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Tonoplast Na^+/H^+ Antiport Activity and Its Energization by the Vacuolar H^+-ATPase in the Halophytic Plant *Mesembryanthemum crystallinum* L[1]

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Tonoplast vesicles were isolated from leaf mesophyll tissue of the inducible Crassulacean acid metabolism plant *Mesembryanthemum crystallinum* to investigate the mechanism of vacuolar Na^+ accumulation in this halophilic species. In 8-week-old plants exposed to 200 mM NaCl for 2 weeks, tonoplast H^+-ATPase activity was approximately doubled compared with control plants of the same age, as determined by rates of both ATP hydrolysis and ATP-dependent H^+ transport. Evidence was also obtained for the presence of an electroneutral Na^+/H^+ antiporter at the tonoplast that is constitutively expressed, since extravesicular Na^+ was able to dissipate a pre-existing transmembrane pH gradient. Initial rates of H^+ efflux showed saturation kinetics with respect to extravesicular Na^+ concentration and were 2.1-fold higher from vesicles of salt-treated plants compared with the controls. Na^+-dependent H^+ efflux also showed a high selectivity for Na^+ over K^+, was insensitive to the transmembrane electrical potential difference, and was more than 50% inhibited by 200 μM *N*-amidino-3,5-diamino-6-chloropyrazinecarboxamide hydrochloride. The close correlation between increased Na^+/H^+ antiport and H^+-ATPase activities in response to salt treatment suggests that accumulation of the very high concentrations of vacuolar Na^+ found in *M. crystallinum* is energized by the H^+ electrochemical gradient across the tonoplast.

The halophyte *Mesembryanthemum crystallinum* (Aizoaceae) shifts its pathway of carbon assimilation from C_3 photosynthesis to CAM as a response to salt and water stress (Winter and von Willert, 1972). This metabolic switch has been shown to be caused by changes in the expression of several genes encoding enzymes in the CAM pathway (Lüttge, 1993; Bohnert et al., 1994; Cushman and Bohnert, 1995). The kinetics of CAM induction suggests that this is a long-term adaptation to reduced water availability but is not in itself a mechanism for tolerance of high tissue levels of salt (Lüttge, 1993). Many days before the CAM pathway is fully induced, *M. crystallinum* is seen to be a strong salt includer, accumulating high levels of Na^+ and Cl^- within the cell vacuoles, balanced by compatible solutes within the cytoplasm (Adams et al., 1992; Bremberger and Lüttge, 1992). Furthermore, the capacity for CAM induction in *M. crystallinum* is fully expressed only in older leaves, whereas the ability to tolerate salt stress appears to be independent of leaf age (Cushman et al., 1990; Herppich et al., 1992). This suggests that the cellular mechanisms for salt tolerance can be separated from those related to CAM induction. For this reason, *M. crystallinum* provides a valuable system for the investigation of both metabolic adaptations and the expression and regulation of transport proteins in plants that tolerate high salinity.

The abilities to maintain a relatively high K^+/Na^+ ratio in the cytoplasm and to sequester NaCl away from the sites of metabolism are two of the most critical requirements for plant growth under saline conditions (Wyn Jones, 1981). Compartmentation of NaCl in the cell vacuole must be particularly effective in *M. crystallinum*, which actually shows a growth optimum at about 100 mM NaCl in the root medium and can thus be regarded as a halophilic species (Winter and Lüttge, 1976). When plants of *M. crystallinum* have been exposed to salt for several weeks, bulk tissue concentrations of NaCl can approach 1.0 M, and they can even exceed this value in the large epidermal bladder cells that cover the shoot surface (Lüttge et al., 1978; Adams et al., 1992). To our knowledge, how such concentrations of NaCl are accumulated and maintained in the vacuole has not yet been experimentally investigated in *M. crystallinum*. Given the inside-positive membrane potentials characteristic of plant vacuoles, Na^+ accumulation clearly requires active transport from the cytosol into the vacuole, but Cl^- might be maintained close to thermodynamic equilibrium across the tonoplast (Tyerman, 1992; Barkla et al., 1994).

Current evidence suggests that the principal mechanism of vacuolar Na^+ accumulation in plants is provided by a

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Abbreviations: amiloride, *N*-amidino-3,5-diamino-6-chloropyrazinecarboxamide hydrochloride; g_{av}, average g; PPiase, inorganic pyrophosphatase; quinacrine, 6-chloro-9-{[4-(diethylamino)-1-methylbutyl]amino}-2-methoxyacridine dihydrochloride.

Na^+/H^+ exchanger, or antiporter, at the tonoplast (reviewed by Barkla et al., 1994). Secondary active transport of Na^+ ions via the Na^+/H^+ antiporter would be energized by the electrochemical gradient of protons generated by one (or both) of the primary H^+ pumps at the tonoplast, i.e. the vacuolar H^+-ATPase (Sze et al., 1992) or the H^+-translocating PPiase (Rea and Poole, 1993). Indications of a role for the tonoplast Na^+/H^+ antiport in the ability of plants to tolerate salt have been found in a number of species. For example, Blumwald and Poole (1987) showed that growth of sugar beet suspension-culture cells in increasing concentrations of NaCl resulted in increased Na^+/H^+ antiport activity. In barley, Garbarino and DuPont (1988) observed a rapid induction of Na^+/H^+ antiport activity on exposure of roots to NaCl; in *Plantago* spp., tonoplast Na^+/H^+ antiport activity was observed only in the salt-tolerant species *Plantago maritima* and not in the salt-sensitive species *Plantago media* (Staal et al., 1991). Studies aimed at the biochemical identification of the tonoplast Na^+/H^+ antiport have been carried out (Barkla et al., 1990), and a 170-kD tonoplast polypeptide has been suggested to be associated with the antiporter (Barkla and Blumwald, 1991).

In the present study, we have investigated the mechanism of Na^+ transport at the vacuolar membrane of leaf mesophyll cells of *M. crystallinum* to determine whether Na^+/H^+ antiport activity could be responsible for Na^+ accumulation in this halophilic species. Previous work with *M. crystallinum* has shown that in response to salt treatment the activity of the vacuolar H^+-ATPase increases, whereas that of the PPiase declines (Bremberger et al., 1988; Bremberger and Lüttge, 1992). We show here that a substrate-specific Na^+/H^+ antiport is constitutively expressed at the tonoplast in this species and that the activity of the Na^+/H^+ antiporter is increased considerably as a response to salt treatment in parallel with that of the vacuolar H^+-ATPase.

MATERIALS AND METHODS

Plant Material

Mesembryanthemum crystallinum L. plants were grown from seed (derived from material originally collected in Caesarea, Israel, by Dr. K. Winter; Winter et al., 1978) in John Innes No. 3 compost in a propagation tray. All plants were watered daily with distilled water and were grown in a glasshouse under natural solar irradiation supplemented by illumination from sodium-vapor lamps to provide a minimum photoperiod of 12 h. Minimum temperature was maintained at 15°C, and maximum temperature ranged between 25 and 35°C. Two weeks after germination, plants were transferred to individual 90-mm-diameter pots. Salt treatment was initiated 6 weeks after germination by increasing the NaCl concentration in 50 mM increments daily until reaching a final concentration of 200 mM, which was then maintained until the end of the experiment. Control plants continued to be supplied with only distilled water. The second and third leaf pairs from the apex of 8-week-old control and salt-treated plants were used for all experiments.

Isolation of Tonoplast Vesicles

Leaves from *M. crystallinum* were harvested and sliced into small pieces following the removal of major veins. Leaf material (60 g fresh weight) was placed directly into 300 mL of ice-cold homogenization medium, and all subsequent operations were carried out at 4°C. The homogenization medium consisted of 400 mM mannitol (600 mM for salt-treated plants), 10% (v/v) glycerol, 5% (w/v) PVP-40, 0.5% (w/v) BSA, 1 mM PMSF, 30 mM Tris, 2 mM DTT, 5 mM EGTA, 5 mM $MgSO_4$, 0.5 mM butylated hydroxytoluene, 0.25 mM dibucaine, 1 mM benzamidine, and 26 mM K^+-metabisulfite, adjusted to pH 8.0 with H_2SO_4. Leaf tissue was homogenized in a commercial blender, filtered through two layers of cheesecloth, and centrifuged at $10{,}000g_{av}$ (20 min at 4°C) using a Sorvall SS-34 rotor in a Sorvall RC5C superspeed centrifuge. Pellets were discarded and the supernatants centrifuged at $80{,}000g_{av}$ (50 min at 4°C) using a Sorvall TFT 50.38 fixed-angle rotor in a Beckman L8-M ultracentrifuge. The supernatant was aspirated and the microsomal pellet was resuspended using a 10-mL glass tissue homogenizer in a suspension medium consisting of 400 mM mannitol, 10% (v/v) glycerol, 6 mM Tris/Mes (pH 8.0), and 2 mM DTT. For experiments using K^+/valinomycin, vesicles were loaded with K^+ by the addition of 15 mM K-gluconate to this suspension medium.

The microsomal suspension was then layered onto discontinuous Suc gradients consisting of a top layer of 14 mL of 22% (w/v) Suc over a bottom layer of 14 mL of 34% (w/v) Suc, all in the appropriate suspension medium. After centrifugation at $100{,}000g_{av}$ (2 h at 4°C) using a Beckman SW 28 swinging bucket rotor in a Beckman L8-M ultracentrifuge, membranes at the 0/22% Suc interface (tonoplast) were removed with a Pasteur pipette. These membranes were diluted with the appropriate suspension solution and sedimented at $80{,}000g_{av}$ (1 h at 4°C) using a Sorvall TFT 50.38 fixed-angle rotor in a Beckman L8-M ultracentrifuge, and the final membrane pellet was resuspended in 200 μL of the same solution. Membranes were frozen directly in liquid N_2 and stored at −80°C in 50-μL aliquots. Those used for quinacrine fluorescence measurements were subject to only a single freeze/thaw cycle because additional cycles increased the leakiness of the vesicles.

Protein Determination

Protein in tonoplast vesicles used for fluorescence assays was measured by a modification of the dye-binding method (Bradford, 1976) in which membrane protein was partially solubilized with 0.5% (v/v) Triton X-100 for 5 min before the addition of the dye reagent concentrate. BSA was used as the protein standard. Protein in tonoplast vesicles used in measurements of ATP hydrolytic activity was measured by the method of Lowry et al. (1951).

ATPase Hydrolytic Activity

Rates of ATP hydrolysis were measured as the liberation of Pi according to the method of Smith et al. (1984) at 30°C on samples containing 2 to 4 μg of tonoplast protein, with modifications as described by Zingarelli et al. (1994).

H^+-Transport Assays

The fluorescence quenching of quinacrine was used to monitor the formation and dissipation of inside-acid pH gradients across the membrane of tonoplast vesicles. Purified tonoplast vesicles (30 μg of protein) were added to 500 μL of a buffer containing 250 mM mannitol, 10 mM Tris/Mes (pH 8.0), 6 mM $MgSO_4$, 50 mM tetramethylammonium chloride or 50 mM bis Tris propane-malate (pH 8.0), and 3 μM quinacrine. Proton translocation was initiated in vesicles by the addition of 3 mM Tris/ATP (pH 8.0). Fluorescence quenching was monitored in a thermostated cell at 25°C using a Perkin Elmer LS-5B fluorescence spectrometer at excitation and emission wavelengths of 427 and 495 nm, respectively, both with a slit width of 5 nm. For measurements of Na^+-dependent dissipation of a preformed, inside-acid pH gradient, the ATP-dependent H^+-transport activity was partially inhibited by the addition of 200 nM bafilomycin A_1 (Bowman et al., 1988) in 0.001% (v/v) DMSO in 250 mM mannitol and 10 mM Tris/Mes (pH 8.0). After a constant rate of fluorescence recovery (H^+ efflux) was obtained, aliquots of Na^+ (5–300 mM) were added to the cell and the initial rate of Na^+-dependent fluorescence recovery was determined. As shown by Bennett and Spanswick (1983), the rate of fluorescence quench or recovery is directly proportional to proton flux. Thus, initial rates of fluorescence quenching or recovery represent initial rates of proton transport. The initial rates of dissipation of the pH gradient by Na^+ were expressed minus the background rates of fluorescence recovery observed after addition of bafilomycin A_1. All rates were expressed as the percentage change in quinacrine fluorescence per min per mg protein.

Chemicals

All chemicals were of standard analytical grade and were purchased from Sigma. Na_2ATP was converted to Tris-ATP by cation exchange with Dowex 50W (Bio-Rad). Bafilomycin A_1 was a gift of Dr. A.J. Pope (SmithKline Beecham, Welwyn, UK).

RESULTS

Tonoplast vesicles were isolated from leaves of 8-week-old *M. crystallinum* plants grown in the presence or absence of 200 mM NaCl for 2 weeks, and all measurements were made on plants of identical age. The purity of the tonoplast preparations isolated by density gradient centrifugation was estimated by measuring ATP-hydrolytic activity in the presence of different effectors and inhibitors according to the method of Sze et al. (1992). The tonoplast H^+-ATPase is sensitive to nitrate and bafilomycin A_1 but insensitive to azide and vanadate, inhibitors of the mitochondrial and plasma membrane ATPases, respectively. In membranes isolated from the 0/22% Suc interface, the azide-resistant, nitrate-sensitive ATP hydrolysis activity at pH 8.0 was 66% of the total ATP-hydrolytic activity of membrane vesicles isolated from control plants and 71% of the total ATP-hydrolytic activity of membrane vesicles isolated from salt-treated plants (data not shown). Azide-sensitive activity was low in membrane vesicles isolated from both control plants and from salt-treated plants (3.4 and 10.2%, respectively), and values for vanadate-sensitive activity were 12.9% for membrane vesicles isolated from control plants and 13.5% for those from salt-treated plants. Also, 50 mM nitrate inhibited H^+-transport activity by 88% in membrane vesicles isolated from control plants and by 89% in membrane vesicles from salt-treated plants (data not shown). Previous work by Struve and Lüttge (1987) also demonstrated that a highly enriched tonoplast fraction can be obtained from *Mesembryanthemum* by collecting membranes at the 0/24% Suc interface. These results are good evidence that the membrane fraction used in this study represents primarily tonoplast. Furthermore, comparison of azide-resistant, nitrate-sensitive H^+-ATPase activity at pH 8.0 in the mixed microsomal fraction to the activity in the fraction isolated from the 0/22% Suc interface indicated a severalfold enrichment of activity in the tonoplast preparation from both control and salt-treated plants (data not shown).

Acidification of tonoplast vesicles and dissipation of this transmembrane pH gradient was monitored by quinacrine fluorescence quenching and fluorescence recovery (Fig. 1). The initial rates of quenching or recovery of quinacrine fluorescence can be used to compare initial rates of H^+-transport activity into or out of the vesicles (Bennett and Spanswick, 1983), reflecting the activity of the tonoplast H^+-ATPase and Na^+/H^+ antiport, respectively.

The H^+-transport activity of the tonoplast H^+-ATPase, taken as the initial rate of quinacrine fluorescence quenching during the first 60 s following the addition of ATP, was 2-fold higher in tonoplast vesicles isolated from the leaves of salt-treated plants than in vesicles from leaves of control plants (Fig. 2; Table I). The ratio of these activities was similar to that obtained from measurements of the hydro-

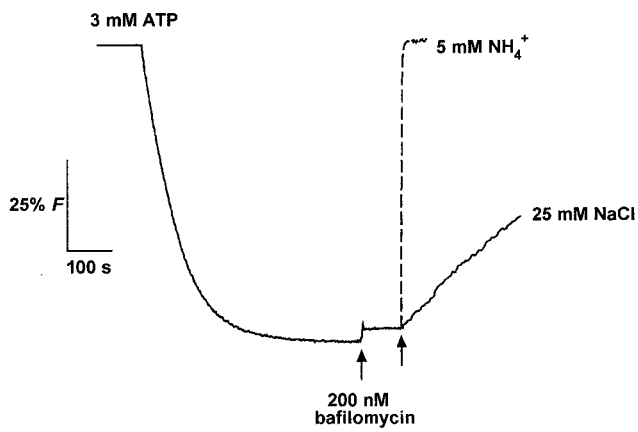

Figure 1. Characteristics of ATP-dependent quenching and Na^+-dependent recovery of quinacrine fluorescence. A pH gradient (inside-acid) was generated in tonoplast vesicles (30 μg of protein) isolated from leaves of *M. crystallinum* upon addition of ATP to the reaction medium. Following the addition of 200 nM bafilomycin A_1, a slow, time-dependent dissipation of the pH gradient was achieved with the addition of Na^+ ions or an instantaneous collapse of the pH gradient was achieved by the addition of NH_4^+ ions. Data shown represent original trace recordings. *F*, Fluorescence intensity relative to that prior to addition of ATP.

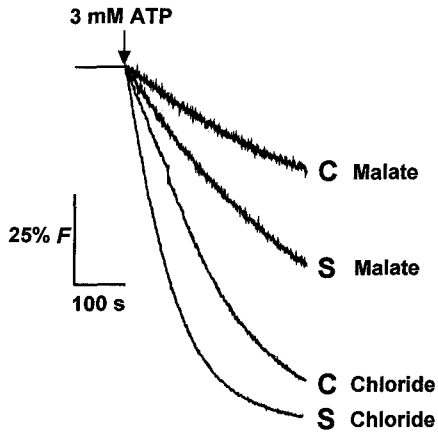

Figure 2. ATP-dependent H^+ transport into tonoplast vesicles isolated from leaves of 8-week-old control (C) or salt-treated (S) *M. crystallinum* plants watered with 200 mM NaCl for 2 weeks. Vesicle acidification was monitored by the quenching of quinacrine fluorescence as described in "Materials and Methods" in the presence of tonoplast vesicles (30 μg of protein) and either 50 mM Cl^- or 50 mM malate. The results are original traces from one experiment representative of a total of five. *F*, Fluorescence intensity relative to that prior to addition of ATP.

lytic activity of the H^+-ATPase in vesicles isolated from the same tonoplast preparation (Table I). However, the magnitude of the final steady-state level of quinacrine quenching, reflecting the pH gradient generated by the pump, was the same for tonoplast vesicles isolated from control or salt-treated plants.

It has been shown that Cl^-, as well as having a direct role in activating the tonoplast H^+-ATPase, can, along with certain other anions, act to dissipate an inside-positive membrane potential associated with H^+-ATPase activity and therefore stimulate H^+ transport (Bennett and Spanswick, 1983). In the present study, H^+ transport was greatly dependent on the nature of this permeant anion. In tonoplast vesicles isolated from leaves of both control and salt-treated *M. crystallinum* plants, Cl^- supported higher rates of vesicle acidification than did malate (Fig. 2), as observed previously for tonoplast vesicles isolated from *M. crystallinum* (Struve and Lüttge, 1987). This is in contrast to the tonoplast H^+-ATPase in another CAM plant, *Kalanchoë daigremontiana*, in which higher rates of H^+ transport were observed in the presence of malate than in the presence of Cl^- (White and Smith, 1989). The rates of H^+ transport in the presence of different anions reflect the relative permeability of the tonoplast to these anions and could indicate a greater tonoplast permeability to Cl^- than to malate in *M. crystallinum*. These differences in anion permeability between the CAM plants *M. crystallinum* and *K. daigremontiana* may be due to the ability of *M. crystallinum*, a halophytic species, to accumulate and compartmentalize high concentrations of both Na^+ and Cl^- ions and thus reflect an adaptation to salt tolerance in these plants.

The effect of Na^+ on the dissipation of a transmembrane pH gradient was tested in isolated tonoplast vesicles. Following the generation of an inside-acid pH gradient by activation of the tonoplast H^+-ATPase, the pump activity was partially inhibited by the addition of 200 nM bafilomycin A_1, a specific inhibitor of the tonoplast H^+-ATPase (Bowman et al., 1988). This concentration of bafilomycin partially inhibited H^+ transport by 75% in membrane vesicles isolated from control plants and by 70% in membrane vesicles from salt-treated plants (data not shown). Bafilomycin was used at a concentration below that required for complete inhibition of the H^+-ATPase H^+-transport activity to allow accurate quantification of the Na^+-induced reversal of quinacrine fluorescence. Addition of increasing concentrations of Na^+ ions resulted in increasing rates of quinacrine fluorescence recovery (Fig. 3A), consistent with dissipation of the transmembrane pH gradient as a result of Na^+-dependent movement of H^+ out of the vesicles. Higher levels of quinacrine fluorescence recovery were observed in tonoplast vesicles isolated from salt-treated plants compared to tonoplast vesicles from control plants for the same Na^+ concentration (Fig. 3A). Recovery of quinacrine fluorescence to the level observed before the addition of ATP was obtained with Na^+ concentrations greater than 200 mM NaCl in the tonoplast vesicles from salt-treated plants or with the addition of 5 mM NH_4^+ ions.

The initial rate of Na^+/H^+ exchange displayed saturation kinetics with respect to extravesicular Na^+ concentration in tonoplast vesicles isolated from both control and salt-treated *M. crystallinum* plants (Fig. 3B). Calculation of the kinetics parameters from the Michaelis-Menten curve fitted to the initial rates of Na^+-dependent H^+ transport showed that the apparent K_m value did not differ significantly between tonoplast vesicles from control plants and salt-treated plants (44 and 51 mM, respectively). However, the value for V_{max} more than doubled, from 4.8 to 10.1% fluorescence intensity min^{-1} mg^{-1} protein, when plants were treated with NaCl.

The selectivity of the Na^+/H^+ antiport was tested by replacement of Na^+ with another monovalent cation, K^+. In contrast to Na^+, the addition of K^+ gave low rates of fluorescence recovery in tonoplast vesicles isolated from both control and salt-treated plants (Fig. 4A). This indicates that the stimulation of H^+ efflux showed a high degree of Na^+ specificity. To determine the effects of the accompanying anion on the rate of Na^+-dependent fluorescence recovery, Cl^- was exchanged for the nonpermeant anion gluconate. As shown in Figure 4A, NaCl and Na-gluconate gave similar rates of fluorescence recovery in tonoplast vesicles isolated from both control and salt-treated plants.

Table I. *Rates of nitrate-sensitive and azide- and vanadate-resistant ATP hydrolysis and H^+ transport for the tonoplast H^+-ATPase in tonoplast vesicles isolated from leaves of control and salt-treated M. crystallinum plants*

Assays were performed in the presence of 50 mM Cl^-. Values are means ± SD (n = 5 independent experiments for ATP hydrolytic activity and 25 independent experiments for H^+-transport activity). F, Fluorescence intensity.

Plants	ATP Hydrolytic Activity	H^+-Transport Activity
	μmol Pi mg^{-1} protein h^{-1}	*% F mg^{-1} protein min^{-1}*
Control	5.67 ± 0.90	591 ± 88
Salt-treated	14.8 ± 1.8	1150 ± 160

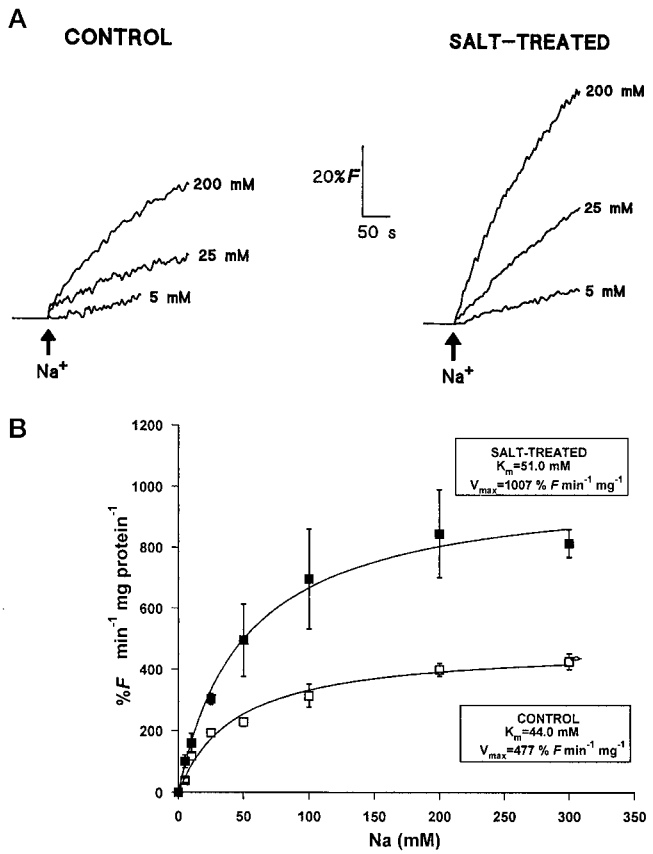

Figure 3. A, Na^+-dependent H^+ efflux from tonoplast vesicles isolated from leaves of 8-week-old control or salt-treated *M. crystallinum* plants. A pH gradient (inside-acid) was generated in tonoplast vesicles (30 μg of protein) by activation of the tonoplast H^+-ATPase; when a steady-state pH gradient was obtained, the H^+-ATPase activity was inhibited by the addition of 200 nM bafilomycin A_1 as shown in Figure 1. The recovery of quinacrine fluorescence was measured upon addition of increasing concentrations of Na^+ (5–300 mM). For clarity, only three of the seven Na^+ concentrations tested are illustrated. Results are original trace recordings from one experiment representative of a total of five. B, Initial rates of Na^+-dependent H^+ fluxes in tonoplast vesicles isolated from leaves of control (□) and salt-treated (■) plants. Initial rates of quinacrine fluorescence recovery were measured immediately after addition of different concentrations of Na^+ (5–300 mM) from traces similar to those in Figure 3A. Curves were fitted by nonlinear least squares analysis. *F*, Fluorescence intensity relative to that prior to addition of ATP.

To analyze whether the Na^+-dependent H^+ fluxes in tonoplast vesicles isolated from leaves of control and salt-treated plants were dependent on a transmembrane electrical potential difference, assays were carried out in the presence of 1 μM valinomycin with equimolar K^+ on either side of the tonoplast vesicle to abolish the membrane potential across the tonoplast. No significant difference was observed in the initial rates of fluorescence recovery upon addition of 200 mM NaCl for tonoplast vesicles from control or salt-treated plants in the presence or absence of K^+ and valinomycin (Fig. 4B). However, an increase in the initial rate of H^+ transport by the tonoplast H^+-ATPase was observed (data not shown), demonstrating that valinomycin in the presence of equimolar K^+ was effective in reducing the transmembrane electrical potential across the vesicles. These results suggest that the dissipation of the preset pH gradient by Na^+ is not due to electrically driven H^+ movement through conductive pathways but is consistent with an electroneutral Na^+/H^+ antiport.

The diuretic drug amiloride has been shown to inhibit Na^+ uptake via the Na^+/H^+ exchange system in animals rapidly, reversibly, and competitively by binding at or near the Na^+-binding site(s) (Rocco et al., 1987). In plants, amiloride has been shown to inhibit competitively the

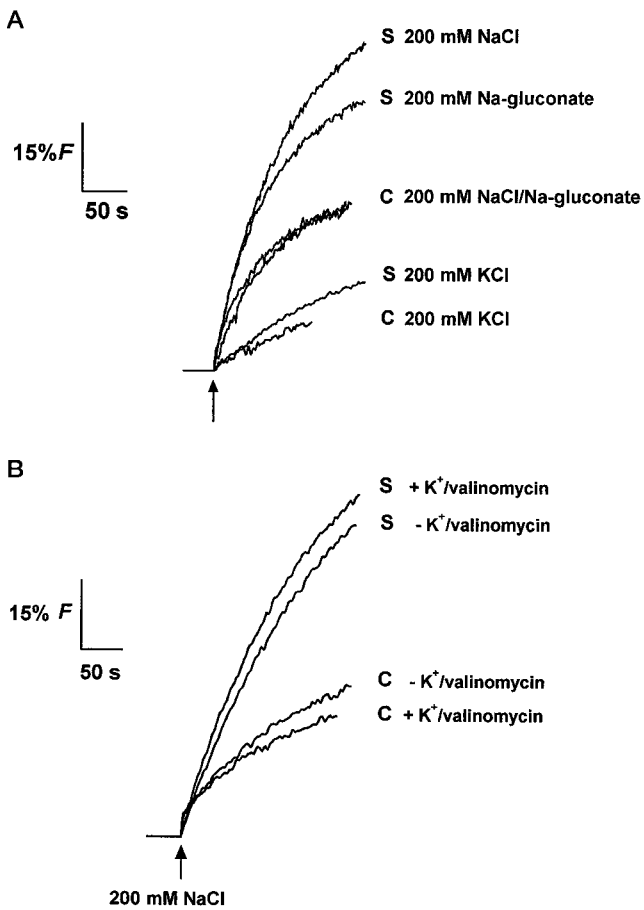

Figure 4. A, Effect of KCl and Na-gluconate on the Na^+-dependent H^+ fluxes in tonoplast vesicles isolated from leaves of control (C) and salt-treated (S) *M. crystallinum* plants. A pH gradient (inside-acid) was generated in tonoplast vesicles (30 μg of protein) by activation of the tonoplast H^+-ATPase, and the pump activity and then stopped by the addition of 200 nM bafilomycin A_1 as shown in Figure 1. The recovery of quinacrine fluorescence was measured upon the addition of 200 mM NaCl, 200 mM Na-gluconate, or 200 mM KCl. The results are original trace recordings from one experiment representative of a total of eight. B, Effect of K^+/valinomycin on the Na^+-dependent H^+ fluxes. Tonoplast vesicles were prepared using homogenization and suspension buffers supplemented with 15 mM K-gluconate. Five minutes before addition of ATP to generate the inside-acid pH gradient, 2 μL of 100 μM valinomycin in ethanol were added to the assay medium to give a final concentration of 1 μM valinomycin. Data shown represent original trace recordings. *F*, Fluorescence intensity relative to that prior to addition of ATP.

Na^+/H^+ antiport in *Beta vulgaris* (Blumwald and Poole, 1985), with a K_i for Na^+-dependent H^+ fluxes in tonoplast vesicles of 140 μM. In contrast, in tonoplast vesicles isolated from salt-treated barley roots, concentrations of up to 500 μM amiloride gave no significant inhibition of the Na^+/H^+ antiport activity (Garbarino and DuPont, 1988). In *M. crystallinum*, 200 μM amiloride inhibited the initial rate of Na^+-dependent quinacrine fluorescence recovery by approximately 56% in tonoplast vesicles isolated from salt-treated plants and by approximately 60% in tonoplast vesicles from control plants (Fig. 5). This indicates that amiloride inhibits the Na^+/H^+ antiport in *M. crystallinum* with a potency similar to that observed for the tonoplast Na^+/H^+ antiport in *B. vulgaris* (Blumwald and Poole, 1985).

DISCUSSION

The ability of salt-tolerant species to sequester high concentrations of NaCl in the cell vacuole is physiologically important for two reasons (Flowers et al., 1977; Wyn Jones, 1981). First, Na^+ and Cl^- ions are removed from the cytoplasm, where they might otherwise reach cytotoxic concentrations; second, the low internal water potential generated by salt accumulation helps to maintain water uptake by the plant from a saline root medium. These characteristics should be particularly well developed in a halophilic species such as *M. crystallinum*, which actually displays its maximum growth rate under moderately saline conditions (Winter and Lüttge, 1976).

Tonoplast vesicles from the leaf mesophyll tissue of *M. crystallinum* showed Na^+-induced H^+ fluxes, with properties that support the existence of an Na^+/H^+ antiporter at this membrane. We believe this transporter is likely to represent the principal mechanism of vacuolar Na^+ accumulation in this species. Na^+/H^+ antiport activity could be assayed in tonoplast vesicles using a three-stage protocol: first generating a steady-state transmembrane pH gradient by activating the H^+-ATPase, then adding bafilomycin A_1 to compromise the ability of the H^+-ATPase to respond to subsequent pH perturbations, and finally adding Na^+ to induce H^+ efflux from the vesicles. The specific activity of the Na^+/H^+ antiporter was more than twice as high (2.1-fold) in salt-treated plants compared with controls of the same age (Fig. 3), but its presence in control plants indicates that the transporter is constitutively expressed in *M. crystallinum*. This also appears to be the case in *B. vulgaris* (Blumwald and Poole, 1987), but in other tissues such as barley roots the activity of the Na^+/H^+ antiporter can be detected only after exposure to NaCl (Garbarino and DuPont, 1989). Constitutive expression of the Na^+/H^+ antiporter in *M. crystallinum* also contrasts with the expression pattern of specific isozymes of the CAM pathway, which are present at very low or undetectable levels in plants of this age not exposed to salt stress (Bohnert et al., 1994).

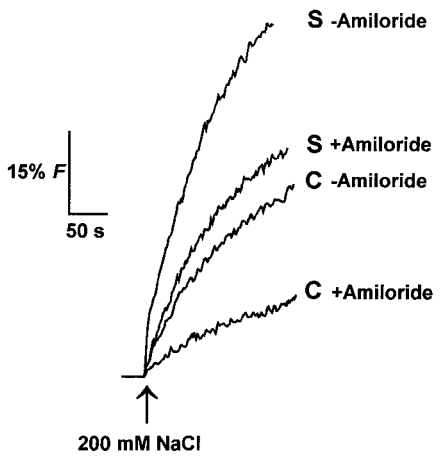

Figure 5. Effect of amiloride on Na^+-dependent H^+ fluxes in tonoplast vesicles isolated from leaves of control (C) and salt-treated (S) *M. crystallinum* plants. Tonoplast vesicles (30 μg of protein) were preincubated for 5 min in the presence or absence of 200 μM amiloride before activation of the tonoplast H^+-ATPase and its subsequent inhibition by the addition of 200 nM bafilomycin A_1 as illustrated in Figure 1. The recovery of quinacrine fluorescence was measured upon the addition of 200 mM NaCl. The results are original trace recordings from one experiment representative of a total of eight. *F*, Fluorescence intensity relative to that prior to addition of ATP.

Although the specific activity of the Na^+/H^+ antiporter was higher in salt-treated plants compared with the controls, kinetics analysis showed no significant difference in the apparent K_m for Na^+, which averaged 48 mM for the two groups of plants (Fig. 3B). This is the highest K_m reported for a tonoplast Na^+/H^+ antiporter, the values in other species ranging from 2.4 mM in *P. maritima* (Staal et al., 1991) to 14 mM in the halophyte *Atriplex gmelini* grown on 250 mM NaCl (Matoh et al., 1989). The relatively high K_m value in *M. crystallinum* may reflect the halophilic nature of this species and the high concentrations of NaCl (of the order of 1.0 M) that can accumulate in the leaf tissue (Winter and Lüttge, 1976; Lüttge et al., 1978; Adams et al., 1992). Direct information concerning cytosolic ion concentrations is not available for *M. crystallinum*, but the apparent K_m of 48 mM suggests that cytosolic Na^+ concentrations may reach several tens of mM in plants exposed to salt. If the higher activity of the Na^+/H^+ antiporter is entirely attributable to an increased V_{max}, this implies either increased synthesis of the transport protein in response to salt or increased turnover number of the Na^+/H^+ exchange reaction mediated by the transporter. The former possibility is supported by the fact that when western blots of microsomal membranes from leaves of control and salt-treated *M. crystallinum* were probed with a polyclonal antibody to the *B. vulgaris* 170-kD Na^+/H^+ antiport-associated polypeptide (Barkla and Blumwald, 1991) higher amounts of this polypeptide were shown in membranes from the salt-treated plants (Barkla et al., 1994).

Apart from its saturation kinetics with respect to Na^+ concentration, Na^+-linked H^+ efflux from tonoplast vesicles of *M. crystallinum* showed other characteristics expected of a substrate-specific transporter. These included a high degree of selectivity for Na^+ over K^+, as well as a lack of any effect of the accompanying anion on Na^+/H^+ exchange (Fig. 4A). Furthermore, the rate of Na^+-dependent H^+ efflux was not sensitive to the electrical potential dif-

ference across the membrane, because there was no significant effect of a K^+/valinomycin clamp used to abolish this membrane potential (Fig. 4B). This result indicates that Na^+/H^+ exchange is an electroneutral process with a 1:1 stoichiometry, and it also precludes the possibility that the H^+ efflux from the vesicles occurred simply as a response to an inside-positive Na^+ diffusion potential generated on addition of Na^+ to the external medium. Finally, Na^+/H^+ exchange in *M. crystallinum* was inhibited by the diuretic drug amiloride, a known inhibitor of the Na^+/H^+ antiporter in animals and plants (Blumwald and Poole, 1985; Rocco et al., 1987), with a sensitivity similar to that observed for the Na^+/H^+ antiporter in *B. vulgaris* (Blumwald and Poole, 1985).

In leaf cells accumulating NaCl, the driving force for secondary active Na^+ transport into the vacuole would be provided by the H^+-electrochemical potential difference generated across the tonoplast by the primary H^+ pumps. Previous work with *M. crystallinum* has shown that the activity of the tonoplast H^+-PPiase declines as a consequence of salt treatment (Bremberger et al., 1988; Bremberger and Lüttge, 1992). The activity of the H^+-PPiase is also K^+ dependent and is competitively inhibited by Na^+ (White et al., 1990; Rea and Poole, 1993). This suggests that the H^+-ATPase will play the principal role in energizing Na^+/H^+ antiport activity in cells accumulating significant quantities of NaCl. Similar observations of an increase in tonoplast H^+-ATPase activity in response to salinity have been made for roots of barley (Matsumoto and Chung, 1988) and for suspension-culture cells of *Nicotiana tabacum* (Reuveni et al., 1990), and in roots of *Vigna mungo* this is also associated with a decline in the H^+-PPiase activity (Nakamura et al., 1992). In the present study with *M. crystallinum*, the increase in Na^+/H^+ antiport activity in the salt-treated plants as compared to the control plants (2.1-fold) was very closely correlated with that of the H^+-ATPase (1.9-fold for H^+-transport activity and 2.6-fold for ATP hydrolytic activity). These increases in tonoplast H^+-ATPase specific activity agree with the earlier observations of Struve et al. (1985), Struve and Lüttge (1987), and Rockel et al. (1994) on *M. crystallinum*, although Ratajczak et al. (1994) more recently suggested that this increase in specific activity is dependent not on salt stress per se but rather on leaf age. Our results do not appear to support this interpretation, in so far as the differences in tonoplast H^+-ATPase specific activity caused by salt treatment in the present experiments were observed in plants of exactly the same age.

A relatively close coupling between the increases in tonoplast Na^+/H^+ antiport activity and H^+-ATPase activity in response to salt treatment may be essential for effective vacuolar salt sequestration in *M. crystallinum*. In the absence of a compensatory increase in tonoplast H^+-ATPase activity, higher Na^+ concentrations in the cytosol would tend to dissipate the transmembrane pH difference and cause net efflux of H^+ (and potentially other cytotoxic solutes) from the vacuole into the cytosol (cf. Niemietz and Willenbrink, 1985; Guern et al., 1989). Although transport of Na^+ into the vacuole is thermodynamically uphill, it is not yet clear whether active transport of Cl^- is also required in *M. crystallinum*. If a sufficiently large, inside-positive electrical potential difference can be sustained across the tonoplast, then the passive conductance of Cl^- would appear to be sufficiently high to allow equivalent rates of Cl^- influx into the vacuole (Fig. 2). As yet we have no data concerning the cytosolic concentrations of Na^+ and Cl^- in *M. crystallinum* or the corresponding electrochemical potential differences for these ions across the tonoplast. Such information, allied to attempts to identify the tonoplast Na^+/H^+ antiporter at the molecular level, will be important for a more complete understanding of the mechanism of salt tolerance in *M. crystallinum*.

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