Increased vacuolar and plasma membrane H\(^+\)-ATPase activities in *Salicornia bigelovii* Torr. in response to NaCl

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Received 26 June 1995; Accepted 15 September 1995

Abstract

The halophyte *Salicornia bigelovii* Torr. shows optimal growth and Na\(^+\) accumulation in 200 mM NaCl and reduced growth under lower salinity conditions. The ability to accumulate and compartmentalize Na\(^+\) may result, in part, from stimulation of the H\(^+\)-ATPases on the plasma membrane (PM-ATPase) and vacuolar membranes (V-ATPase). To determine if these two primary transport systems are involved in salt tolerance, shoot fresh weight (FW) and activity of the PM- and V-ATPases from shoots in *Salicornia* grown in 5 and 200 mM NaCl were compared. Higher PM-ATPase activity (60%) and FW (60%) were observed in plants grown in 200 mM NaCl and these stimulations in growth and enzyme activity were specific for Na\(^+\) and not observed with Na\(^+\) added in vitro. V-ATPase activity was significantly stimulated *in vivo* and *in vitro* (26% and 46%, respectively) after exposure to 200 mM NaCl, and stimulation was Na\(^+\)-specific. Immunoblots indicated that the increases in activity of the H\(^+\)-ATPases from plants grown in 200 mM NaCl was not due to increases in protein expression. These studies suggest that the H\(^+\)-ATPases in *Salicornia* are important in salt tolerance and provide a biochemical framework for understanding mechanisms of salt tolerance in plants.

Key words: *Salicornia*, H\(^+\)-ATPases, salt tolerance.

Introduction

A common characteristic of most glycophytes is a decline in growth and yield when exposed to saline conditions. In contrast, many plants are highly adapted to salinity (halophytes) and exhibit maximum growth between 100 and 300 mM NaCl (Flowers et al., 1977). In halophytes the ability to grow in high levels of salt is often accompanied by an increase in plant size and succulence (Poljakoff-Mayber, 1975) and is dependent on regulation of intracellular ion concentrations (Ungar, 1991); however, there is very little information about the specific physiological mechanisms leading to salt tolerance.

Studies of halophytes have shown that increases in plant size and succulence in saline environments are the result of increases in cell size (Strogonov, 1964; Waisel, 1972; Poljakoff-Mayber, 1975), and studies of glycophytes have implicated acidification of the apoplast in cell wall loosening required for cell extension and growth (Rayle and Cleland, 1992). The plasma membrane H\(^+\)-ATPase (PM-ATPase) hydrolyses ATP and acidifies the apoplast by transporting protons to the cell wall space. In addition, the electrochemical gradient generated by the movement of protons constitutes the driving force for nutrient and solute transport, and the regulation of cellular ion concentrations (Sze, 1985; Briskin, 1990a). Halophytes can also be distinguished from glycophytes based on their capacity to accumulate large quantities of ions in their cells (Jeffries, 1981; Flowers et al., 1986). By sequestering sodium (Na\(^+\)) and chloride (Cl\(^-\)) in vacuoles of halophyte leaf cells, the osmolality of the cells is increased and salt-sensitive metabolic reactions in the cytoplasm are protected (Flowers et al., 1986). Vacuolar Na\(^+\) accumulation is thought to be mediated by a Na\(^+\)/H\(^+\) antipporter that uses the pH gradient generated by the vacuolar H\(^+\)-ATPase (V-ATPase) to couple the active accumulation of Na\(^+\) with the passive efflux of protons (Blumwald and Poole, 1987; Garbarino and DuPont, 1988; Staal et al., 1991; Barkla and Blumwald, 1992).

If salt tolerance is a result of the ability to regulate the osmotic potential and intracellular Na\(^+\) levels, one mech-
anism that may help to confer this ability is Na⁺ stimulation of the H⁺-ATPases on the vacuolar and plasma membranes. To determine if the PM-ATPase and the V-ATPase are regulated by Na⁺, plant growth and the activity of these two ATPases in Salicornia bigelovii (Chenopodiaceae) grown in 5 and 200 mM NaCl were compared. This succulent C₃ annual, native to coastal saltmarshes, grows optimally in 200 mM NaCl and shows a dramatic reduction in growth at low levels of NaCl. Shoots of plants grown in 200 mM NaCl are one and a half to two times larger and accumulate three times as much Na⁺ as plants grown in 5 mM NaCl (Ayala and O'Leary, 1995). This differential growth response allows a direct comparison of the physiological mechanisms conferring salt tolerance in plants with the same genetic background. In addition, Salicornia adapts to high levels of Na⁺ without the production of specialized salt-sequestering structures or differential production of osmotic solutes (Weeks, 1986), suggesting that Salicornia will be an excellent system in which to understand the biochemical basis of salt tolerance. In this report, evidence is provided that the PM- and V-ATPases are not only tolerant of, but are stimulated by Na⁺ in Salicornia, indicating that these transport systems may be important in the regulation of intracellular Na⁺ levels. In addition, it is shown that Na⁺ stimulation of the PM-ATPase is highly and positively correlated with increases in plant growth suggesting that this transporter plays an important role in salt tolerance.

Materials and methods

Plant material

S. bigelovii seeds were collected in the autumn of 1992 from Estero Morúa, a coastal estuary near Puerto Peñasco, Mexico (31° 17'N, 113° 24'W). A series of experiments was conducted under greenhouse conditions at the University of Arizona from December 1992 to December 1993. Night temperatures ranged from 15–26°C and day temperatures ranged from 26–32°C. Photosynthetically active radiation measured at noon varied from 450 to 1350 μmol m⁻² s⁻¹ and relative humidity varied from 25 to 50% during the day and from 70 to 85% at night. Seeds were sown in an equal mixture of organic soil and sand in flats lacking drainage and were irrigated with tap water. After 48–65 d, seedlings (40–120 per experiment) approximately 2 cm tall were transplanted to containers where they were grown in 6 l of aerated nutrient solution (composition in mM: 3 Ca(NO₃)₂, 2 KNO₃, 2 KHP₄, 2 MgSO₄, 7H₂O; in μM: 7.6 MnCl₂, 40 H₂BO₃, 0.3 CuCl₂, 1.3 ZnSO₄, 3 MnO₂). Iron was supplied as the EDTA complex at 4.2 mg Fe I⁻ per l, respectively. Vanadate-sensitive ATPase activity was calculated from the mitochondrial and plasma membrane ATPases, respectively. Vanadate-sensitive ATPase activity was calculated as the difference in activity in the presence and absence of 0.2 mM sodium vanadate.

Reagents

ATP was purchased from Boehringer Mannheim (Indianapolis, IN) as the disodium salt and converted to ATP-BTP using Dowex 50W X8. Baflomycin A₁ was obtained from Dr Karlheinz Alendon, University of Osnabrück, Osnabrück, FRG. All other reagents were obtained from Sigma (St Louis, MO).

Preparation of membrane vesicles

 Entire shoots were harvested after 14 d growth at the indicated salinity levels. After recording the fresh weight (FW), internode length and diameter, all procedures were carried out at 4°C. Plant material was homogenized with a mortar and pestle in a buffer containing in mM: 250 sorbitol, 25 HEPES-BTP (pH 7.4), 3 EGTA, 1 DTT, 1 iodoacetamide, 0.1 PMSF, 0.01 peptide A, and 0.25 g polyvinylpoly-pyrrolidone (PVPP) per gram FW. Three ml of homogenization buffer were used per gram of plant material. Contamination of vacuolar and plasma membranes with thylakoids from broken chloroplasts was minimized by the addition of 0.2% fatty acid-free BSA to the homogenization buffer. The homogenized material was filtered through cheesecloth and the homogenate centrifuged for 15 min at 13,000 g. The 13,000 g supernatant was centrifuged for 30 min at 60,000 g (Beckman SW 28 rotor, rmax) for 2 h. The 0–6% interface was collected and is referred to as vacuolar-enriched vesicles; the 6–12% dextran interface was collected and is referred to as plasma membrane-enriched vesicles.

ATPase assays and protein determination

Protein concentration was determined after precipitation with TCA by the Lowry method (Lowry et al., 1951) using BSA as the standard. ATPase activity, measured as release of inorganic phosphate from hydrolysis of ATP (Fiske and SubbaRow, 1925; Hodges and Leonard, 1974; Schumaker and Sze, 1986), was measured in 0.5 ml volume. Reactions were initiated with the addition of membrane protein and conducted at 35°C for 60 min. Inorganic phosphate release was linear for at least 60 min (data not shown). For measurement of vanadate-sensitive ATPase activity (plasma membrane) reaction mixes contained in mM: 10 PIPES (buffered with TRIS Base to pH 6.7), 5 MgCl₂, 5 phosphoenol pyruvate (PEP, monopotassium salt), 5 Na₂ATP, 4.5 units of pyruvate kinase, and 20–25 μg of membrane protein. Activities were similar in the presence or absence of Triton X-100 (data not shown), so detergent was omitted from subsequent reactions. Unless otherwise indicated, sodium azide (5 mM) and bafilomycin A₁ (0.1 μM) were added to inhibit ATPase activity from the mitochondrial and vacuolar membrane ATPases, respectively. Vanadate-sensitive ATPase activity was calculated as the difference in activity in the presence and absence of 0.2 mM sodium vanadate.

For measurement of bafilomycin A₁-sensitive ATPase activity (vacuolar membrane) reaction mixes contained in mM: 30 HEPES (buffered with BTP to pH 7.5), 5 MgSO₄, 5 PEP, 5 ATP (buffered to pH 7.5 with BTP), 4.5 units of pyruvate kinase, 0.01% Triton X-100, and 15 μg of membrane protein. Unless otherwise indicated, sodium azide (5 mM) and sodium orthovanadate (0.2 mM) were added to inhibit ATPase activity from the mitochondrial and plasma membrane ATPases.
respectively. Bafilomycin-sensitive activity was calculated in the absence and presence of 0.1 μM bafilomycin A₁.

**SDS-polyacrylamide gel electrophoresis**
After TCA precipitation (12.5% final concentration), membrane proteins were resuspended in a buffer containing 62 mM TRIS-HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol. Samples were incubated at 30 °C for 2 min and run on 10% polyacrylamide gels according to Laemmli (1970). To visualize proteins, gels were silver-stained (Oakley et al., 1980).

**Immunoblotting**
After separation by SDS-PAGE, polypeptides were transferred electrophoretically to an Immobilon-P membrane (Millipore, Bedford, MA) and reacted with antiserum directed against the 100 kDa PM-ATPase (PMA-1) from Arabidopsis thaliana (PM-ATPase) or with monoclonal antiserum directed against the 70 kDa catalytic subunit of the V-ATPase from oat roots (V-ATPase). Blots were developed by treatment with alkaline phosphatase-conjugated goat antiserum to rabbit IgG (PM-ATPase) or mouse IgG (V-ATPase) and then stained for alkaline phosphatase activity (Burnette, 1981; Ward et al., 1992, respectively).

**Determination of Na⁺ and K⁺**
Shoot samples were dried at 65 °C to constant weight, digested with nitric acid, and Na⁺ and K⁺ concentrations were determined by atomic absorption spectroscopy (Association of Official Analytical Chemists, 1984).

**Data analysis**
Analysis of variance for specific V-ATPase activity due to salts added in vitro was analysed using the GLM procedure in SAS (SAS Institute, 1988). Analyses of variance for all other variables were done using CoStat (CoHort Software, Minneapolis, MN). Throughout, P≤0.05 was used to define statistical significance. Whenever significant effects of salinity were observed, mean separation was accomplished using Duncan’s Multiple Range Test.

**Results**

**Characteristics of the H⁺-ATPases in Salicornia**
Plasma membrane-enriched vesicles recovered from a 6–12% dextran interface from shoots of plants grown in 200 mM NaCl were used to characterize ATPase activity (Briskin et al., 1987). Activity was inhibited by sodium orthovanadate with 50% of the activity inhibited at 20 μM and stimulated by potassium (K⁺) (Kᵣ = 15 mM), but was insensitive to bafilomycin A₁, nitrate, DCCD, and sodium azide (Table 1). This indicates that the major activity in this membrane fraction was associated with the plasma membrane (Sze, 1985; Brüggemann and Janisch, 1989; Briskin, 1990a). Large amounts of PVPP were required to allow measurement of ATPase activity (both PM- and V-ATPase activities) in membrane preparations from Salicornia shoots, suggesting that secondary compounds may be responsible for the relatively low levels of ATPase activity observed. Activity due to Ca²⁺-ATPase was minimized by performing assays at pH 6.7 (the pH optimum for Ca²⁺-ATPases in plants is between 7 and 7.5) and in the absence of calcium (Briskin, 1990b). All subsequent assays contained 0.1 μM bafilomycin A₁ and 5 mM sodium azide.

Vacuolar-enriched vesicles from plants grown in 200 mM NaCl and recovered from a 0–6% dextran interface were used to characterize ATPase activity. Activity was stimulated by Cl⁻ (Kᵣ = 3 mM) and inhibited by bafilomycin A₁, nitrate, and DCCD (Table 1) with 50% of the activity inhibited at 0.2 μM, 100 mM and 7 μM, respectively. To measure optimal V-ATPase activity, 10 mM Cl⁻-BTP, 5 mM sodium azide, and 0.2 mM sodium orthovanadate were included in all subsequent assays, which are reported as bafilomycin A₁-sensitive, vanadate- and azide-insensitive.

**Plant growth in Salicornia is stimulated by Na⁺**
Plants grown in 200 mM NaCl showed increased growth and had longer internodes with larger diameters compared with plants grown in 5 mM NaCl (data not shown). It has previously been found that the relative response to salinity in Salicornia is the same whether expressed on a fresh, dry, or ash-free dry weight basis (Ayala and O’Leary, 1995). Data are reported as shoot fresh weight to allow comparison of increased growth and ATPase activity in the same plants. Shoot fresh weight of plants grown in 200 mM NaCl averaged more than 50% greater than in plants grown in 5 mM NaCl (Table 2). Growth of plants in 200 mM KCl was used as a cation control to demonstrate that ATPase stimulation is specifically associated with sodium (Na⁺)-induced growth. KCl-grown plants showed no growth and had high mortality rates (85±10%) compared to plants grown in 200 mM NaCl. Plants that survived KCl treatment were severely stunted and could not be used for membrane isolation and

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of stimulation seen in vitro (46%) relative to that observed in vivo (26%) may reflect differences in actual concentrations of Na⁺ at the membrane surface. While equivalent amounts of Na⁺ were added to membranes in vitro and to solutions during plant growth, ATPases in the whole plant may have been exposed to lower levels of Na⁺ depending on the efficiency of Na⁺ uptake into the plant. As with measurements of in vitro Na⁺-stimulation of PM-ATPase in 200 mM NaCl-grown plants, the V-ATPase from salt-grown plants was insensitive to Na⁺ added in vitro (data not shown).

## Discussion

Some solute accumulation is needed in cells of halophytes to lower their water potential below that of the external water potential for continued uptake of water and growth (Ungar, 1991). However, salt tolerance in halophytes depends ultimately upon the ability of cells to regulate intracellular Na⁺ concentrations to keep cytoplasmic levels low enough to protect salt-sensitive metabolic processes (Flowers et al., 1986). Increased activity of the PM-ATPase in 200 mM NaCl-grown Salicornia plants may be an important component in the regulation of intracellular Na⁺ levels. Increased activity leads to a greater pH gradient across the plasma membrane which may provide the driving force for a plasma membrane Na⁺/H⁺ exchanger to move Na⁺ from the cytoplasm into the apoplast. The differential growth response seen in Salicornia will allow the correlation of the activities of the PM-ATPase and the Na⁺/H⁺ exchanger by comparing apoplastic pH and Na⁺-induced dissipation of the pH gradient in 5 and 200 mM NaCl-grown plants. In addition, increased ATPase activity may be needed for increased cell elongation leading to larger cell size which, in combination with increased succulence, may enable the plant to dilute high intracellular Na⁺ concentrations and may facilitate expansion of the vacuole to allow increased Na⁺ sequestration.

Increased activity of the PM-ATPase with increasing salinity in Salicornia is in contrast to decreased activity seen in the halophyte Plantago maritima (L.) (Brüggeman and Janishe, 1989). The lack of Na⁺ stimulation of the ATPase in Plantago may reflect decreases in growth seen with increasing salt (Erdei and Kuiper, 1979). In the halophyte Atriplex nummularia Lindl., Na⁺-stimulation of the proton transport capacity of the PM-ATPase has been reported (Niu et al., 1993) while only a slight Na⁺-stimulation of ATP hydrolysis was seen (Mills and Hodges, 1988). The Na⁺-stimulation of H⁺-pumping in Atriplex may be due to increased abundance of PM-ATPase mRNA presumably leading to increased levels of the protein (Braun et al., 1986). Immunoblot data presented here suggests that increased PM-ATPase activity in Salicornia is not due to increased expression of the ATPase protein. Any differences in expression between Salicornia and Atriplex may reflect tissue differences (as studies in Atriplex were done with salt-grown roots) or isoform differences. Currently, it is not known if Salicornia has multiple isoforms of the PM-ATPase as has been found for Arabidopsis thaliana (Sussman and Harper, 1989). Final conclusions relating activity and expression will depend on the identification of isoforms and the availability of isoform specific antibodies.

In order to understand the mechanisms of Na⁺-stimulation of the PM-ATPase, ATPase activity in plasma membrane vesicles in the presence of increasing Na⁺ added in vitro was measured. No stimulation of ATPase activity was observed, leading to the conclusion that Na⁺-stimulation in vivo is accomplished by pathways requiring cytoplasmic factors. One mechanism of Na⁺-stimulation may involve Na⁺-dependent inactivation of the auto-inhibitory domain of the C-terminal portion of the 100 kDa PM-ATPase. This domain may be the target for hormones and toxins that function as regulators of H⁺-pumping across the plasma membrane in vivo (Palmgren, 1991). Johansson et al. (1993) suggested that in vivo activation of the H⁺-ATPase by the fungal toxin fusicocin proceeds by a mechanism involving a displacement of the C-terminal inhibitory domain. A second mechanism for Na⁺ regulation of the PM-ATPase may involve a change in the phosphorylation state of the enzyme. The plant plasma membrane H⁺-ATPase has been shown to be phosphorylated both in vivo and in vitro (Schaller and Sussman, 1988); what remains is to correlate phosphorylation of the H⁺-ATPase with changes in activity of the enzyme. Antibodies specific to the C-terminal sequence (Palmgren et al., 1991) and in vitro and in vivo phosphorylation should permit determination of whether either mechanism defines Na⁺ stimulation of the PM-ATPase.

The accumulation of Na⁺ in the shoots of Salicornia was greater in 200 mM NaCl-grown plants than in 5 mM NaCl-grown plants. Most of the Na⁺ accumulated in 200 mM NaCl-grown plants must be stored in vacuoles (Harvey et al., 1981; Matoh et al., 1987) because metabolic enzymes in the cytoplasm of most plants studied are salt-sensitive (Flowers, 1972; Greenway and Osmond, 1972; Osmond and Greenway, 1972). V-ATPase activity was significantly higher (P<0.05) in the shoots of Salicornia plants grown in 200 mM NaCl. As a control for Na⁺ specificity, Salicornia was also grown in 200 mM KCl. Measurements of K⁺ concentration in plants grown in NaCl show that even when K⁺ levels are higher (5 mM NaCl-grown plants compared to 200 mM NaCl-grown plants), there is neither a corresponding increase in V-ATPase activity, nor an increase in plant growth. Increased V-ATPase activity may provide the driving force for Na⁺ sequestration into the vacuole (DuPont, 1992) and is consistent with the estimates made in the halophyte Suaeda maritima that compartmentation of...
NaCl in leaf vacuoles can be sustained by about 30% of ATP-dependent proton pumping capacity (Maathuis et al., 1992). The ability to sequester Na\(^+\) in the vacuole may be an essential component of salt tolerance as studies with the glycophyte *Phaseolus vulgaris* L., one of the most salt-sensitive plant species known, showed a lack of effective intracellular ion compartmentation in plants grown in 150 mM NaCl (Seemann and Critchley, 1985). It will be important to link increased V-ATPase activity and increases in driving force for Na\(^+\)/H\(^+\) exchange.

Conflicting reports exist relative to the effects of Na\(^+\) on the V-ATPase in other systems. When the glycophyte, barley, was grown in NaCl, proton transport activity of the V-ATPase (isolated from roots) was increased compared to that of a no-salt control (Matsumoto and Chung, 1988; DuPont and Morrisey, 1991) with no change in ATP hydrolysis (DuPont and Morrisey, 1991). In cultured salt-grown cells of the glycophyte *Nicotiana*, both the ATP hydrolysis and the proton transport capacity of the V-ATPase activity increased (Reuveni et al., 1990). In *Plantago maritima* and the glycophyte *P. media*, specific V-ATPase activities in roots were similar and did not change after exposure to 50 mM NaCl (Staal et al., 1991). Varied Na\(^+\) effects on the V-ATPase in these systems may be due to the fact that these studies were done using roots or cultured cells while Na\(^+\) compartmentation studies have generally indicated that a substantial portion of the Na\(^+\) accumulates in the shoots (Cheeseman, 1988).

In *Salicornia*, additions of 200 mM NaCl in *in vitro* stimulated the V-ATPase beyond the Cl\(^-\)-stimulated activity. Biochemical mechanisms that might account for V-ATPase regulation are unknown. This ATPase is composed of 10 subunits in oat roots (Ward and Sze, 1992). It has been suggested that salt adaptation could involve post-translational or allosteric regulation affecting ATP hydrolysis and proton transport activities (Reuveni et al., 1990) or removal of an inhibitory subunit.

ATPase activity from *Salicornia* shoots of plants grown in 200 mM NaCl for 14 d showed similar properties (pH optima, stimulator and inhibitor sensitivities) when compared with those of various glycophyles grown in Na\(^+\)-free conditions (Table 2; Sze and Hodges, 1977; Churchill and Sze, 1984; Poole et al., 1984; Sze, 1985; Randall and Sze, 1986; Matsumoto and Chung, 1988; Briskin, 1990a). These results indicate that, other than its response to Na\(^+\), the ATPases from *Salicornia* have biochemical characteristics similar to their counterparts in glycophyles, and that *Salicornia* will be an excellent system in which to understand the role of the ATPases in salt tolerance.

These findings suggest that the two primary H\(^+\)-ATPases are important in the increases in growth and salt tolerance observed in *Salicornia* grown in high salt. In addition, the work described provides a starting point for understanding mechanisms that regulate intracellular Na\(^+\) levels in *Salicornia*, and in combination with whole plant studies, may ultimately help us to improve the salt tolerance of crop plants.

**Acknowledgements**

This work was supported in part by grants from US Department of Agriculture, Southwest Plant Genetics/Water Consortium grant number 92-34186-7249 (to JWO and KSS) and CONACYT and Universidad de Sonora (to FA). We thank Dr MR Sussman (University of Wisconsin) for providing the *Arabidopsis* PM-ATPase antiserum, Dr H Sze (University of Maryland) for providing the V-ATPase antiserum, and Michael Gizinski, Daniel Parker, and Sandra Salus for technical assistance. We also thank Drs M Dietrich, RT Leonard, S Smith, and J Verbeke for comments on the manuscript.

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