued growth. If Na⁺/H⁺ exchange is a key component of the mechanism allowing *S. bigelovii* to thrive in extremely saline environments, it must be highly responsive to and efficient at dealing with changes in Na⁺ levels; the low K_m and high V_{\max} values for the exchanger provide evidence that this is the case.

Vacuolar Na⁺/H⁺ exchange is specific for Na⁺

Na⁺/H⁺ exchange in *S. bigelovii*, as in other organisms, is highly selective. Dissipation of ΔpH by both Na⁺ and Li⁺ may indicate that both ions are transported via the Na⁺/H⁺ exchanger or that one or both ions inhibit the V-H⁺PPase (thus reducing the ΔpH). However, studies of substrate hydrolytic activity of the *S. bigelovii* V-H⁺PPase demonstrated that neither Na⁺ nor Li⁺ inhibit the V-H⁺PPase in the range of concentrations used in this experiment (Fig. 2, data not shown, respectively). Therefore, the Na⁺/H⁺ exchanger in *S. bigelovii* may be able to transport both Na⁺ and Li⁺. Ion specificity of Na⁺/H⁺ exchange activity appears to vary in different plants. While most Na⁺/H⁺ exchangers also transport Li⁺, there are some examples to the contrary (Katz *et al.*, 1986; Garbarino and Dupont, 1988; Staal *et al.*, 1991).

Under conditions used to assay Na⁺/H⁺ exchange in vacuolar membrane vesicles isolated from *S. bigelovii* (50 mM KCl added for maximum stimulation of the V-H⁺PPase), no K⁺/H⁺ exchange activity was measured. This is in contrast to the K⁺/H⁺ exchange activity that has been measured in vacuolar membrane vesicles in *Beta vulgaris* (Blumwald and Poole, 1985). If a K⁺/H⁺ exchanger is present in the vacuolar membrane of *S. bigelovii*, several lines of evidence suggest that it is not a major transport activity relative to Na⁺/H⁺ exchange. (1) The ΔpH formed in vacuolar membrane vesicles under conditions where K⁺/H⁺ exchange would be operating (in the presence of 50 mM KCl, Fig. 3), but not under conditions where Na⁺/H⁺ exchange has been demonstrated (50 mM KCl and 20 mM NaCl, data not shown). (2) After ΔpH formation reached steady-state, Na⁺ addition led to a rapid dissipation of the ΔpH while an equivalent K⁺ addition had no effect on or led to increased ΔpH formation (data not shown). (3) If a K⁺/H⁺ exchanger is present and is using (dissipating) the ΔpH, reducing K⁺ concentrations in the assay would be expected to lead to increased ΔpH formation. In experiments where the extent of the ΔpH formation was equalized in vesicles isolated from plants grown in 5 and 200 mM NaCl (Figs 5, 7), the opposite was true;

reducing KCl concentrations led to the formation of a smaller ΔpH .

MIA inhibits vacuolar Na⁺/H⁺ exchange

The diuretic drug amiloride has been shown competitively to inhibit Na⁺ transport processes mediated by Na⁺ channels and Na⁺/H⁺ exchangers in numerous organisms including plants. While amiloride (concentrations up to 750 μM) did not inhibit Na⁺/H⁺ exchange in *S. bigelovii* (data not shown), amiloride sensitivity of Na⁺/H⁺ exchange has been reported for isolated vacuoles and vacuolar membrane vesicles isolated from storage tissue of *Beta vulgaris* (Blumwald and Poole, 1985; Blumwald *et al.*, 1987), for leaf cell vacuoles of *M. crystallinum* (Barkla *et al.*, 1995), and for plasma membrane vesicles of *Dunaliella salina* (Katz *et al.*, 1986). However, amiloride-insensitivity has been reported for leaf microsomes (mainly vacuole) of another halophyte, *Atriplex gmelini* (Garbarino and DuPont, 1988) and for vacuolar membrane vesicles isolated from barley root (Matoh *et al.*, 1989).

While Na⁺/H⁺ exchange in *S. bigelovii* is insensitive to amiloride, some sensitivity to a more potent derivative of amiloride, MIA, is evident but at a lower level than reported for some other plants. In *S. bigelovii*, the initial rate of dissipation of ΔpH by 2.5 mM NaCl was inhibited approximately 40% in the presence of 20 μM MIA (Fig. 5). In vacuolar vesicles isolated from *B. vulgaris* cell suspensions and *O. sativa* roots, 5.9 μM and 2.2 μM MIA led to half-maximal inhibition, respectively (Barkla *et al.*, 1995; Fukuda *et al.*, 1998).

Vacuolar Na⁺/H⁺ exchange is stimulated by growth in high levels of NaCl

Analysis of the effect of growth in NaCl on Na⁺/H⁺ exchange revealed significant NaCl-dependent regulation of activity. Na⁺/H⁺ exchange is clearly stimulated when plants are grown in the presence of high NaCl concentrations. By contrast, initial rates of dissipation of ΔpH were low in vacuolar membrane vesicles isolated from *S. bigelovii* grown in 5 mM NaCl. These rates increased only slightly over the range of NaCl (substrate) concentrations tested *in vitro* indicating that the exchanger is present either at low levels or in a relatively inactive state or both. A comparison of the K_m values for the Na⁺/H⁺ exchanger in plants grown at the two salinities indicates that the mechanism underlying the activity of the transporter in response to NaCl involves, at least in part, alterations in the protein that change its affinity for substrate.

Concluding remarks

The research presented here demonstrates that in the highly salt-tolerant plant, *S. bigelovii*, activities of

transporters involved in vacuolar Na⁺ accumulation increase when plants are grown in high levels of salt. Increased vacuolar Na⁺ accumulation is likely the result of both more driving force for Na⁺/H⁺ exchange (2-fold increase in V-H⁺PPase activity; Table 1) and increased exchange activity itself (9-fold increase in V_{max} and 3-fold decrease in K_m ; Fig. 7). These findings provide the critical biological framework for future biochemical and molecular studies of the role and regulation of these transporters in salt adaptation in *S. bigelovii*. Knowledge of how this euhalophyte deals so effectively with salt will contribute to a broader understanding of salt adaptation in vascular plants and will provide information and tools for plant improvement.

Dedication

The authors would like to dedicate this work to our colleague, Dr James W O'Leary, on the occasion of his retirement. His long-standing interest in and enthusiasm for halophytes as model organisms for the study of plant adaptation to salt continues to be the inspiration for our work.

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