

Regulation of Vacuolar Na⁺/H⁺ Exchange in *Arabidopsis thaliana* by the Salt-Overly-Sensitive (SOS) Pathway*

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For plants growing in highly saline environments, accumulation of sodium in the cell cytoplasm leads to disruption of metabolic processes and reduced growth. Maintaining low levels of cytoplasmic sodium requires the coordinate regulation of transport proteins on numerous cellular membranes. Our previous studies have linked components of the Salt-Overly-Sensitive pathway (SOS1-3) to salt tolerance in *Arabidopsis thaliana* and demonstrated that the activity of the plasma membrane Na⁺/H⁺ exchanger (SOS1) is regulated by SOS2 (a protein kinase) and SOS3 (a calcium-binding protein). Current studies were undertaken to determine if the Na⁺/H⁺ exchanger in the vacuolar membrane (tonoplast) of *Arabidopsis* is also a target for the SOS regulatory pathway. Characterization of tonoplast Na⁺/H⁺ exchange demonstrated that it represents activity originating from the AtNHX proteins since it could be inhibited by 5-(*N*-methyl-*N*-isobutyl)amiloride and by anti-NHX1 antibodies. Transport activity was selective for sodium (apparent $K_m = 31$ mM) and electroneutral (one sodium ion for each proton). When compared with tonoplast Na⁺/H⁺-exchange activity in wild type, activity was significantly higher, greatly reduced, and unchanged in *sos1*, *sos2*, and *sos3*, respectively. Activated SOS2 protein added *in vitro* increased tonoplast Na⁺/H⁺-exchange activity in vesicles isolated from *sos2* but did not have any effect on activity in vesicles isolated from wild type, *sos1*, or *sos3*. These results demonstrate that (i) the tonoplast Na⁺/H⁺ exchanger in *Arabidopsis* is a target of the SOS regulatory pathway, (ii) there are branches to the SOS pathway, and (iii) there may be coordinate regulation of the exchangers in the tonoplast and plasma membrane.

In order to avoid the adverse effects of salt stress on growth and development, plants have developed mechanisms to maintain low levels of salt in the cytoplasm (1, 2). One mechanism involves removal of sodium (Na⁺) from the cytoplasm by trans-

port into the vacuole or out of the cell. This transport is catalyzed by Na⁺/H⁺ exchangers (antiporters), membrane proteins localized in either the vacuolar (tonoplast) or plasma membrane (2–4). Na⁺/H⁺-exchange activity is driven by the electrochemical gradient of protons (H⁺) generated by the H⁺-pumps such as the plasma membrane H⁺-ATPase or the tonoplast H⁺-ATPase and H⁺-pyrophosphatase (4).

Recently, the molecular identity of a plant plasma membrane Na⁺/H⁺ exchanger and one mechanism underlying its regulation have been reported (5). In a genetic screen designed to identify components of the cellular machinery that contributes to salt tolerance in *Arabidopsis*, three salt-overly-sensitive mutants (*sos1*, *sos2*, and *sos3*)¹ were characterized (6–12). Mutations in *SOS1* rendered *Arabidopsis* extremely sensitive to growth in high levels of NaCl (7, 13) as these plants accumulated more Na⁺ than wild-type plants (11). Subsequent studies demonstrated that *SOS1* encodes a plasma membrane Na⁺/H⁺ exchanger (5, 11). (i) The predicted *SOS1* protein sequence shares significant sequence and domain homology with plasma membrane Na⁺/H⁺ exchangers from animal, bacterial, and fungal cells (11). (ii) When the *SOS1* gene was expressed in cells of *Saccharomyces cerevisiae* with mutations in endogenous Na⁺ transporters, growth in NaCl was restored as Na⁺ content decreased (14). (iii) Studies with *SOS1*-GFP fusion protein (14) and anti-*SOS1* antibody (15) have shown that *SOS1* localizes to the plasma membrane. (iv) *SOS1* Na⁺/H⁺-exchange activity was demonstrated using plasma membrane vesicles (5). This Na⁺/H⁺-exchange activity was induced by salt stress, was specific for and had a low affinity for Na⁺ and exchanged one Na⁺ for each H⁺ (15).

The molecular identities of other known components of the SOS regulatory pathway have also been determined (12). *SOS2* is a serine/threonine kinase with a catalytic domain similar to the yeast SNF1 and the mammalian AMPK kinases (10). The kinase domain in *SOS2* has been localized to the N terminus of the protein; while a regulatory domain that inhibits kinase activity has been identified in the C-terminal portion of the protein (16). *SOS3* is a Ca²⁺-binding protein that contains EF-hand domains in the C terminus and a myristoylation site in the N-terminal portion of the protein (6).

Molecular genetic and biochemical studies have demonstrated that *SOS2* and *SOS3* are part of a novel signal transduction pathway involved in the perception and transduction of salt stress signals in the plant. (i) The phenotypes of *sos2* and *sos3* mutant plants are similar, suggesting that the two genes function in the same pathway to regulate Na⁺/K⁺ homeostasis (7). (ii) Yeast two-hybrid and *in vitro* binding assays have

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¹ The abbreviations used are: sos, salt-overly-sensitive; ΔpH, pH gradient; ΔΨ, electrical or membrane potential; MIA, 5-(*N*-methyl-*N*-isobutyl)amiloride; PBS, phosphate-buffered saline.

shown that the SOS2 protein kinase physically interacts with and is activated by SOS3 in the presence of Ca^{2+} (8). The SOS3 binding site has been localized to a 21-amino acid motif (FISL) within the regulatory domain of the SOS2 protein. When Thr¹⁶⁸ in the activation loop of the SOS2 kinase domain was changed to Asp, the kinase was constitutively activated in a SOS3-independent manner (16). (iii) *S. cerevisiae* cells expressing all three SOS pathway members (*SOS1*, *SOS2*, and *SOS3*) were much more salt tolerant than cells expressing *SOS1* alone (17).

SOS1 has been shown to be an output or target of the SOS pathway whose activity is controlled by SOS2/SOS3. *SOS1* expression was up-regulated when plants were exposed to high levels of NaCl, and this salt regulation was partly mediated by SOS2 and SOS3 (11). Transport experiments have shown that plasma membrane Na^+/H^+ exchange was reduced in *sos1* plants relative to activity in wild-type plants, and this activity could not be restored by *in vitro* addition of activated SOS2 protein (5). Mutations in the *SOS2* and *SOS3* genes led to reductions in plasma membrane Na^+/H^+ -exchange activity; however, transport in these mutants could be restored by adding activated SOS2 protein (5). *SOS1* has been shown to be a phosphorylation substrate for the SOS2/SOS3 kinase complex and these two regulatory proteins are necessary and sufficient for *in vivo* activation of the *SOS1* transporter (17).

The transport activity of a plant tonoplast Na^+/H^+ exchanger has been well characterized (3, 4, 18, 19). In addition, the molecular mechanism underlying tonoplast Na^+ compartmentation by the exchanger and its function in plant salt tolerance have been recently demonstrated (20, 21). When *AtNHX1*, an *Arabidopsis* homolog of the *S. cerevisiae* Na^+/H^+ exchanger *NHX1*, was overexpressed in *Arabidopsis*, tonoplast exchange activity increased (relative to activity in wild-type plants) and the plants were better able to grow in high levels of salt (20, 21). The transport activity of *AtNHX1* has been demonstrated by expressing the gene in yeast (18), tomato (22), and *Brassica* (23) and by reconstituting it into liposomes (19).

To understand how intracellular Na^+ levels are regulated during salt stress, it will be necessary to determine how the individual proteins that transport Na^+ function coordinately. With this as our goal, we asked whether the tonoplast Na^+/H^+ exchanger is a target of the SOS pathway. Tonoplast Na^+/H^+ -exchange activity was measured using purified membrane vesicles isolated from cell cultures of wild-type and *sos* plants. When compared with tonoplast Na^+/H^+ -exchange activity in wild type, activity was significantly higher, greatly reduced, and unchanged in *sos1*, *sos2*, and *sos3*, respectively. Activated SOS2 protein increased tonoplast Na^+/H^+ -exchange activity in vesicles isolated from *sos2* but did not have any effect on activity in vesicles isolated from wild-type, *sos1*, or *sos3*. These results (i) functionally identify another target of the SOS regulatory pathway, (ii) demonstrate that the activity of the tonoplast Na^+/H^+ exchanger is controlled by the SOS2 kinase, (iii) provide evidence that there can be coordination of the activities of the exchangers in the tonoplast and plasma membrane in *Arabidopsis*, and (iv) indicate that the activity of the SOS2 protein kinase may be regulated by more than one upstream regulatory component in the salt signal transduction pathway.

EXPERIMENTAL PROCEDURES

Plant Materials—*Arabidopsis thaliana* ecotype Columbia was used in all experiments. *sos1-1*, *sos2-2*, and *sos3-1* plants were as described (7). For callus induction, seedlings were germinated on Murashige and Skoog salts (MS, Sigma-Aldrich) and allowed to grow for 2 weeks. When the cotyledons were fully expanded, the seedlings were transferred to callus initiation medium (43 g/liter MS salts, 30 g/liter sucrose, 1× MS vitamins, 3 mg/liter 2,4-dichlorophenoxyacetic acid, 0.05 mg/liter kinetin, 1 g/liter casein hydrolysate, and 7 g/liter agar at pH 5.7), placed in

the dark and subcultured onto new medium every 2 weeks. After 3–5 passages, friable callus formed and was transferred from plates to liquid culture (4.3 g/liter MS salts, 30 g/liter sucrose, 1× MS vitamins, 3 mg/liter 2,4-dichlorophenoxyacetic acid, 0.05 mg/liter kinetin, and 1 g/liter casein hydrolysate at pH 5.0). Cells were cultured in the dark at 24 °C with shaking at 130 rpm and were subcultured every 5–7 days. One-week-old cells were harvested and used for membrane isolation.

Tonoplast Isolation, Purification, and Characterization—Tonoplast vesicles were isolated using dextran gradients as described previously (24). The purity of these membrane preparations was examined by assaying ATPase activity in the absence and presence of a number of inhibitors. To characterize the activities of the H^+ -ATPases, their substrate hydrolytic activity was determined by measuring the release of P_i from ATP according to Poole *et al.* (25) and Qiu (26). Activities for the plasma membrane, mitochondrial and tonoplast ATPases were measured using optimal conditions for each enzyme (27, 28). The purity and latency of the membrane preparations were determined as described (25, 29).

Plasma Membrane Isolation—Plasma membrane vesicles were isolated using aqueous two-phase partitioning as described in Qiu *et al.* (5).

Proton Transport Assays—The pH gradient (ΔpH) was established by the activity of the tonoplast H^+ -ATPase and was measured as a decrease (quench) in the fluorescence of the pH-sensitive fluorescent probe quinacrine (5, 25, 26). Assays (1 ml) contained 5 μM quinacrine, 3 mM ATP, 100 mM BTPCl, 25 mM BTP-Hepes (pH 7.5), 250 mM mannitol, and 50 μg tonoplast protein. Assays were initiated with the addition of MgSO_4 (3 mM) and were conducted as described in Qiu *et al.* (5).

Control experiments were conducted to monitor potential effects of organic solvents used with inhibitors and ionophores on H^+ -transport; no solvent effects were observed (data not shown).

Na^+/H^+ Exchange Assays— Na^+/H^+ -exchange activity was measured as a Na^+ -induced dissipation of ΔpH (*i.e.* a Na^+ -induced increase in quinacrine fluorescence; 30). For these experiments, ΔpH was established by the H^+ -pyrophosphatase (22, 24). The reaction medium (1 ml) contained 2.5 μM quinacrine, 0.3 mM PP_i -BTP, 50 mM CsCl, 30 mM BTP-Hepes (pH 8.5), 250 mM mannitol, and 50 μg tonoplast protein. Assays were initiated with the addition of MgSO_4 (2 mM). While the *in vitro* activity (hydrolysis and H^+ -transport) of the tonoplast H^+ -pyrophosphatase is stimulated by K^+ (31, 32), the presence of K^+ in the transport assays prevented the formation of a stable ΔpH (likely due to phosphate inhibition of the H^+ -pump when it is operating maximally).² When Cs^+ (which stimulates the H^+ -pyrophosphatase to a lesser extent, see Ref. 33) was used in place of K^+ , the activity of the H^+ -pyrophosphatase (H^+ -transport) was reduced but stable, so Cs^+ was used in all assays. The reactions and assays were conducted as described in Qiu *et al.* (5) with the modifications indicated. To determine initial rates of Na^+/H^+ exchange (change in fluorescence per minute; $\Delta\% \text{F min}^{-1}$), changes in relative fluorescence were measured during the first 15 s after addition of Na^+ . Specific activity was calculated by dividing the initial rate by the mass of tonoplast protein in the reaction ($\Delta\% \text{F mg}^{-1} \text{ protein min}^{-1}$).

Membrane Potential Assays—Oxonol V, an inside-positive electrical or membrane potential ($\Delta\Psi$)-sensitive fluorescent probe, was used to measure the tonoplast $\Delta\Psi$ (15, 34). Assays (1 ml) contained 3 μM Oxonol V (Sigma-Aldrich), 0.3 mM PP_i -BTP, 2 mM MgSO_4 , 10 mM potassium iminodiacetic acid, 30 mM BTP-Hepes (pH 8.5), 250 mM mannitol, and 50 μg tonoplast protein. Assays were initiated with the addition of MgSO_4 (2 mM) and formation of $\Delta\Psi$ was measured as described above for ΔpH at excitation and emission wavelengths of 580 and 650 nm, respectively.

Preparation of Constitutively Active Recombinant SOS2 Protein—A constitutively active form of the serine/threonine kinase SOS2, T/DOSOS2DF, was used to study regulation of tonoplast Na^+/H^+ -exchange activity. Recombinant wild-type and constitutively active (activated) SOS2 protein was expressed, purified, and assayed as described (5, 9, 16).

When used in transport assays, T/DOSOS2DF (at the concentrations indicated) was incubated with membrane vesicles for 7 min at room temperature before ΔpH formation was initiated with the addition of MgSO_4 .

Preparation of *AtNHX1* Antibody—A BclI-BamHI fragment of the *AtNHX1* cDNA containing the last 122 C-terminal amino acids of the *AtNHX1* polypeptide was ligated into the BamHI site of the expression vector pEX2 (35) to produce an in-frame translational fusion to β -galactosidase. The construct was verified by DNA sequencing and trans-

² Q.-S. Qiu and K. S. Schumaker, unpublished data.

TABLE I
Determination of vesicle purity and sidedness

Membrane vesicles were isolated from culture cells of wild-type *Arabidopsis*. ATPase activity was assayed as described under "Experimental Procedures." Numbers in parenthesis represent percent of activity relative to the value with BTPCI. ATPase activity in the absence of BTPCI is $0.56 \pm 0.01 \mu\text{mol P}_i \text{ mg}^{-1} \text{ protein min}^{-1}$. Data represent means \pm S.E. of three replicate experiments. Each replicate was performed using independent membrane preparations.

	ATPase activity $\mu\text{mol P}_i \text{ mg}^{-1} \text{ protein min}^{-1}$
+BTPCI (50 mM)	0.91 ± 0.02 (100)
Triton X-100 (0.02%)	0.93 ± 0.03 (2.2%) ^a
Nitrate (50 mM)	0.50 ± 0.01 (45.1%) ^b
Vanadate (100 μM)	0.83 ± 0.02 (8.8%) ^b
Azide (1 mM)	0.83 ± 0.02 (8.8%) ^b
Molybdate (100 μM)	0.88 ± 0.01 (3.3%) ^b

^a Stimulation of ATPase activity.

^b Inhibition of ATPase activity.

formed into *Escherichia coli* POP2136 (35). Inclusion bodies with the recombinant protein were obtained after induction at 43 °C and resolved using a preparative 6% SDS-PAGE gel. A ground polyacrylamide gel slice containing 2 mg of the fusion protein was used to immunize rabbits by intradermal injections.

When used in transport assays, anti-AtNHX1 antibody was diluted 100-fold and incubated with the membrane vesicles for 7 min at room temperature before ΔpH formation was initiated with the addition of MgSO_4 .

Immunoblot Analysis—For immunoblot analysis, 6 μl of 3 \times protein loading buffer (200 mM Tris-HCl, pH 6.8, 8% SDS, 30% glycerol, 1.5% β -mercaptoethanol, and 0.3% bromophenol blue) was added to 50 μg of either plasma membrane vesicles (PM) or tonoplast vesicles (VM) in a 20- μl volume. The samples were boiled for 5 min, and the proteins separated on a 10% SDS-PAGE gel. The proteins were transferred from the gel to a pure nitrocellulose membrane (Bio-Rad Laboratories) at 80 volts for 60 min. The membrane was blocked in 1 \times PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , and 1.4 mM NaH_2PO_4 , pH 7.4) with 5% fat free milk overnight at 4 °C; and then rinsed one time with 1 \times PBS and incubated with a 1:1000 dilution of the AtNHX1 antibody for 3 h at room temperature. After three washes with 1 \times PBS buffer, the membrane was incubated with anti-rabbit IgG secondary antibody diluted 1:2000 (Amersham Biosciences) for 1 h at room temperature. After five washes in 1 \times PBS, the immunoreactive bands were detected using the chemiluminescent ECL detection substrate (Amersham Biosciences) and exposed to x-ray film.

Protein Determination—The protein content of membrane vesicles was determined by the method of Bradford (36) with bovine serum albumin as a standard.

RESULTS

Arabidopsis Tonoplast Vesicles Are Transport Competent—In order to study the transport activity of the tonoplast Na⁺/H⁺ exchanger, membrane vesicles were isolated from cell cultures of *Arabidopsis* using dextran gradients. The purity of the membrane preparations was analyzed by comparing the substrate hydrolytic and H⁺-transport activities of the ATPases on the mitochondrial, tonoplast, and plasma membranes, and the results are shown in Table I and Fig. 1. ATP hydrolysis was unaffected by molybdate (an inhibitor of non-specific phosphatases) or inhibitors of the plasma membrane (vanadate) and mitochondrial (azide) membrane ATPases, but was sensitive to the tonoplast H⁺-ATPase inhibitor, nitrate (45.1% inhibition at 50 mM), demonstrating that the vesicles were enriched in tonoplast. The sidedness of the membrane preparations was determined as described (29). When Triton X-100 (0.02%) was added to the assays, activity increased only 2.2% indicating that the vesicles were oriented mainly right-side-out relative to the orientation of the tonoplast *in vivo* (Table I).

Quenching of quinacrine fluorescence was observed when ATP/Mg²⁺ was added to the vesicles (Fig. 1), and quinacrine

fluorescence recovered if either the protonophore gramicidin or the uncoupler NH_4Cl were added after ΔpH had formed (Fig. 1A and data not shown, respectively). Fluorescence quench was inhibited if the H⁺ channel inhibitor N,N'-dicyclohexylcarbodiimide was added at the start of the reaction (Fig. 1B). These results indicate that this ATP/Mg²⁺-induced fluorescence quench was caused by the transport of H⁺ and reflects the formation of a ΔpH . A significant reduction in H⁺ transport activity in the presence of nitrate and insensitivity to vanadate (Fig. 1, C and D) provided additional evidence that the vesicles were enriched in tonoplast.

Wild-type Arabidopsis Cells Have Tonoplast Na⁺/H⁺-Exchange Activity—Dissipation of a ΔpH (formed by the tonoplast H⁺-pyrophosphatase) was induced by the addition of Na⁺ to tonoplast vesicles that had been isolated from wild-type cells (Fig. 2A). To determine the ion specificity of the exchange reaction, the ability of various salts to dissipate ΔpH was monitored. Similar rates of exchange were observed when either NaCl or sodium gluconate was added (Fig. 2, A and E), suggesting that Na⁺ and not the anion is responsible for the dissipation of ΔpH . Addition of Li⁺ also led to dissipation of ΔpH , but at a slower rate (Fig. 2, B and E). When salts of cesium, 1,3-bis[tris(hydroxymethyl)methylamine] (BTP)-propane or K⁺ were used instead of Na⁺, no dissipation of ΔpH was observed (Fig. 2, C–E). When K⁺ was added prior to Na⁺, Na⁺-induced dissipation of ΔpH was reduced (Fig. 2D) suggesting either that K⁺ is a competitive inhibitor of Na⁺ transport or that Na⁺ transport is masked due to K⁺ stimulation of the H⁺-pump and the resulting increase in ΔpH formation. Taken together, these results suggest that the tonoplast exchanger transports Na⁺ and to a much lesser extent Li⁺.

The effects of the diuretic compound amiloride and its analogs on Na⁺/H⁺-exchange activity in tonoplast vesicles from wild-type *Arabidopsis* were determined. As the concentration of the potent amiloride analog 5-(N-methyl-N-isobutyl)amiloride (MIA) in the assay was increased, exchange activity decreased. Exchange activity was reduced 50% with 10 μM MIA and completely eliminated with 20 μM (Fig. 3, A and B). This is in contrast to amiloride, which did not affect Na⁺/H⁺ exchange at any of the concentrations tested (data not shown). The inhibitory effects of amiloride and its analogs on AtNHX1 expressed in yeast and reconstituted in liposomes have been reported recently (18, 19). In comparative studies of yeast cells expressing AtNHX1 from *Arabidopsis* and overexpressing NHX1 from yeast, Darley *et al.* (18) showed that, activity originating from the *Arabidopsis* exchanger was completely inhibited with 120 μM amiloride while activity originating from the yeast exchanger was inhibited 20–40% at the same concentration. When AtNHX1 was reconstituted into liposomes, exchange activity was inhibited by 57 and 42% with 10 μM ethylisopropyl-amiloride (EIPA) or 10 μM MIA, respectively (19). At this same concentration, amiloride did not have any inhibitory effect (19). The MIA sensitivity of Na⁺/H⁺-exchange in our vesicle preparations provides evidence that this transport originates from NHX protein(s) as these are the only *Arabidopsis* exchangers known to possess an amiloride binding site (37).

To provide information about the relative number of ions (Na⁺ and H⁺) being transported during the exchange reaction (stoichiometry), changes in Oxonol V fluorescence were monitored. Oxonol V accumulates inside membrane vesicles in response to an increase in positive charge (an inside-positive $\Delta\Psi$, 38). If one Na⁺ is exchanged for one H⁺, the reaction is electroneutral (no net charge transfer across the membrane and no $\Delta\Psi$ is generated). If the exchange of Na⁺ for H⁺ is more than one for one (or *vice versa*), the reaction is electrogenic (generating a $\Delta\Psi$). Oxonol V accumulated inside the vesicles when H⁺

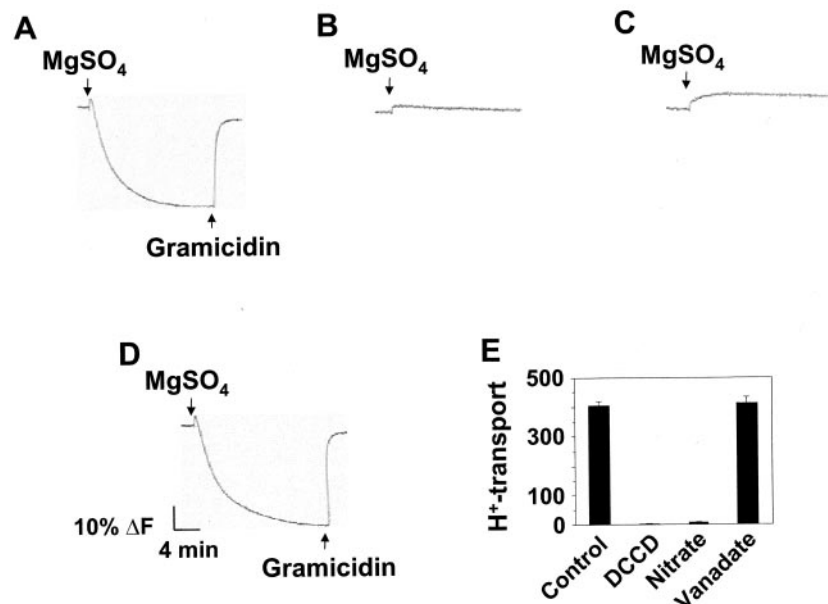


FIG. 1. Vesicles isolated from wild-type *Arabidopsis* cells are transport-competent and enriched in tonoplast. Tonoplast vesicles were isolated from the cell cultures of wild-type *Arabidopsis* using dextran gradients. The pH gradient (ΔpH) was established by the activity of the tonoplast H^+ -ATPase and was measured as a decrease (quench) in the fluorescence of the pH-sensitive fluorescent probe quinacrine (5, 25, 26). Assays (1 ml) contained 5 μM quinacrine, 3 mM ATP, 100 mM BTPCl, 25 mM BTP-Hepes (pH 7.5), 250 mM mannitol, and 50 μg of tonoplast protein. Assays were initiated with the addition of MgSO_4 (3 mM) and were conducted as described in Qiu *et al.* (5). To measure ΔpH formation, reactions were illuminated at 430 nm and quinacrine fluorescence was monitored at 500 nm. A, when added after ΔpH formation reached steady state, the protonophore gramicidin (5 μg) dissipated the existing ΔpH . B, when added at the start of the reaction, the proton channel inhibitor *N,N'*-dicyclohexylcarbodiimide (DCCD, 20 μM) prevented formation of ΔpH . C, when added at the start of the reaction, 50 mM nitrate (a tonoplast H^+ -ATPase inhibitor) inhibited ΔpH formation, while (D) control levels of ΔpH formation were measured in the presence of 100 μM vanadate (a plasma membrane H^+ -ATPase inhibitor). For panels A–D, one representative experiment of three replicates is shown. Each replicate experiment was performed using independent membrane preparations. E, initial rates of H^+ -transport (means \pm S.E.) of the three replicates; units are $\Delta\% \text{ F mg}^{-1} \text{ protein min}^{-1}$.

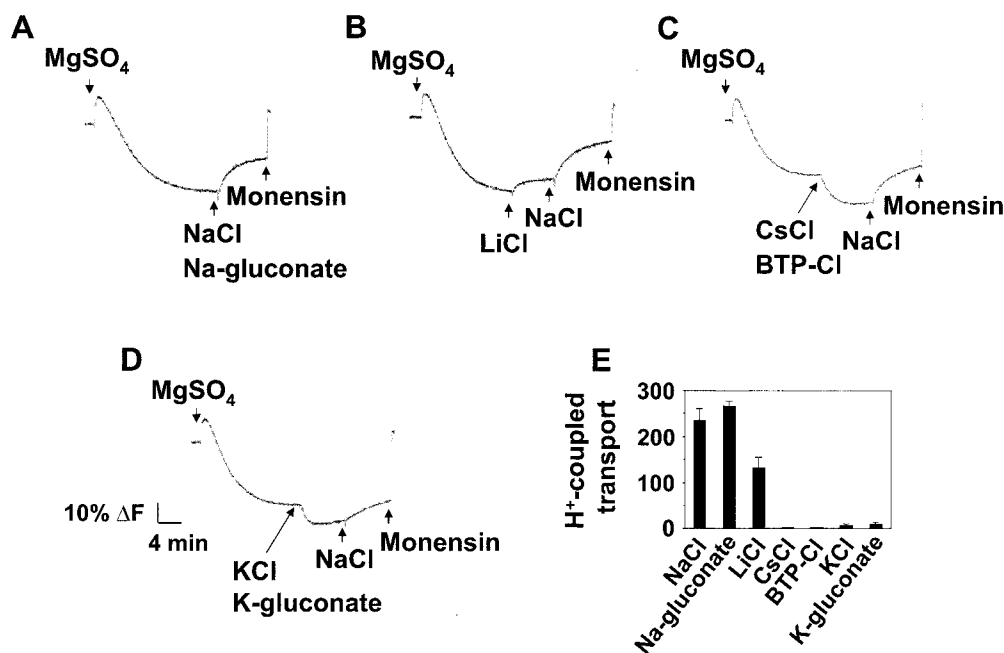


FIG. 2. Ion specificity of tonoplast H^+ -coupled exchange activity in wild-type *Arabidopsis* cells. Tonoplast vesicles were isolated from cell cultures of wild-type *Arabidopsis* using dextran gradients. ΔpH was established by the activity of the tonoplast H^+ -pyrophosphatase and was measured as a decrease (quench) in the fluorescence of the pH-sensitive fluorescent probe quinacrine. The reaction medium (1 ml) contained 2.5 μM quinacrine, 0.3 mM PP_i -BTP, 50 mM CsCl, 30 mM BTP-Hepes (pH 8.5), 250 mM mannitol, and 50 μg of tonoplast protein. Reactions were illuminated at 430 nm and quinacrine fluorescence was monitored at 500 nm. A, after the formation of ΔpH , 50 mM NaCl or sodium gluconate was added to initiate Na^+/H^+ exchange (dissipation of ΔpH). The electroneutral Na^+/H^+ exchanger, Monensin (150 μM), was added at the end of each assay to determine if a ΔpH was present. B, after the formation of ΔpH , 50 mM LiCl was added and, once a new steady state was reached, NaCl (50 mM) was added to initiate Na^+/H^+ exchange. C, after the formation of ΔpH , 50 mM CsCl or BTP-Cl was added and, once a new steady state was reached, NaCl (50 mM) was added to initiate Na^+/H^+ exchange. D, after the formation of ΔpH , 50 mM KCl was added and, once a new steady state was reached, NaCl (50 mM) was added to initiate Na^+/H^+ exchange. For panels A–D, one representative experiment of three replicates is shown. Each replicate experiment was performed using independent membrane preparations. E, initial rates of H^+ -coupled transport (means \pm S.E.) of the three replicates; units are $\Delta\% \text{ F mg}^{-1} \text{ protein min}^{-1}$.

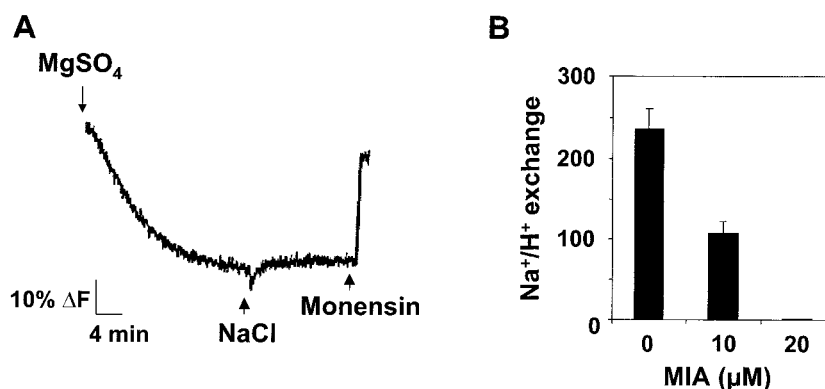


FIG. 3. **Tonoplast Na^+/H^+ exchange in wild-type *Arabidopsis* cells is sensitive to methyl-isobutyl amiloride.** Tonoplast vesicles were isolated from the cell cultures of wild-type *Arabidopsis*. Reaction mixes and assay conditions were as described in the legend to Fig. 2. *A*, a more potent form of amiloride, MIA (20 μM) was added before ΔpH formation was initiated. After the formation of ΔpH , NaCl (50 mM) was added to initiate Na^+/H^+ exchange. Monensin (150 μM), was added at the end of the assay to determine if a ΔpH was present. *B*, initial rates of Na^+/H^+ exchange as a function of MIA concentration. Data represents means \pm S.E. of the three replicates; units are $\Delta\% \text{ F mg}^{-1} \text{ protein min}^{-1}$.

transport was established by the tonoplast H^+ -pyrophosphatase (shown as a quench of Oxonol V fluorescence, Fig. 4). In these experiments, potassium iminodiacetic acid and sodium gluconate rather than CsCl and NaCl were used to stimulate the H^+ -pyrophosphatase and dissipate the ΔpH , respectively in order to limit the concentrations of permeant anions while maintaining the required concentrations of cations. Once a steady state ΔpH was formed, Na^+ was added to initiate Na^+/H^+ exchange. Under conditions of optimum Na^+/H^+ exchange, no change in Oxonol V fluorescence was observed (Fig. 4), demonstrating that Na^+/H^+ exchange did not alter the $\Delta\psi$ and that the exchange reaction is electroneutral.

*AtNHX1 Contributes the Major Portion of Na^+/H^+ -Exchange Activity in Tonoplast Vesicles Isolated from Wild-type *Arabidopsis* Cells*—Recent research indicates that there are six *AtNHX* gene family members in *Arabidopsis* (37). Phylogenetically, the proteins have been categorized into two subgroups, one containing four (*AtNHX1–4*) and the other two (*AtNHX5* and 6) members. Both *AtNHX1* and *AtNHX2* have been localized to the tonoplast of plant cells (21, 37, 39). In order to determine the molecular identity of the transporter responsible for Na^+/H^+ -exchange activity in tonoplast vesicles isolated from *Arabidopsis* cell cultures, we compared transport in the absence and presence of anti-*AtNHX1* antibody. As shown in Fig. 5A, the antibody cross-reacted with a tonoplast band of 50 kDa, while no cross-reacting plasma membrane proteins were observed. When added to transport assays, increasing amounts of antibody progressively reduced Na^+/H^+ -exchange (Fig. 5, B and C). Initial rates of Na^+/H^+ -exchange activity were inhibited 36.4, 45.1, and 56.2% in the presence of 10, 20, and 40 μl of the diluted (1:100) antibody, respectively (Fig. 5C). This antibody inhibition of activity was specific as Na^+/H^+ exchange was largely unaffected when equivalent amounts of pre-immune serum were added to the assays (Fig. 5C). Taken together, these results indicated that the major portion of Na^+/H^+ -exchange activity observed in tonoplast vesicles isolated from *Arabidopsis* cell cultures was contributed by *AtNHX1* and/or *AtNHX2*, which shares 87.5% sequence similarity with *AtNHX1* and cross-reacts with anti-*AtNHX1* antibody (37).

*Tonoplast Na^+/H^+ -Exchange Activity Is Higher in *sos1* Cells Than in Wild-type Cells*—In tonoplast vesicles isolated from wild-type *Arabidopsis* cells, Na^+ -induced dissipation of ΔpH was dependent on Na^+ (substrate) concentration as initial rates of exchange increased with increasing NaCl up to 100 mM (Fig. 6A). Kinetic analysis of the data showed that the tonoplast exchanger has an apparent K_m for Na^+ of 31 mM and V_{max} of 370 units ($\Delta\% \text{ F mg}^{-1} \text{ protein min}^{-1}$, Fig. 6, B and C). When

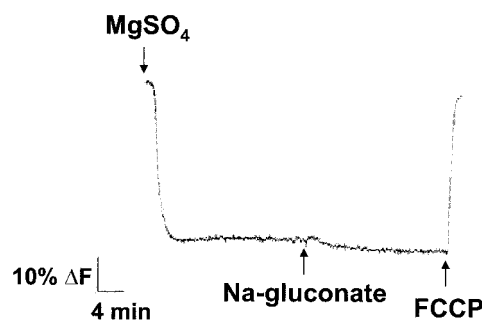


FIG. 4. **Tonoplast Na^+/H^+ exchange in wild-type *Arabidopsis* cells is electroneutral.** Assays (1 ml) contained 3 μM Oxonol V (Sigma-Aldrich), 0.3 mM PP_i -BTP, 2 mM MgSO_4 , 10 mM potassium iminodiacetic acid, 30 mM BTP-Hepes (pH 8.5), 250 mM mannitol, and 50 μg tonoplast protein. Reactions were illuminated at 580 nm and Oxonol V fluorescence was monitored at 650 nm. An inside-acid positive $\Delta\psi$ was formed when tonoplast protein (50 μg) was added to the reaction mix. Once $\Delta\psi$ reached steady state, sodium gluconate (50 mM) was added to initiate Na^+/H^+ exchange. At the end of the experiment, the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (*FCCP*, 5 μM) was added to determine if a $\Delta\psi$ was present. One representative experiment of three replicates is shown; each replicate experiment was performed using independent membrane preparations.

assaying Na^+/H^+ -exchange activity in vacuoles isolated from *Arabidopsis* leaves in plants over-expressing the *Arabidopsis* tonoplast Na^+/H^+ exchanger *AtNHX1*, Apse *et al.* (21) reported an apparent K_m for Na^+ of 7 mM. Similarly, when assaying Na^+/H^+ -exchange activity in tonoplast vesicles isolated from yeast cells overexpressing *AtNHX1*, Darley *et al.* (18) found that the exchanger has an apparent K_m for Na^+ of 11 mM. In contrast, Venema *et al.* (19) reported that *AtNHX1* reconstituted in liposomes displayed a K_m for Na^+ of 42 mM. It is not clear if and how these values are affected by the identity of the exchanger (*AtNHX1* versus other tonoplast exchangers), the assay systems (isolated vacuoles, tonoplast vesicles, liposomes) or levels of expression (wild-type versus overexpression) of the exchanger.

To understand how intracellular Na^+ levels are regulated during salt stress, it is necessary to determine how the individual proteins that transport Na^+ function together. Our previous studies have demonstrated that plasma membrane Na^+/H^+ -exchange activity is significantly reduced in *sos1* plants when compared with activity in wild-type plants. To determine if there is coordination of the activities of the Na^+/H^+ exchangers on the tonoplast and plasma membranes, tonoplast Na^+/H^+ -exchange activity was compared in vesicles isolated from

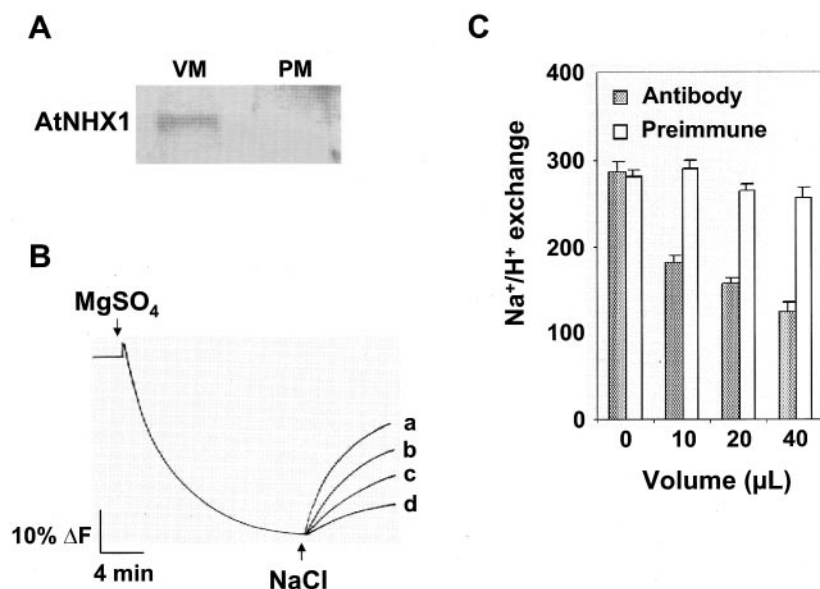
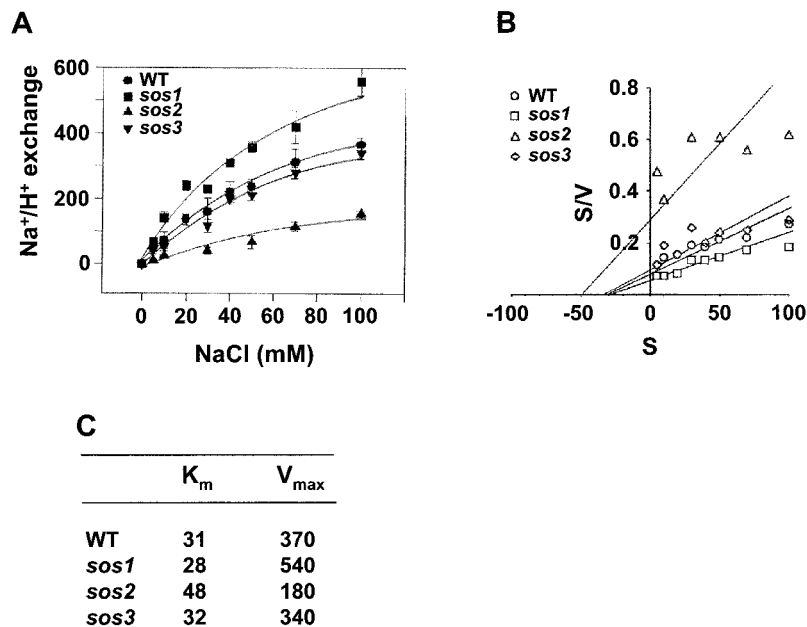


FIG. 5. Tonoplast Na^+/H^+ exchange in wild-type *Arabidopsis* cells is contributed by AtNHX. A, anti-AtNHX1 antibody cross-reacts with a 50 kDa tonoplast protein. Tonoplast and plasma membrane proteins were isolated from cell cultures of *Arabidopsis* using dextran gradients and aqueous two-phase partitioning, respectively. Membrane proteins (50 μg) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Anti-AtNHX1 antibody (a 1:1000 dilution) was used to detect cross-reacting proteins. B and C, anti-AtNHX1 antibody inhibits tonoplast Na^+/H^+ exchange. B, tonoplast vesicles were isolated from cell cultures of wild-type *Arabidopsis* using dextran gradients. Increasing amounts (10, 20, and 40 μl , traces b, c, and d, respectively) of a 1:100 dilution of the anti-AtNHX1 antibody were added to the reaction medium before ΔpH formation was initiated. Na^+/H^+ exchange was compared with assays without antibody (trace a) or with 40 μl of preimmune serum (trace a). Traces are aligned at the point of NaCl addition. One representative experiment of three replicates is shown; each replicate experiment was performed using independent membrane preparations. C, initial rates of Na^+/H^+ -exchange activity (means \pm S.E.) of the three replicates; units are $\Delta\% \text{ F mg}^{-1} \text{ protein min}^{-1}$.

FIG. 6. Tonoplast Na^+/H^+ exchange is higher, greatly reduced and unchanged in *sos1*, *sos2*, and *sos3* cells relative to activity in wild-type *Arabidopsis* cells. Tonoplast vesicles were isolated from the cell cultures of wild-type (WT), *sos1*, *sos2*, and *sos3*. Reaction mixes and assay conditions were as described in the legend to Fig. 2. A, initial rates of Na^+ -induced dissipation of ΔpH in vesicles isolated from wild-type (\bullet), *sos1* (\blacksquare), *sos2* (\blacktriangle), or *sos3* (\blacktriangledown) cells were calculated over a range of Na^+ concentrations from 0–100 mM. Units of Na^+/H^+ exchange are $\Delta\% \text{ F mg}^{-1} \text{ protein min}^{-1}$. All data represent means \pm S.E. of at least three replicate experiments. Each replicate experiment was performed using independent membrane preparations. B and C, data shown in A were transformed using a Hanes-Woolf plot in order to determine kinetic parameters ($-X$ intercept = K_m , K_m/Y intercept = V_{max}). Units for K_m and V_{max} are mM and $\Delta\% \text{ F mg}^{-1} \text{ protein min}^{-1}$, respectively).



wild-type and *sos1* cells. Na^+/H^+ exchange was significantly higher in *sos1* at all Na^+ concentrations tested (Fig. 6A); with 100 mM Na^+ , the transport activity was increased by 52.4%. Kinetic analysis of the data indicated that the apparent affinity of the exchanger for substrate did not change substantially but its maximum velocity was higher in the *sos1* mutant ($K_m = 28$ mM and $V_{\text{max}} = 540$ units, respectively; Fig. 6, B and C) than in wild type. These results provide strong evidence for coordination of the activities of the Na^+/H^+ exchangers on the tonoplast and plasma membranes.

sos2 Cells Have Reduced Tonoplast Na^+/H^+ -Exchange Activity—Our previous studies have shown that *sos2* and *sos3* plants

have reduced plasma membrane Na^+/H^+ -exchange activity and that addition of activated SOS2 protein *in vitro* to membrane vesicles isolated from these plants restored this transport activity (5). From these studies we concluded that SOS2 and SOS3 regulate the activity of the plasma membrane Na^+/H^+ exchanger (SOS1). In the present study, Na^+/H^+ -exchange activity was compared in tonoplast vesicles isolated from wild-type and *sos2* cells to determine whether this kinase also regulates the transport activity of the tonoplast Na^+/H^+ exchanger. Exchange activity in vesicles isolated from *sos2* cells was greatly reduced over the range of Na^+ concentrations tested (Fig. 6A); with 100 mM Na^+ , the transport activity was

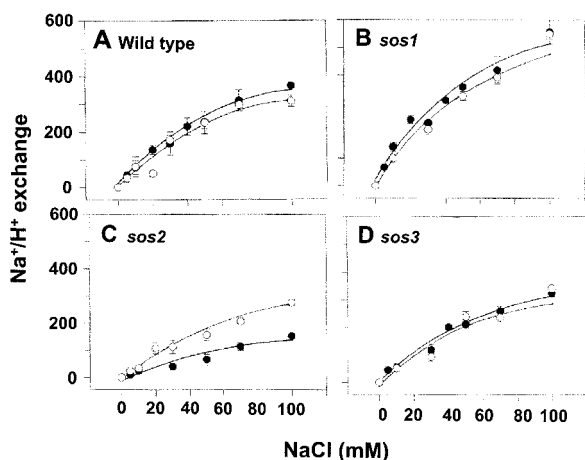


FIG. 7. Constitutively active recombinant SOS2 protein stimulates tonoplast Na^+/H^+ -exchange activity in vesicles isolated from *sos2* cells, but not in vesicles isolated from wild-type, *sos1*, or *sos3* cells. Reaction mixes and assay conditions were as described in the legend to Fig. 2. ΔpH was formed in the absence (●) or presence (○) of constitutively active recombinant SOS2 protein (0.2 μg GST-T/DSOS2DF). When ΔpH reached steady state, NaCl was added over a range of final concentrations (0–100 mM) and initial rates of dissipation were measured. A, wild type; B, *sos1*; C, *sos2*; D, *sos3*. Units of Na^+/H^+ exchange are $\Delta\% \text{ F mg}^{-1} \text{ protein min}^{-1}$. All data represent means \pm S.E. of at least three replicate experiments. Each replicate experiment was performed using independent membrane preparations.

reduced by 60.3% relative to that in wild type. Kinetic analysis of the data indicated that the transporter in the *sos2* mutant had an apparent K_m for Na^+ of 48 mM and V_{max} of 180 units (Fig. 6, B and C), suggesting that *sos2* mutants had a lower affinity for substrate relative to wild-type plants. This reduced activity at the tonoplast suggests that this transporter is also regulated by SOS2.

Tonoplast Na^+/H^+ -Exchange Activity Is Unchanged in *sos3* Cells—Previous genetic analyses have shown that the three identified SOS genes function in the same salt-tolerance pathway (12). Subsequent studies demonstrated that plasma membrane Na^+/H^+ -exchange activity was reduced in *sos3* plants and that this activity could be restored with the addition of activated SOS2 protein (15), by-passing the requirement for SOS3. To determine if SOS3 is involved in the regulation of the tonoplast Na^+/H^+ exchanger, exchange activity was monitored in vesicles isolated from *sos3* cells. In contrast to what was found for *sos2*, the exchange activity in *sos3* was similar to what was measured in wild type (Fig. 6A) indicating that SOS3 is not involved in the regulation of the tonoplast Na^+/H^+ exchanger. Kinetic analysis of the data indicated that the properties of the transporter in the *sos3* mutant (Fig. 6, B and C; $K_m = 32$ mM and $V_{\text{max}} = 340$ units) were similar to those in wild type.

Constitutively Active SOS2 Does Not Stimulate Tonoplast Na^+/H^+ -Exchange Activity in Wild-Type, *sos1*, or *sos3* Cells *in Vitro*—In the presence of constitutively active (activated) SOS2 protein (T/DSOS2DF, Ref. 5), plasma membrane Na^+/H^+ -exchange activity in vesicles isolated from wild-type plants increased 2-fold relative to activity without added protein (5). Pathway regulation of the tonoplast Na^+/H^+ exchanger was also studied by addition of the activated SOS2 protein directly to the assay medium. No change in activity was observed in the vesicles isolated from wild-type cells relative to activity without added protein (Fig. 7A).

In the absence of activated SOS2 protein added *in vitro*, tonoplast Na^+/H^+ exchange activity (in the presence of 100 mM NaCl) is 45.9% higher in vesicles isolated from *sos1* when compared with activity in vesicles isolated from wild-type cells

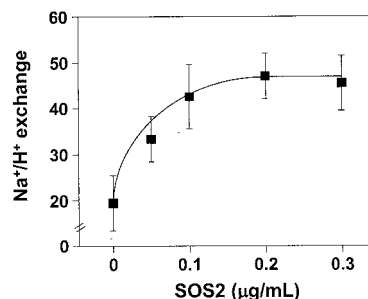


FIG. 8. SOS2 stimulation of tonoplast Na^+/H^+ -exchange activity in *sos2* cells is dependent on SOS2 protein concentration. Reaction mixes and assay conditions were as described in the legend to Fig. 2. At each concentration of constitutively active recombinant SOS2 protein, when ΔpH reached steady state, 10 mM NaCl was added to initiate Na^+/H^+ exchange. Initial rates of dissipation were measured and plotted as a function of the concentration of SOS2 protein. Units of Na^+/H^+ -exchange are $\Delta\% \text{ F mg}^{-1} \text{ protein min}^{-1}$. All data represent means \pm S.E. of at least three replicate experiments. Each replicate experiment was performed using independent membrane preparations.

(Fig. 6A). When activated SOS2 protein was added *in vitro* to membranes isolated from *sos1*, no stimulation of activity was measured (Fig. 7B) suggesting that either the exchanger is already operating maximally or that the sensitivity of the exchanger to SOS2 regulation was altered in the *sos1* mutant *in vivo*.

When activated SOS2 protein was added to vesicles isolated from *sos3* cells, no stimulation of tonoplast Na^+/H^+ -exchange activity was observed (Fig. 7D); this lack of stimulation is in contrast to what was found for the plasma membrane exchanger where activated SOS2 protein stimulated activity 2-fold (5). These results provide additional evidence that the activity of the tonoplast exchanger is not influenced by SOS3.

Constitutively Active SOS2 Stimulates Tonoplast Na^+/H^+ -Exchange Activity of *sos2* Cells *in Vitro*—Tonoplast Na^+/H^+ -exchange activity was significantly reduced in *sos2* cells (Fig. 6). To determine if this is due to a direct regulation of the transporter by the SOS2 kinase, activated SOS2 protein was added to vesicles isolated from *sos2*. Exchange activity increased with increasing NaCl concentration and, with 100 mM NaCl, a 2-fold stimulation was measured relative to the activity without added protein (Fig. 7C). Stimulation of exchange activity increased as a function of the amount of added SOS2 protein up to 0.2 $\mu\text{g/mL}$ (Fig. 8). Kinetic analysis of the data indicated that, in the presence of activated SOS2 protein, both the apparent affinity and the activity ($K_m = 38$ mM and $V_{\text{max}} = 300$ units, respectively) of the tonoplast exchanger in the *sos2* mutant were restored to wild-type levels.

SOS2 protein in which kinase activity had not been activated (either unmodified wild-type SOS2 recombinant protein or boiled T/DSOS2DF protein) did not have any effect on exchange activity (data not shown). Stimulation of exchange activity in *sos2* cells by *in vitro* addition of activated SOS2 protein provides additional evidence that SOS2 regulates the tonoplast Na^+/H^+ exchanger.

To determine if the mechanism of NHX transport activation is due to phosphorylation by SOS2, *in vitro* phosphorylation assays were conducted. Under conditions where SOS2 kinase phosphorylates SOS1 protein *in vitro* (17), no SOS2 phosphorylation of NHX proteins was observed (data not shown). Direct SOS2 stimulation of transport activity in the absence of a corresponding *in vitro* phosphorylation of the transporter has also been found for CAX1, a tonoplast $\text{Ca}^{2+}/\text{H}^+$ exchanger in Arabidopsis (40); several interpretations for these results are possible. For example, it may be that conditions required for the *in vitro* phosphorylation of the tonoplast exchangers are

different than those for the exchanger at the plasma membrane. Alternatively, it may be that SOS2 does not directly phosphorylate the tonoplast exchangers and that an important, but as yet unidentified, membrane-associated intermediate exists between SOS2 and the transporters.

DISCUSSION

The Tonoplast Na^+/H^+ Exchanger Is a Target of the SOS Pathway—In the present study, tonoplast Na^+/H^+ -exchange activity was measured as Na^+ -induced dissipation of a pH gradient established by the tonoplast H^+ -pyrophosphatase. These studies were performed using purified tonoplast vesicles isolated from cell cultures of *Arabidopsis* (Table I and Fig. 1). Na^+/H^+ -exchange activity was greatly reduced (51.4% reduction at 100 mM NaCl) in the tonoplast vesicles isolated from *sos2* relative to activity in wild type (Fig. 6). This activity approached wild-type levels when constitutively active SOS2 protein was added to the assay (Fig. 7C). These results clearly show that the tonoplast Na^+/H^+ exchanger is regulated by the SOS2 kinase. Our previous studies demonstrated that the SOS2 kinase regulates the Na^+/H^+ -exchange activity of SOS1, a plasma membrane Na^+/H^+ exchanger (5, 17). Together, these results indicate that the SOS2 protein kinase plays an important role in regulating transporters that are involved in cellular Na^+ homeostasis in plants and that the signal transduction pathways involving the SOS2 kinase are critical for the plant's response to salt stress.

Kinetic analyses of tonoplast exchange activity as a function of substrate concentration in the presence and absence of activated SOS2 protein suggests that SOS2 stimulates transport activity through a change in the structure of the Na^+ -binding site of the exchanger. In the absence of activated SOS2 protein, the exchange reaction in *sos2* had a lower affinity for substrate than the reaction in wild type (Fig. 6C). When activated SOS2 protein was added to vesicles isolated from *sos2*, both the velocity ($V_{\text{max}} = 300$ units) and the affinity (decreased apparent $K_m = 38$ mM) of the exchanger for substrate increased significantly and approached levels measured in wild type.

We have previously shown that the activity of the plasma membrane exchanger in wild-type plants was stimulated 2-fold by activated SOS2 protein added *in vitro* (5). We suggested that two possible explanations for this increase might be that activated SOS2 protein is limiting exchange activity *in vivo*, or that the activity of the exchanger is tightly regulated *in vivo*. Under the same experimental conditions, the activity of the tonoplast Na^+/H^+ exchanger was unaffected by addition of activated SOS2 (Fig. 7A). These results indicate that, although the tonoplast and plasma membrane Na^+/H^+ exchangers are both regulated by SOS2, the mechanisms of regulation are clearly different.

Our previous studies demonstrated that in *sos3* plants, the activity of the plasma membrane Na^+/H^+ exchanger was reduced compared with activity in wild-type plants (5). When activated SOS2 protein was added to vesicles isolated from *sos3* plants, plasma membrane exchange activity was increased to levels above those measured in wild-type plants without activated SOS2 protein (5). These results provided evidence that both SOS2 and SOS3 regulate the exchange activity of the plasma membrane Na^+/H^+ exchanger, and that they operate in the same pathway. In contrast, no significant change in tonoplast Na^+/H^+ -exchange activity was observed in *sos3* (Fig. 6A) and activated SOS2 protein did not stimulate exchange activity in this mutant (Fig. 7D). These results indicate that the tonoplast Na^+/H^+ exchanger is not regulated by SOS3 and that an unknown component must regulate SOS2 in this pathway. This differential regulation of the exchangers by SOS3 indicates that the signaling pathways involved are different and that

they diverge upstream of SOS2. The identity of the upstream component in the tonoplast SOS pathway remains to be determined.

Multiple SOS Pathways Exist—SOS2 represents a novel protein kinase that functions in salt stress (10, 16). A search of the data base indicates that there are many sequences with significant similarity to SOS2 in both the catalytic and regulatory domains. In *Arabidopsis*, there are at least 23 SOS2-like protein kinases (PKS). All of these PKS proteins contain a putative FISL motif near the kinase domain and belong to CaMKII/SNF1/AMPK kinase family (16). Similarly, there are many sequences in the data base that share significant similarity with the SOS3 protein; all of these SOS3-like calcium-binding proteins (SCaBP) contain three EF-hands as part of their structure. Using yeast two-hybrid assays, Guo *et al.* (16) found that there are interactions between these PKSs and the SCaBPs. For example, SOS2 was found to interact with SOS3, SCaBP1, SCaBP3, SCaBP5, and SCaBP6 while SOS3 was found to interact with SOS2, PKS2, PKS3, PKS6, and PKS7. Differential regulation of the Na^+/H^+ exchangers on the tonoplast and plasma membranes along with the presence of and interactions between the PKSs and SCaBPs suggest that multiple networks that function in signal transduction exist in *Arabidopsis*. Future studies will determine if any of these other interacting partners are also involved in the regulation of ion homeostasis.

There Is Coordination between the Na^+ Transporters in the Tonoplast and Plasma Membranes—In plants, regulation of cellular Na^+ homeostasis is critical for plant growth and development. The tonoplast and plasma membrane Na^+/H^+ exchangers play an important role in Na^+ regulation by removing this ion from the cytoplasm through transport into the vacuole or out of the cell. When these transporters operate, how they are regulated, and if there is coordinate regulation of the transporters remain fundamental questions in plant stress biology. Our previous studies demonstrated that the activity of the plasma membrane Na^+/H^+ exchanger is salt-induced; no plasma membrane activity was measured in *Arabidopsis* plants grown without salt. This exchange activity was induced when plants were grown in 250 mM NaCl and increased with prolonged salt exposure up to 8 days (15). In contrast, the activity of the tonoplast transporter is present in cells grown in the absence of salt (21, Fig. 2). This differential salt induction implies diverse roles for these transporters in ion homeostasis and salt tolerance. It is possible that the tonoplast exchanger plays a major role in maintaining Na^+ homeostasis during normal growth and when the plant first experiences increasing levels of Na^+ . With prolonged exposure to high levels of Na^+ , the transporter on the plasma membrane may begin to play a greater role in reducing cellular Na^+ levels and ultimately in the plant's ability to continue to grow during salt stress.

We have recently shown that the mRNA levels of *NHX* genes were up-regulated in the *sos1* mutant. Densitometric analysis of RNA blots of *NHX1* and *NHX2* levels in wild-type *versus sos1* plants indicates an up-regulation of about 2-fold in the *sos1* mutant (Ref. 37, data not shown). This increase is consistent with the enhanced tonoplast Na^+/H^+ -exchange activity measured in *sos1* cells (relative to activity in wild-type cells) reported in the present study (Fig. 6). From previous studies, we have shown that the plasma membrane exchanger in the *sos1* mutant has greatly reduced activity (5). These results indicate that there can be coordination of activity between the exchangers on the tonoplast and plasma membranes; when the activity of one exchanger is missing or reduced, the activity of the other may be enhanced to compensate for the lost activity. This compensation could provide an adaptive mechanism to enable the plant to maintain the low levels of intracellular Na^+ required for growth. The mecha-

nisms underlying the coordinate regulation of these two exchangers await further experimentation.

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