Salt Sensitivity and the Activities of the H^+-ATPases in Cotton Seedlings

Howard Lin, Sandra S. Salus, and Karen S. Schumaker*

ABSTRACT

Salinity is a major problem confronting agriculture in arid environments. Sensitivity to high levels of salt in plants is associated with an inability to effectively remove Na^+ ions from the cell cytoplasm. The ability to compartmentalize Na^+ may result, in part, from stimulation of the H^+-ATPases on the plasma membrane (PM-ATPase) and vacuolar membrane (V-ATPase). These H^+-pumping ATPases may provide the driving force for Na^+ transport via Na^+-H^+ exchangers. In a salt-sensitive line of cotton (*Gossypium hirsutum* L.), greater relative reductions in root length and root fresh weight than in hypocotyl length of seedlings grown in 75 m*M* NaCl indicated that the root was most affected by salt stress. To determine if the H^+-ATPases are involved in the response to salt, we compared activities of the PM- and V-ATPases from roots in salt-sensitive cotton seedlings grown with or without 75 m*M* NaCl. Higher PM-ATPase activity (42%) was observed in seedlings grown in 75 m*M* NaCl. This stimulation was specific for Na^+, was not observed when Na^+ was added to membrane fractions, and was not due to an increase in PM-ATPase protein levels. V-ATPase protein accumulation was unaffected by growth in the presence of Na^+, and activity was unaffected by Na^+ in the growth medium or by Na^+ added to membrane fractions. These studies suggest that although the PM-ATPase responds to increased Na^+, activity of the transport proteins on the plasma membrane alone may be insufficient to regulate intracellular Na^+ levels. In addition, the inability of the V-ATPase to respond to increased levels of Na^+ indicates that salt sensitivity in cotton seedlings may result, in part, from a lack of effective driving force for compartmentalization of Na^+.

FOR CENTURIES, a major problem facing agriculture in arid environments has been the increase in soil salinity, which is typically associated with irrigation (Bernstein and Hayward, 1958). When irrigation water contains a high concentration of solutes, and when leaching is limited, salt concentrations in the soil solution reach levels that are injurious to salt-sensitive species (Flowers et al., 1977). With increasing use of irrigation, more crop plants are being grown under saline conditions. As a consequence, plant breeders have devoted considerable effort to the isolation of salt-tolerant cultivars from many agriculturally important crops (Shannon, 1985). Although there has been some success in developing salt-tolerant cultivars (Johnson et al., 1991), there is very little information about the molecular mechanisms conferring salt tolerance. Understanding these mechanisms may ultimately accelerate the development of salt-tolerant genotypes (Noble and Rogers, 1992).

Under nonsaline conditions, the cytosol of higher plant cells contains 100 to 200 m*M* K^+ and 1 m*M* Na^+ (Pierce and Higinbotham, 1970; Binzel et al., 1988), while Na^+ ions are often found in high concentration in the plant under saline conditions (Greenway and Munns, 1980). When high levels of salt are present, abnormally high ratios of Na^+ to K^+ in the cell lead to inactivation of enzymes and inhibition of protein synthesis (Flowers et al., 1977; Greenway and Osmond, 1972). At high concentrations, Na^+ has also been shown to displace calcium from the plasma membrane of cotton root hairs resulting in a change in plasma membrane permeability that can be detected as leakage of K^+ to the environment (Cramer et al., 1985). In addition, photosynthesis is inhibited when high concentrations of Na^+ accumulate in chloroplasts (Yeo et al., 1985; Bethke and Drew, 1992).

Enzymes extracted from salt-tolerant species are equally as sensitive to the presence of NaCl as enzymes from salt-sensitive species suggesting that resistance to salt is not solely a consequence of a salt-resistant metabolic machinery (LaRosa et al., 1991). Instead, it appears that other mechanisms are involved (Wyn Jones et al., 1979; Jeschke, 1984); for example, plants may avoid injury by exclusion or compartmentation of harmful ions.

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Abbreviations: ABA, abscisic acid; FW, fresh weight; kD, kilodalton; PM-ATPase, plasma membrane H^+-ATPase; V-ATPase, vacuolar membrane H^+-ATPase.

While Na^+ ions can enter roots passively (moving down an electrochemical potential gradient), removal of Na^+ from the cytoplasm can be accomplished by sequestration of the ion into the vacuole or by transport across the plasma membrane and out of the cell (Behl and Jeschke, 1981). Movement of Na^+ across membranes must involve specific transport proteins embedded in the lipid bilayer.

Plant cells have a relatively small number of primary energy transducing proteins in their cell membranes (Maathius and Sanders, 1992). Primary ion transporters generate transmembrane gradients by transferring specific ions energetically uphill. The major primary active transporter in the plant cell is the H^+-translocating ATPase. H^+-ATPases have been identified on membranes of most intracellular organelles as well as on the plasma membrane (Sze, 1985). The H^+-ATPases on the plasma membrane (PM-ATPase) and vacuolar membrane (V-ATPase) have been shown to hydrolyze ATP and acidify the apoplast and vacuolar lumen, respectively. As H^+ are translocated by these ATPases, a proton motive force composed of a pH component and an electrical component is generated. These gradients can be used as the direct source of energy for the transmembrane flux of numerous other metabolites and ions if there are porter molecules in the membrane which couple the two fluxes. Evidence exists for transport mechanisms that allow plants to maintain low cytoplasmic concentrations of Na^+ (Cheeseman, 1988). Na^+ gradients may be regulated by the operation of secondary Na^+-H^+ antiporters on the tonoplast and plasma membranes (Blumwald and Poole, 1985; Barkla et al., 1995). These transport systems could function to remove Na^+ ions from the cytoplasm across the plasma membrane and out of the cell, or to compartmentalize it in the vacuolar lumen. The combined operation of H^+-ATPases and Na^+-H^+ antiporters could accomplish this.

If plants avoid injury by exclusion or compartmentation of harmful ions, understanding the mechanisms by which this is accomplished could be of great practical importance. One mechanism that may help to confer this ability is direct Na^+-stimulation of the ATPases on the vacuolar and plasma membranes. Our objectives in this research were to characterize the transport properties of the H^+-ATPases on the vacuolar and plasma membranes in salt-sensitive cotton seedlings and to determine the effect of Na^+ on these enzymes.

MATERIALS AND METHODS

Plant Material

A salt-sensitive line of cotton was derived from two cycles of mass selection for reduced germination and emergence in soil irrigated with 91.3 m*M* NaCl (Ledbetter, 1986). Seeds from this line were germinated at 31°C in the dark. Forty seeds were rolled in germination paper (Anchor Paper Co., St. Paul, MN) and kept moist by placing the lower portion of the paper in water (200 mL in a 1-L beaker). For experiments requiring non-salt-stressed (control) tissue, seedlings were transferred to an aerated nutrient solution containing in millimolars: 3 $Ca(NO_3)_2$, 2 KNO_3, 2 KH_2PO_4, 2 $MgSO_4$; in micromolars: 7.6 $MnCl_2$, 40 H_3BO_3, 0.3 $CuCl_2$, 1.3 $ZnSO_4$, 3 MoO_3. Iron was supplied as the EDTA complex at 4.2 mg Fe L^{-1} (Ayala et al., 1996). Solutions were changed daily and plants were grown for 72 h before harvest. For salt treatments, seeds that had germinated for 24 h without salt were transferred to an aerated nutrient solution with 75 m*M* NaCl or KCl. Salt solutions were changed daily and the plants were harvested after a 72-h salt exposure.

Preparation of Vacuolar-enriched Membrane Vesicles

Cotton roots (10–30 g) were cut into upper (closer to the hypocotyl) and lower halves and, after recording fresh weights (FW) of each, all procedures were carried out at 4°C. Root sections were homogenized separately with a mortar and pestle in buffer (3 mL g^{-1} plant material) containing in millimolars: 250 sorbitol, 25 Hepes {(*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid])}-BTP [1,3-bis[tris(hydroxymethyl)-methyl amino]propane)] at pH 7.4, 3 EGTA [ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid], 1 DTT (dithiothreitol), 1 iodoacetamide, 0.1 PMSF (phenylmethylsulfonylfluoride), 0.01 pepstatin A, and 2.94×10^{-3} mol L^{-1} fatty acid-free BSA (bovine serum albumin) and 0.25 g PVPP (polyvinylpolypyrrolidone) per gram FW. The homogenized material was filtered through cheesecloth and the homogenate centrifuged for 15 min at 13 000 g. The supernatant was centrifuged for 30 min at 60 000 g (Beckman SW rotor, r_{max}), and the resulting pellet was gently resuspended in a resuspension buffer containing in millimolars: 250 sorbitol, 2.5 Hepes-BTP (pH 7.4), and 1 DTT. The suspensions (1 mL) were layered on gradients (12 mL each) containing 8.22×10^{-4} mol kg^{-1} or 1.64×10^{-3} mol kg^{-1} dextran (average mol wt-73 000) made in resuspension buffer to isolate vacuolar and plasma membranes, respectively. After centrifugation at 70 000 g (Beckmann SW 28.1 rotor, r_{max}) for 2 h, vesicles at the 0 to 8.22×10^{-4} mol kg^{-1} and 8.22×10^{-4} mol kg^{-1} to 1.64×10^{-3} mol kg^{-1} interfaces (vacuolar and plasma membranes, respectively) were recovered.

ATPase Assays and Protein Determination

ATPase activity, measured as release of inorganic phosphate from hydrolysis of ATP (Briskin et al., 1987; Schumaker and Sze, 1986), was monitored in a 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 in plasma membranes, reaction mixes contained in millimolars: 10 PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid), buffered with Tris Base to pH 6.7], 5 $MgCl_2$, 5 PEP [phospho(enol) pyruvate, hexamethylammonium salt], 5 Na_2ATP, 4.5 units of pyruvate kinase and 10 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 m*M*) and bafilomycin A_1 (0.1 μ*M*) 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 absence and presence of 0.2 m*M* sodium vanadate.

For measurement of bafilomycin A_1-sensitive ATPase activity in vacuolar membranes, reaction mixes contained in millimolar: 30 Hepes (buffered with BTP to pH 7.5), 5 $MgSO_4$, 5 PEP, 5 ATP (buffered to pH 7.5 with BTP using Dowex 50W), 4.5 units of pyruvate kinase, 0.12 μL mL^{-1} Triton X-100 and 15 to 20 μg of membrane protein. Unless otherwise indicated, 5 m*M* sodium azide and 0.2 m*M* sodium ortho-

vanadate were added to inhibit ATPase activity from the mitochondrial and plasma membrane ATPases, respectively. Bafilomycin A_1-sensitive activity was calculated as the difference in activity in the absence and presence of 0.1 μM bafilomycin A_1. In experiments measuring the effect of DCCD (*N,N'*-dicyclohexylcarbodiimide) on V-ATPase activity, membrane protein was pre-incubated with DCCD at 22°C for 10 min and assays were carried out in glass tubes. DCCD levels were adjusted to keep concentrations constant during incubation and assay.

Protein concentration was estimated after precipitation with 100 $\mu L\ mL^{-1}$ TCA (trichloroacetic acid) by the method of Lowry et al. (1951) using BSA as the standard.

SDS-Polyacrylamide Gel Electrophoresis

After TCA precipitation (0.76 mol L^{-1} final concentration) and a wash with 13.5 mol L^{-1} acetone, membrane proteins (3–5 μg) were resuspended in a buffer containing 62 m*M* Tris-HCl (pH 6.8), 0.07 mol L^{-1} SDS (sodium dodecyl sulfate), 1.37 mol L^{-1} glycerol, 8 *M* urea, 2.89×10^{-5} mol L^{-1} bromophenol blue, and 0.71 mol L^{-1} 2-mercaptoethanol. Samples were denatured by boiling for 4 min (vacuolar membranes) or incubation at 30°C for 2 min (to prevent aggregation of plasma membrane proteins due to boiling) and separated on polyacrylamide gels (1.41 mol L^{-1})according to Laemmli (1970). To visualize proteins, gels were silver-stained (Oakley et al., 1980).

Immunoblotting

After separation by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), polypeptides were transferred electrophoretically to an Immobilon-P membrane (Millipore, Bedford, MA) and reacted with antiserum directed against the 100-kilodalton (kD) PM-ATPase (PMA-1) from *Arabidopsis thaliana* (L.) Heynh. (PM-ATPase) or with monoclonal antiserum directed against the 70-kD 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).

Data Analysis

Analyses of variance for all variables were done with PROC ANOVA of SAS (SAS Instit., 1988). Whenever significant effects of salinity were observed, mean separation was accomplished using a *t*-test. Throughout, $P \leq 0.05$ was used to define statistical significance.

Western blot data were analyzed by means of a COHU High Performance CCD Camera (San Diego, CA) and a Mitsubishi Video Copy Processor (Somerset, NJ). Quantitation was performed on a Mac Power PC computer (8 bit) by the public domain NIH Image program (version 1.57) developed at the U.S. National Institutes of Health.

RESULTS AND DISCUSSION

Growth of Cotton Seedlings is Inhibited by Na^+

Exposure to high levels of NaCl has been shown to affect the growth of shoots, roots or both depending on the species (Poljakoff-Mayber, 1975). To determine if and where salt is affecting the growth of cotton, seedlings were germinated without salt, then transferred to solutions containing 75 m*M* NaCl or 75 m*M* KCl and root fresh weight, and root and hypocotyl lengths were compared with those of plants grown without salt. Within 72 h of exposure to 75 m*M* NaCl, seedlings showed a 40 to 51% reduction in root fresh weight compared with those grown without NaCl (Table 1), and seedlings died when plants were kept in NaCl solutions beyond 72 h (data not shown). Reduced root fresh weight was also associated with a reduction in root length, while NaCl had little effect on hypocotyl length (Table 1). These results indicate that the cotton seedling root is the organ most severely affected by exposure to salt. Growth of plants in 75 m*M* KCl did not differ significantly from control plants (Table 1) suggesting that reduction in growth in NaCl grown plants was due specifically to sodium (Na^+).

Characteristics of the H^+-ATPases in Cotton Seedlings

Initial characterizations of ATPase activity from vacuolar enriched membranes (0–8.22 $\times 10^{-4}$ mol kg^{-1} dextran gradients) from whole root tissue indicated low levels of bafilomycin A_1-sensitive (vacuolar) ATPase activity. Examination of hand sections of root tissue indicated that the upper half of the root contained highly vacuolated cells while the cells nearer the root tip were densely cytoplasmic (data not shown). Characterization of vacuolar ATPase (V-ATPase) activity from membranes isolated from each half of the root indicated that the properties of the ATPase were identical; however, the yields of vacuolar membranes (and V-ATPase activity) were much higher in preparations from the upper root. Similar results were seen for plasma membranes and the PM-ATPase in membranes isolated from lower roots. Therefore, to enrich for V- and PM-ATPase activity, vacuolar and plasma membranes were isolated from the upper and lower halves of the root, respectively. To determine optimal assay conditions for comparing activities of the ATPases in control and salt-treated seed-

Table 1. Effect of 75 m*M* KCl or NaCl on root fresh weight, and hypocotyl and root length in cotton seedlings during 72-h exposure to salt treatments.

Exposure time	Control	75 m*M* KCl	75 m*M* NaCl	Ratio (Na^+/Control)
h				
	Root fresh weight (mg $root^{-1}$)			
24	18.6 ± 0.01	17.9 ± 0.02	7.5 ± 0.01*	0.40
48	22.0 ± 0.001	23.1 ± 0.01	12.2 ± 0.01*	0.55
72	26.4 ± 0.01	26.7 ± 0.01	13.5 ± 0.01*	0.51
	Root length (mm)			
24	23.8 ± 2.82	22.9 ± 1.76	8.6 ± 1.73*	0.36
48	35.8 ± 2.40	34.3 ± 1.93	17.0 ± 4.78*	0.48
72	42.0 ± 3.76	43.7 ± 2.29	22.6 ± 6.08*	0.54
	Hypocotyl length (mm)			
24	13.0 ± 1.05	14.5 ± 2.33	11.6 ± 1.65*	0.89
48	29.8 ± 1.44	30.8 ± 0.96	23.0 ± 1.78*	0.77
72	41.1 ± 1.44	43.1 ± 1.56	38.8 ± 3.47	0.95

* Mean significantly different from the mean of the control treatment, $P \leq 0.05$. Values represent the means ± SE from four different experiments. Within a given treatment, each experiment represents the mean of 20 plants.

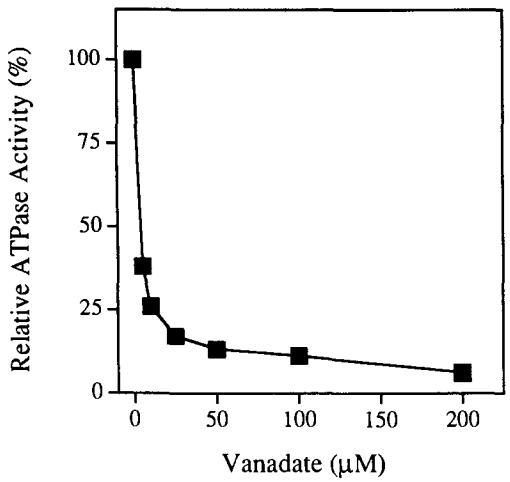

Fig. 1. Inhibition of the PM-ATPase from cotton seedlings by increasing concentrations of sodium ortho-vanadate. Points represent the means of six independent experiments. For all, SEs are less than 2% of the total activity. Control ATPase activity was 11.44 ± 0.99 μg P_i released mg protein^{-1} h^{-1}.

lings, vesicles recovered from a 0 to 8.22 × 10^{-4} mol kg^{-1} or a 8.22 × 10^{-4} to 1.64 × 10^{-3} mol kg^{-1} dextran interface from upper and lower halves of roots of plants grown without NaCl were used to characterize V- and PM-ATPase activities, respectively (Briskin et al., 1987). PM-ATPase activity was inhibited by sodium ortho-vanadate, an inhibitor of ATPases with phosphorylated intermediates in their reaction mechanism, with 50% of the activity inhibited at 3 μ*M* (Fig. 1). Activity was stimulated by potassium (Fig. 2) with a K_m = 2.4 m*M* (Fig. 2 and Table 2), but was insensitive to the V-ATPase inhibitors bafilomycin A_1 (Bowman et al., 1988) and nitrate, and the mitochondrial ATPase inhibitor, sodium azide (Table 2). PVPP was required to allow measurement of ATPase activity (both PM- and V-ATPase activities) in membrane preparations from roots, suggesting that phenolics may be responsible for the relatively low levels of ATPase activity observed. Activity due to Ca^{2+}-ATPases was minimized by performing assays at pH 6.7 (the pH optimum for Ca^{2+}-ATPases in plants is between 7 and 7.5) and in the absence of calcium (Briskin, 1990). Subsequent assays contained 0.1 μ*M* bafilomycin A_1 and 5 m*M* sodium azide. Similar levels of vanadate-sensitive ATPase activity were seen when plasma membranes were isolated by means of aqueous two-phase partitioning (Palmgren et al., 1990) (data not shown); however, yields of purified plasma membranes were so low that this method was impractical for subsequent assays.

Table 2. Properties of the vacuolar and plasma membrane H⁺-ATPases from roots of cotton seedlings.

Parameter	V-ATPase	PM-ATPase
pH optimum†	7.5	6.7
Stimulator‡		
Cl^-	6.3 m*M*, 44%	RS§
K^+	RS	2.4 m*M*, 18%
Inhibitor¶		
Vanadate	NI#	3 μ*M*, 98%
Bafilomycin A_1	53 nM, 62%	NI
Nitrate	15 mM, 70%	NI
DCCD	3.7 μM, 82%	NI

† pH optima for each ATPase were calculated from three independent experiments measuring ATPase activity from pH 5 to 9 in 0.5 pH increments.
‡ K_m values and maximum stimulation of ATPase activity.
§ RS, reduced stimulation, stimulation was less than 5% of the total ATPase activity.
¶ Concentrations of inhibitors required for 50% inhibition and maximum inhibition of ATPase activity.
# NI, not inhibited; inhibition was less than 10% of the total ATPase activity.

V-ATPase activity was insensitive to vanadate but inhibited by bafilomycin A_1, nitrate, and the lipid-soluble carbodiimide, *N,N'*-dicyclohexylcarbodiimide (DCCD) with 50% of the activity inhibited at 53 n*M*, 15 m*M*, and 3.7 μ*M*, respectively (Fig. 3). Low levels of Triton X-100 increased vanadate-insensitive, bafilomycin A_1-sensitive ATPase activity approximately 2.5 fold (data not shown). Triton stimulation of activity suggests that vesicles isolated from the 8.22 × 10^{-4} to 1.64 × 10^{-3} mol kg^{-1} interface were both inside-out and right-side-out in their orientation. V-ATPase activity was stimulated by Cl^- (Fig. 4) with a K_m = 6.3 m*M* (Fig. 4 and Table

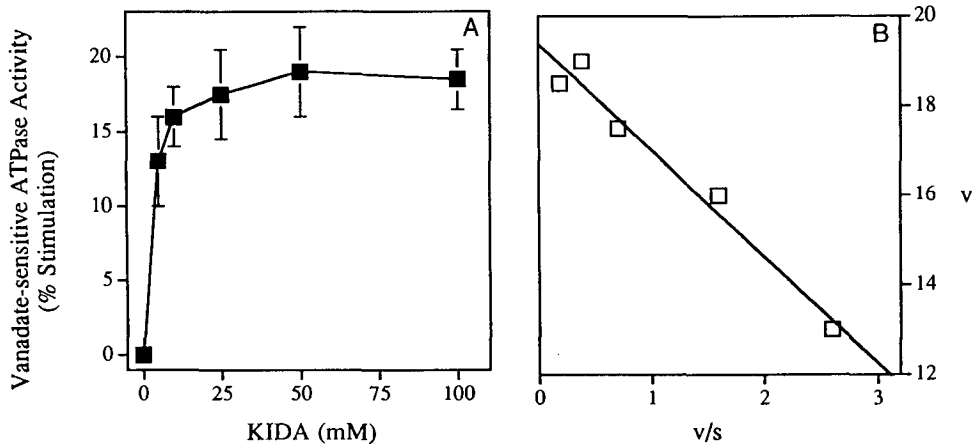

Fig. 2. Stimulation of the PM-ATPase from roots of cotton seedlings by increasing concentrations of potassium. *A*, Vanadate-sensitive ATPase activity. Points represent the mean ± SE of six independent experiments. *B*, An Eadie-Hofstee plot of the data was used to calculate the K_m value (y = 19.391 − (2.371)x, r^2 = 0.969).

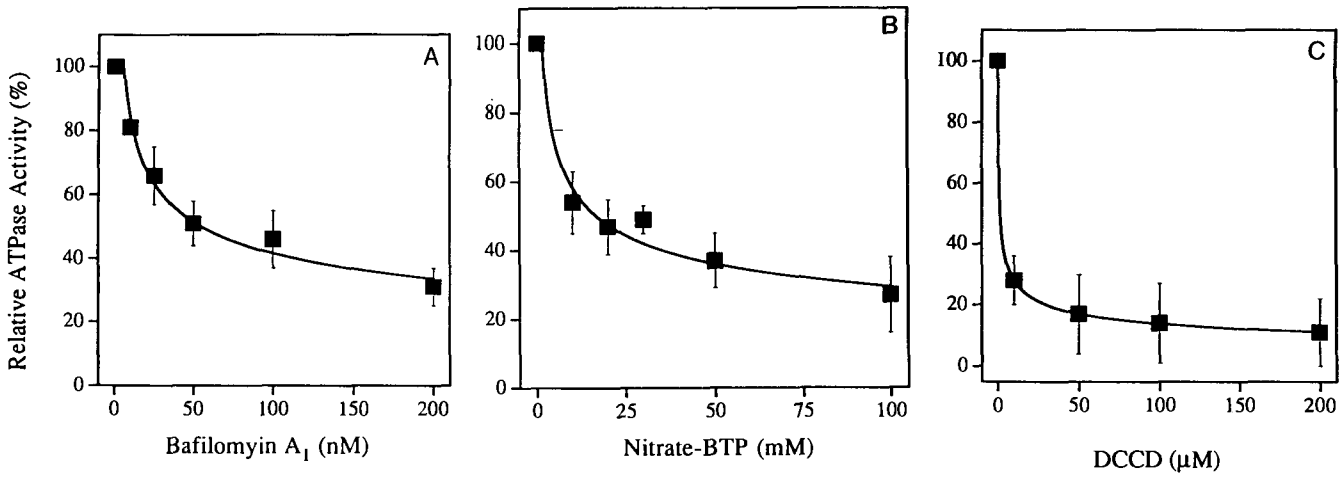

Fig. 3. Inhibition of the V-ATPase from roots of cotton seedlings by bafilomycin A_1, nitrate, and DCCD. ATPase activity was measured with the addition of increasing concentrations of bafilomycin A_1 (A), nitrate (B), or DCCD (C). Points represent mean ± SE of three independent experiments. Control ATPase activity was 6.1 ± 0.8, 7.3 ± 0.66, and 6.33 ± 0.4 μg P_i released mg protein^{-1} h^{-1} for the bafilomycin A_1, nitrate, and DCCD experiments, respectively.

2). This represents a direct stimulation of the protein (not Cl^- dissipation of the membrane potential) as Triton X-100 was included in all assays and would prevent the establishment of a membrane potential across the vesicle.

The PM-ATPase in Cotton Seedlings Grown in NaCl is Stimulated by Na^+

To determine the role of the PM-ATPase in the response of cotton seedlings to salt, PM-ATPase activity was compared in control and salt-grown plants. Specific activity of the vanadate-sensitive, bafilomycin A_1-insensitive PM-ATPase in membranes isolated from 75 m*M* NaCl-grown plants was 42% higher than that of the plants grown without NaCl (Table 3). This stimulation was specific for Na^+ as plants grown in 75 m*M* KCl had ATPase activities similar to control plants (Table 3). Immunoblots of plasma membrane proteins reacted with antibody to the 100-kD PM-ATPase from *A. thaliana* (PMA-1) identified a single reactive protein with an apparent molecular mass of 100 kD. Comparison of proteins isolated from plants grown in the absence and presence of 75 m*M* NaCl indicated that Na^+-stimulation of ATPase activity in plants grown in saline conditions was not due to an increase in levels of this isoform of the PM-ATPase (Fig. 5B). Quantitation of the integrated densities of the immunoreactive bands from four independent Western blots indicated that there were no significant differences in the levels of protein (the mean of the integrated density of Na^+-treated plants as a percent of control plants was 19.1% ± 2.8). Electrophoresis (Laemmli, 1970) of membrane polypeptides (Fig. 5A) suggested that proteins with an apparent molecular mass of 100 kD were not affected by the Na^+ treatment either quantitatively or qualitatively.

To begin to understand the biochemical basis for the increased PM-ATPase activity in cotton seedlings after exposure to 75 m*M* NaCl, we tested the effect of NaCl added to vesicles from shoots of plants grown without NaCl. Because the PM-ATPase is cation-stimulated in vitro (Sze and Hodges, 1977), Na^+-specific stimulation

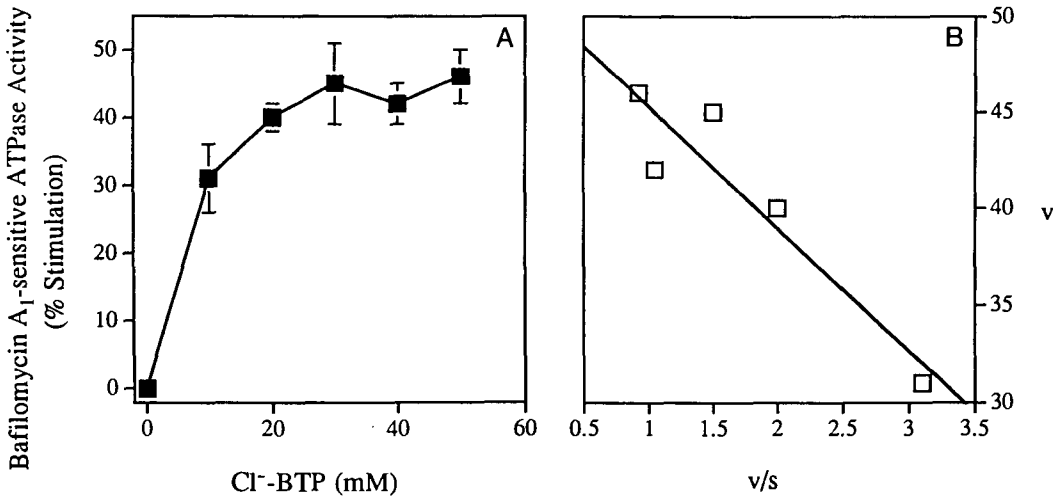

Fig. 4. Stimulation of the V-ATPase from roots of cotton seedlings by increasing concentrations of chloride. *A*. Bafilomycin A_1-sensitive ATPase activity. Points represent the means ± SE of six independent experiments. *B*, An Eadie-Hofstee plot of the data was used to calculate the K_m value (y = 51.587 − (6.29)x, r^2 = 0.865).

Table 3. Effect of 75 m*M* KCl or NaCl on V- and PM-ATPase activities from roots of cotton seedlings.

	ATPase Activity†		
	Control	75 m*M* NaCl	75 m*M* KCl
	μmol P_i mg protein^{-1} h^{-1}		
V-ATPase Activity‡	6.5 ± 1.08	7.1 ± 1.18	8.2 ± 1.65*
PM-ATPase activity§	13.1 ± 0.70	18.6 ± 1.26*	12.1 ± 0.74

* Mean activity significantly different from the mean of the control treatment, $P \leq 0.05$.
† Results represent means ± SE for ATPase activities from membranes isolated after 72 h without salt (Control), or with 75 m*M* KCl or NaCl.
‡ Vanadate-insensitive, bafilomycin A_1-sensitive activity, $n = 6$.
§ Bafilomycin A_1-insensitive, vanadate-sensitive activity, $n = 4$.

in the presence of 5 to 50 m*M* K^+ was measured. Vanadate-sensitive ATPase activity was insensitive to Na^+ added to membrane vesicles at any of the concentrations tested (5–100 m*M*, data not shown) leading to the conclusion that Na^+ stimulation of the ATPase in salt-grown plants is accomplished by pathways requiring cytoplasmic factors. Mechanisms of Na^+ stimulation might include Na^+-induced changes in phosphorylation of the PM-ATPase (Schaller and Sussman, 1988) or Na^+-dependent inactivation of the auto-inhibitory domain of the C-terminal portion of the PM-ATPase (Palmgren, 1991). When vanadate-sensitive ATPase activity was measured in plasma membrane fractions from plants grown in 75 m*M* NaCl, activity was also insensitive to Na^+ added to membrane vesicles (data not shown) indicating that exposure to salt during growth did not alter the sensitivity of the isolated protein to Na^+.

Increases in PM-ATPase activity in salt-sensitive cotton seedlings grown in 75 m*M* NaCl suggest that this proton pump can be regulated by Na^+ (Table 3). We have previously reported that the activity (ATP hydrolysis) of the PM-ATPase in the halophyte *Salicornia bigelovii* Torr. is stimulated when plants are grown in 200 m*M* NaCl (Ayala et al., 1996). In *Salicornia*, increased activity of the PM-ATPase was highly correlated with increased growth under high salt conditions (Ayala et al., 1996). The fact that the PM-ATPase is stimulated in both cotton seedlings and *Salicornia* while the cultivar of cotton used is relatively salt-sensitive and *Salicornia*

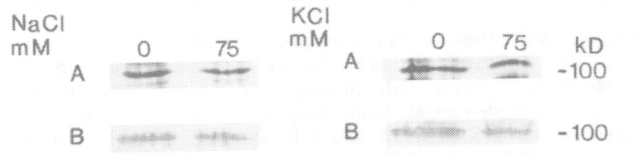

Fig. 5. Effect of salt treatment on expression of the PM-ATPase in roots of cotton seedlings. Results are from one representative experiment of four. A. Plasma membrane proteins. Membrane proteins (1 μg) from plants grown without or with 75 m*M* NaCl or 75 m*M* KCl were resolved on SDS-PAGE (1.41 mol L^{-1}) (Laemmli, 1970) and silver stained (Oakley et al., 1980). Region shown corresponds to proteins with a molecular mass of 100 kD. B. Protein immunoblot analysis of plasma membrane proteins. Membrane proteins (50 μg) from plants grown without or with 75 m*M* NaCl or 75 m*M* KCl were resolved on SDS-PAGE, transferred to Immobilon-P membrane (Millipore, Bedford, MA), and reacted with antiserum directed against the 100-kD PM-ATPase from *Arabidopsis*. Blots were developed by treatment with alkaline phosphatase-conjugated goat antiserum to rabbit IgG and then stained for alkaline phosphatase activity (Burnette, 1981).

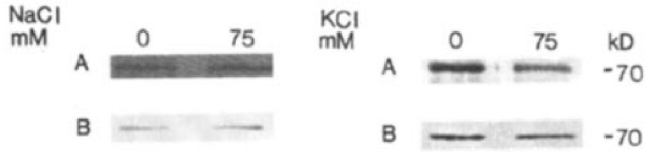

Fig. 6. Effect of salt treatment on expression of the V-ATPase in roots of cotton seedlings. Results are from one representative experiment of three. A. Vacuolar membrane proteins. Membrane proteins (1 μg) from plants grown without or with 75 m*M* NaCl or 75 m*M* KCl were resolved on SDS-PAGE (1.41 mol L^{-1}) and silver stained. Region shown corresponds to proteins with a molecular mass of 70 kD. B. Protein immunoblot analysis of vacuolar vesicles. Membrane proteins (6 μg) from plants grown without or with 75 m*M* NaCl or 75 m*M* KCl were resolved on SDS-PAGE, transferred to Immobilon-P membrane, and reacted with monoclonal antiserum directed against the 70-kD V-ATPase from oat roots. Blots were developed by treatment with alkaline phosphatase-conjugated goat antiserum to mouse IgG and then stained for alkaline phosphatase activity (Ward et al., 1992).

is salt-tolerant, suggests that the activity of the PM-ATPase is not the primary determinant in salt tolerance and activity of the proteins on the plasma membrane alone may be insufficient to regulate intracellular Na^+ levels.

V-ATPase Activity in Cotton is Unaffected by Na^+

The contribution of the V-ATPase in the response of cotton seedlings to salt was measured by comparing V-ATPase activity in control and salt-grown seedlings. There was no increase in the specific activity of the V-ATPase in vesicles isolated from 75 m*M* NaCl-grown roots (Table 3). V-ATPase activity in 75 m*M* KCl grown plants resulted in a small stimulation relative to control (26 ± 16.3%). Immunoblots of tonoplast proteins reacted with antibody to the 70-kD subunit of the V-ATPase from oat (*Avena sativa* L.) identified a single reactive protein with an apparent molecular mass of 70 kD. Comparisons of proteins isolated from plants grown in the absence and presence of 75 m*M* NaCl indicated that exposure to Na^+ during growth did not affect the accumulation of the V-ATPase protein (Fig. 6B). Quantitation of the integrated densities of the immunoreactive bands from three independent Western blots indicated that there were no significant differences in the levels of protein (the mean of the integrated density of NaCl-treated plants as a percent of control plants was 14.9% ± 2.3). Electrophoresis of membrane polypeptides indicated that proteins with an apparent molecular mass of 70 kD were not affected by the Na^+ treatment (Fig. 6A).

Addition of Na^+ to vacuolar vesicles isolated from shoots of seedlings grown without NaCl caused a small increase in V-ATPase activity (data not shown). This stimulation is not specific for Na^+ as KCl at equivalent concentrations resulted in similar levels of stimulation. This was not an anion stimulation as the effects of Na^+ and K^+ were examined in the presence of 0 to 50 m*M* BTP-Cl or with IDA (iminodiacetate) salts of Na^+ and K^+. As with measurements of Na^+-stimulation of the PM-ATPase in 75 m*M* NaCl grown plants, the V-ATPase

from salt-grown plants was insensitive to Na^+ added to membrane vesicles (data not shown).

Studies examining the ion distribution of a number of salt-sensitive plants suggest that the lack of effective intracellular compartmentation leads to sensitivity to salt (Seemann and Critchley, 1985). Our results support this model and suggest that the inability of the V-ATPase in roots of cotton seedlings to respond to in vivo or in vitro exposure to Na^+ may lead to an insufficient driving force for Na^+ transport (via Na^+-H^+ exchangers) into the vacuolar lumen. Studies examining the V-ATPases in halophytes or salt-tolerant (or adapted) glycophytes have shown increases in response to Na^+ in proton transport activity (Matsumoto and Chung, 1988; Reuveni et al., 1990), ATP hydrolysis (Reuveni et al., 1990; Ayala et al., 1996), or accumulation of the V-ATPase transcript (Narasimhan et al., 1991).

Abscisic Acid Does Not Directly Affect the H^+-ATPases in Cotton

A number of plants accumulate the phytohormone abscisic acid (ABA) during exposure to stresses (particularly water stress induced by salt) (Cowan, 1991). In these plants, ABA results in improved ability to acclimate to high salt concentrations (LaRosa et al., 1985; Behl and Raschke, 1986). We measured the effect of ABA, added to membrane fractions, on activities of the H^+-ATPases in salt-sensitive cotton seedlings grown with and without NaCl. The ATPases on the vacuolar and plasma membranes showed no sensitivity to ABA at any of the concentrations tested (5–100 μM, data not shown) indicating that ABA does not directly affect the activity of the transporters.

CONCLUSIONS

Results presented here indicate that root growth in salt-sensitive cotton seedlings is highly sensitive to exposure to salt. The inability of the V-ATPase to respond to increased Na^+ in the cytoplasm with increased activity may result in insufficient proton motive force to allow Na^+ compartmentation in the vacuolar lumen if Na^+-H^+ exchangers exist. A comparison of the effect of Na^+ on the V-ATPase in salt-tolerant cotton with the effect seen in salt-sensitive cotton in this study in combination with studies identifying and characterizing proteins that transport Na^+ will provide important insights into mechanisms that regulate intracellular Na^+ levels in cotton. Providing plant breeders with physiological criteria for salt tolerance to use in combination with agronomic criteria may ultimately allow development of breeding strategies that will maximize salt tolerance improvements.

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