

# Salt Stress in *Thellungiella halophila* Activates Na<sup>+</sup> Transport Mechanisms Required for Salinity Tolerance<sup>1</sup>

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Salinity is considered one of the major limiting factors for plant growth and agricultural productivity. We are using salt cress (*Thellungiella halophila*) to identify biochemical mechanisms that enable plants to grow in saline conditions. Under salt stress, the major site of Na<sup>+</sup> accumulation occurred in old leaves, followed by young leaves and taproots, with the least accumulation occurring in lateral roots. Salt treatment increased both the H<sup>+</sup> transport and hydrolytic activity of salt cress tonoplast (TP) and plasma membrane (PM) H<sup>+</sup>-ATPases from leaves and roots. TP Na<sup>+</sup>/H<sup>+</sup> exchange was greatly stimulated by growth of the plants in NaCl, both in leaves and roots. Expression of the PM H<sup>+</sup>-ATPase isoform AHA3, the Na<sup>+</sup> transporter HKT1, and the Na<sup>+</sup>/H<sup>+</sup> exchanger SOS1 were examined in PMs isolated from control and salt-treated salt cress roots and leaves. An increased expression of SOS1, but no changes in levels of AHA3 and HKT1, was observed. NHX1 was only detected in PM fractions of roots, and a salt-induced increase in protein expression was observed. Analysis of the levels of expression of vacuolar H<sup>+</sup>-translocating ATPase subunits showed no major changes in protein expression of subunits VHA-A or VHA-B with salt treatment; however, VHA-E showed an increased expression in leaf tissue, but not in roots, when the plants were treated with NaCl. Salt cress plants were able to distribute and store Na<sup>+</sup> by a very strict control of ion movement across both the TP and PM.

Much of the recent advances in understanding plant salt tolerance have come from studies that employ the salt-sensitive model plant *Arabidopsis thaliana*. Na<sup>+</sup> transporters, including the NHX/SOS family of Na<sup>+</sup>/H<sup>+</sup> exchangers, and HKT, a Na<sup>+</sup> transporter, as well as components of the signaling pathway that regulate these genes, including SOS2 and SOS3, have been shown to be involved in plant response to sodium (for a recent review, see Horie and Schroeder, 2004). Yet, despite this progress, there is still a consensus that certain novel adaptive responses may be overlooked when using a glycophyte as the exclusive model. Halophytes may have evolved unique mechanisms or regulatory pathways that are not found in glycophytes, which would primarily present a stress response. This belief has fueled the use of the ice plant (*Mesembryanthemum crystallinum*) and other halophytes (e.g. *Salicornia bigelovii* and *Atriplex gmelini*) to further our understanding of plant salt tolerance (Ayala et al., 1995; Adams et al., 1998; Hamada et al., 2001). Unfortunately, none of these are suitable genetic models. While for the ice plant, a large expressed

sequence tag database has been amassed, as well as the start of a mutant collection (Cushman and Bohnert, 1999, 2000), the ability to transform this plant efficiently is still lacking. Recently, several groups have reported on the use of a close *Arabidopsis* relative, salt cress (*Thellungiella halophila*), as an appropriate halophytic model (Amtmann et al., 2005). Apart from being salt tolerant, salt cress exhibits many of the advantages that have made *Arabidopsis* such a popular model plant system. These include a small genome of approximately twice the size of *Arabidopsis*, a short life cycle, self-pollination, and abundant seed production following vernalization, thus allowing for fast and efficient genetic analysis (Bressan et al., 2001). More importantly, the plant has proven easy to transform using the floral-dip method (Bressan et al., 2001) and shares on average 92% sequence identity with *Arabidopsis* (Bressan et al., 2001; Volkov et al., 2003; Inan et al., 2004). In addition, work is in progress to generate t-DNA-tagged mutant lines (Wang et al., 2003; Inan et al., 2004). Also noteworthy is the fact that, although salt cress is classified as a halophyte, it does not appear to rely on specialized morphological features, such as salt glands, bladder cells, or metabolic changes, such as Crassulacean acid metabolism, that are utilized by other halophytes to tolerate high salinity.

In this study, we report that salt cress is able to tolerate high salinity levels for short periods of time; however, when this plant is stressed for longer durations at high salt concentrations in soil medium, growth is inhibited and plants show signs of death. In aerial tissue, salt cress accumulates Na<sup>+</sup> primarily in older leaves, while in the roots accumulation is limited to a large, defined taproot. Activities and/or protein expression of transporters shown to be important

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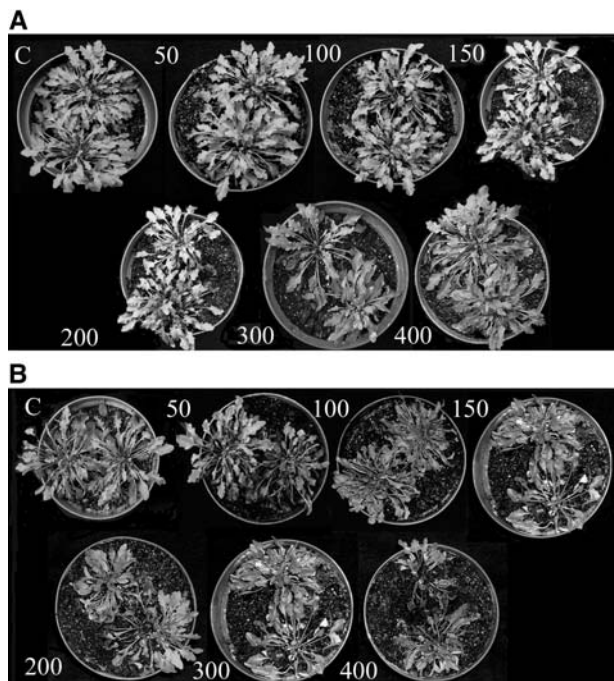
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in  $\text{Na}^+$  transport were also investigated, including vacuolar  $\text{H}^+$ -translocating ATPase (V-ATPase) and vacuolar  $\text{H}^+$ -translocating inorganic pyrophosphatases (V-PPase), and plasma membrane (PM)  $\text{H}^+$ -translocating ATPase (P-ATPase) and the  $\text{Na}^+$  transporter, HKT1, as well as tonoplast (TP) and PM  $\text{Na}^+/\text{H}^+$  exchangers.

## RESULTS

### Effect of NaCl on Salt Cress Survival, Ion Content, and Osmolarity

It has been shown that, with preconditioning (slow stepwise increases in salt concentration), artificial maintenance of a constant water content, or short treatment periods, salt cress plants tolerate higher levels of salinity when compared to *Arabidopsis* (Volkov et al., 2003; Inan et al., 2004; Amtmann et al., 2005; Vinocur and Altman, 2005). In this study, we have tested the ability of salt cress to tolerate immediate and long-term exposure to high levels of salinity in the absence of preconditioning or manipulation of water content, to reproduce the stress conditions that are tolerated by true halophytes, including the ice plant, which has long been used as a model halophyte for plant salinity tolerance (Adams et al., 1998; Barkla et al., 1999). Soil-grown salt cress plants treated with increasing concentrations of NaCl (50–400 mM) were able to grow without any visual signs of stress for the first week (Fig. 1A); however, after 2 weeks of treatment, leaf chlorosis was observed in plants treated with concentrations of NaCl above 200 mM (Fig. 1B), and a loss of



**Figure 1.** Effect of NaCl on growth of salt cress plants. Four-week-old plants were treated with NaCl (0–400 mM) for 1 (A) or 2 (B) weeks.

**Table I.** Chlorophyll content in salt cress leaf tissue

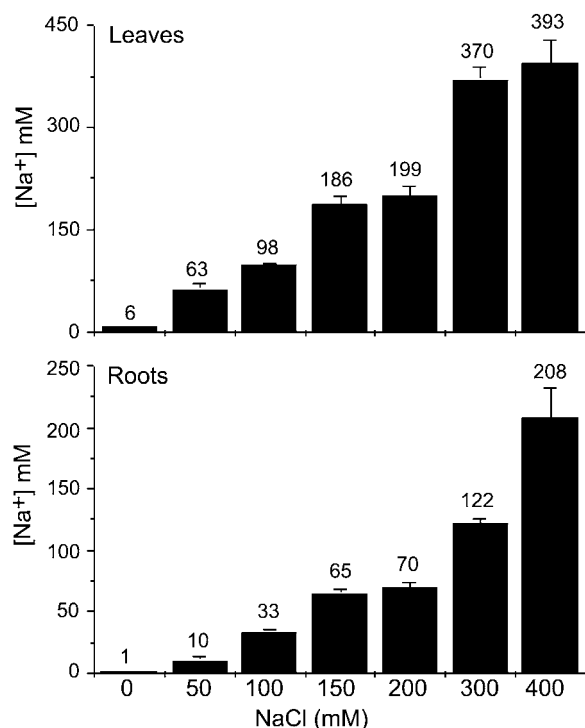
Chlorophyll was measured as described in “Materials and Methods” from salt cress control plants and from plants treated with 200 and 400 mM NaCl for 2 weeks. Units for chlorophyll concentration are  $\mu\text{mol}$  chlorophyll  $\text{mg}^{-1}$  fresh weight. Data represent means  $\pm$  SE of four replicate experiments. Each replicate experiment was performed using independent plants.

Treatment	Total Chlorophyll	Chlorophyll a	Chlorophyll b
Control	31.1 $\pm$ 0.7	21.8 $\pm$ 4.9	2.6 $\pm$ 1.2
200 mM NaCl	18.5 $\pm$ 1.5	17.1 $\pm$ 1.1	2.9 $\pm$ 2.2
400 mM NaCl	8.6 $\pm$ 4.9	5.8 $\pm$ 1.6	0.15 $\pm$ 0.05

viability (40% survival) was observed after 2 weeks of high salt treatment (data not shown). We observed a 41% and 73% decrease in total chlorophyll amounts in plants treated with 200 or 400 mM NaCl for 2 weeks, respectively (Table I).

Measurements of the concentration of  $\text{Na}^+$  in the cell sap of leaves and roots grown for 1 week with increasing concentrations of NaCl reflected an accumulation of  $\text{Na}^+$  from 62.8 to 393.4 mM in leaves and from 10.2 to 208.1 mM in roots for plants grown in the presence of 50 to 400 mM NaCl, respectively (Fig. 2). In leaves, a gradual increase in cell sap  $\text{Na}^+$  was observed as the NaCl concentrations were increased (Fig. 2); however, the  $\text{Na}^+$  concentrations did not vary significantly between the plants treated with 50 or 100 mM, 150 or 200 mM, and 300 or 400 mM NaCl (Fig. 2). In roots, increases in cell sap  $\text{Na}^+$  concentrations as a function of external NaCl were also observed, with a large increment detected between 300 and 400 mM NaCl (Fig. 2). To obtain more specific information on the site for  $\text{Na}^+$  accumulation in salt-treated salt cress, we separated the plant tissues as young and old leaves and taproot and lateral roots (Fig. 3), and measured the concentration of  $\text{Na}^+$  present in the cell sap of each tissue (Table II). After 1 week of salt treatment, there were large increases in  $\text{Na}^+$  accumulation in all tissues, with the highest concentrations detected in cell sap from old leaves (120 mM  $\text{Na}^+$ ), and the lowest levels measured in the taproot (24 mM  $\text{Na}^+$ ). Following 2 weeks of treatment, it was clear that the major site for  $\text{Na}^+$  accumulation was in old leaves (300 mM  $\text{Na}^+$ ), followed by young leaves (200 mM  $\text{Na}^+$ ), and then taproot (160 mM  $\text{Na}^+$ ). The least accumulation occurred in the lateral roots (60 mM  $\text{Na}^+$ ; Table II). Concentrations of  $\text{K}^+$  did not change significantly with respect to the values for the control plants (e.g. 41  $\pm$  4.3 and 36  $\pm$  3.8 mM, control and 200 mM NaCl-treated leaves, respectively; data not shown).

To determine the effects of NaCl treatment on leaf water potential, the osmolarity of the cell sap was measured in leaves from plants treated with different concentrations of NaCl. While cell sap osmolarity was greater in salt-treated plants compared to control plants, it did not increase with increasing concentrations of NaCl (data not shown), only with duration of treatment. A 1.4- and 3.2-fold increase in osmolarity



**Figure 2.** Changes in cell sap Na<sup>+</sup> concentrations in salt cress following 2 weeks of treatment with NaCl. Measurements of Na<sup>+</sup> in cell sap extracted from leaves or roots as described in "Materials and Methods." Values are means  $\pm$  SE of three independent experiments.

was observed in young leaves from salt cress plants treated for 1 and 2 weeks, respectively, while old leaves showed increases of 2.2- and 4.3-fold, respectively, for the same time periods (Table III). The osmolarity in the roots also increased, with taproots showing a 4.2-fold increase following 2 weeks of NaCl treatment and lateral roots showing a 2.0-fold increase for the same time period (Table III). In control plants, there was little change in cell sap osmolarity over the 2-week experimental period; the largest increase was measured in the taproot, which presented a 2.4-fold increase from week 2 compared to week 1 (Table III).

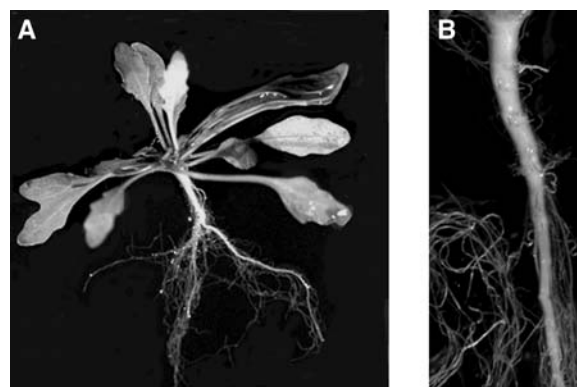
### Characterization of the Purity of TP and PM Fractions

To purify TP and PM fractions from salt cress, microsomal membranes were separated on continuous Suc gradients, and fractions were analyzed for ATPase hydrolytic activity at either pH 6.5 or 8.0, corresponding to the pH optima of the PM P-ATPase and TP V-ATPases, respectively. Assays were performed in the presence of inhibitors that differentiate specific types of H<sup>+</sup>-translocating ATPases (Sze, 1985). Peak P-ATPase activity, measured as vanadate-sensitive, azide-resistant, and nitrate/bafilomycin-resistant activity, was observed in fractions 39 to 61, corresponding to 34% to 38% Suc for both the control and salt-treated plants (data not shown). In these fractions, ATPase hydrolytic activity at pH 6.5 was inhibited 89% by 100  $\mu$ M vanadate, while

the nitrate/bafilomycin and azide sensitivity of this fraction was only 20% of the total activity (data not shown). TP V-ATPase activity, measured as bafilomycin-sensitive, azide-insensitive, and vanadate-insensitive activity, was highest in fractions 13 to 25, corresponding to 20% to 24% Suc for both the control and NaCl-treated plants (data not shown). Activity of the H<sup>+</sup>-ATPase at pH 8.0 in these fractions was inhibited 90% by 200 nM bafilomycin, while the vanadate- and azide-sensitive activity of this fraction was only 10% of the total activity (data not shown). The similar values of enzyme inhibition obtained from the fractions isolated from control and salt-treated plants indicated that salt treatment had no effect on the density of the membranes. Further evidence for the purity of the membrane fractions was obtained from western-blot analysis of membrane proteins. No cross-reactivity with antibodies against TP marker proteins was observed in the PM fraction, and, conversely, antibodies against PM marker proteins did not cross-react with the TP fractions (Fig. 4). Based on these results, PM and TP fractions were collected at the 34% to 40% Suc interface and the 0% to 22% Suc interface, respectively, on discontinuous Suc gradients to ensure purity. To obtain sealed vesicles used for H<sup>+</sup> transport assays, it was also necessary to isolate PM using the method of two-phase partitioning of microsomal membranes (Qiu et al., 2003) because PM vesicles isolated by Suc density centrifugation, while of sufficient purity, were not sealed and could therefore only be used for hydrolytic assays and for western-blot analysis.

### Effect of NaCl Treatment on TP V-ATPase and V-PPase Activity and PM P-ATPase Activity in Salt Cress Leaves and Roots

Quinacrine fluorescence quenching and recovery was used to monitor the rate of formation and dissipation of transmembrane pH gradients (inside acid) generated by activation of the PM P-ATPase and TP V-ATPase or V-PPase in sealed and purified PM and TP vesicles. Total fluorescence quenching for all treatments



**Figure 3.** Salt cress whole plant structure. A, Eight-week-old salt cress plant grown in hydroponics for 5 weeks. B, Close-up of the taproot.

**Table II.** Sodium accumulation in salt cress

Cell sap was extracted from the different tissues of control and 200 mM NaCl-treated plants and the Na<sup>+</sup> content (mM) was measured as described in "Materials and Methods." Data represent the means ± SE of four experiments. Each replicate experiment was performed using independent plants.

Treatment	Young Leaves		Old Leaves		Taproot		Lateral Roots	
	Week 1	Week 2	Week 1	Week 2	Week 1	Week 2	Week 1	Week 2
Control	2.7 ± 0.5	3.5 ± 0.1	3.8 ± 0.7	4.8 ± 0.7	2.2 ± 0.8	4.8 ± 0.9	0.04 ± 0.01	2.3 ± 0.7
Salt	50 ± 1.7	200 ± 23	120 ± 11	300 ± 32	25 ± 0.9	160 ± 1.2	26 ± 0.4	60 ± 1.2

was approximately 70% of the initial fluorescence level (data not shown), indicating that the final, steady-state pH gradients generated by the pumps were not affected by treatment conditions. The initial rates of H<sup>+</sup> transport were calculated from the rates of quinacrine fluorescence quenching taken during the first 40 s following the addition of either ATP (for activation of the V-ATPase or P-ATPase) or inorganic pyrophosphate (for measurements of PPase H<sup>+</sup> transport activity). Hydrolytic activities of the H<sup>+</sup> pumps were also determined to compare with H<sup>+</sup> transport activities according to the method of Ames (1966).

Salt treatment increased both the hydrolytic and H<sup>+</sup> transport activity of salt cress TP V-ATPase from leaves (Tables IV and V). H<sup>+</sup> transport activity of the V-ATPase increased 1.5- and 2.5-fold, while hydrolytic activity showed an increase of 1.5- and 1.9-fold in plants treated with 200 and 400 mM NaCl for 2 weeks, respectively. H<sup>+</sup> transport of salt cress TP V-ATPase from roots increased by 1.2-fold, while the hydrolytic activity showed an increase of 1.5-fold in plants treated with 200 mM NaCl (Tables IV and V). V-PPase hydrolytic activity increased 1.3-fold in leaves of plants treated with 200 mM NaCl, and no further increase was observed when plants were grown in 400 mM NaCl (Table IV). Salt treatment resulted in only a slightly higher V-PPase hydrolytic activity (1.1-fold) in roots of 200 mM grown plants compared to plants grown under control conditions (Table IV). H<sup>+</sup> transport of salt cress TP V-PPase from leaves and roots did not increase upon salt treatment (data not shown).

Vanadate-sensitive P-ATPase hydrolytic activity was higher in PM vesicles isolated from plants treated with NaCl as compared to those isolated from untreated control plants (Table IV). In leaves, treatment with 200 mM NaCl produced a 1.3-fold increase in activity, while plants treated with 400 mM NaCl showed a 1.7-fold stimulation over values for control plants (Table IV). Similar increases in P-ATPase H<sup>+</sup>

transport were also observed: a 1.4-fold increase for 200 mM NaCl and a 1.6-fold increase for 400 mM NaCl (Table VI). In roots, treatment with 200 mM NaCl produced a 1.3-fold increase in hydrolytic activity over control values (Table IV), and similar increases in P-ATPase H<sup>+</sup> transport were observed (Table VI).

To determine whether Na<sup>+</sup> was directly regulating the activity of the proton pumps (P-ATPase, V-ATPase, and V-PPase), hydrolytic activity was also measured in the presence of Na<sup>+</sup> (200 and 400 mM NaCl). Under these conditions, Na<sup>+</sup> showed no direct effect on the hydrolysis of ATP by these enzymes (data not shown).

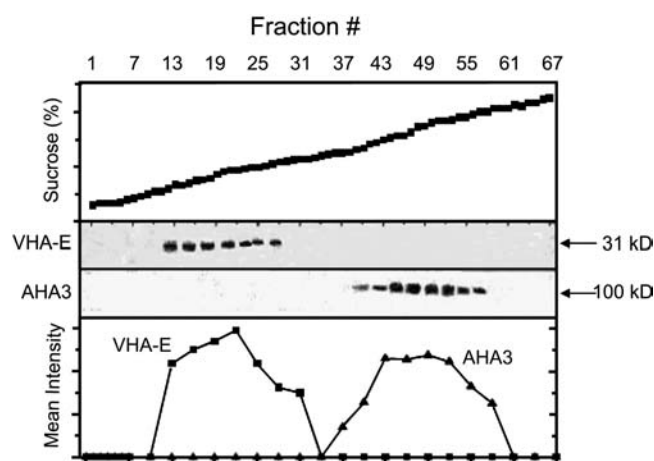
#### TP and PM Na<sup>+</sup>/H<sup>+</sup> Exchange in Salt Cress Leaf and Root Tissue

The ability of Na<sup>+</sup> to dissipate a preformed transmembrane pH gradient was tested in TP or PM vesicles isolated from leaves of salt cress plants. Following the generation of a preset, inside acid pH gradient, by activation and subsequent inhibition of the V-ATPase or P-ATPase (Barkla et al., 1999), Na<sup>+</sup> was added to the reaction media and the initial rate of quinacrine fluorescence recovery was measured over the first 100 s. The initial rate of Na<sup>+</sup>/H<sup>+</sup> exchange in TP vesicles from leaves and roots of control-treated plants was very low; however, Na<sup>+</sup>/H<sup>+</sup> exchange was greatly stimulated by growth of the plants in NaCl. In leaves, treatment with 200 or 400 mM NaCl resulted in a 13- and 19-fold increase in activity, respectively (Table VII). In roots, the induction by NaCl was even greater, with a 60-fold increase in Na<sup>+</sup>/H<sup>+</sup> exchange activity following treatment with 200 mM NaCl (Table VII). The Na<sup>+</sup>/H<sup>+</sup> exchange activity of the PM vesicles from both roots and leaves was also investigated; however, no Na<sup>+</sup>-dependent pH recovery of a preformed pH gradient was detected for any of the growth treatments for all Na<sup>+</sup> (or K<sup>+</sup>) concentrations tested (10 mM to 500 mM; data not shown).

**Table III.** Osmolarity measurements in salt cress

Cell sap was extracted from the different tissues of control and 200 mM NaCl-treated plants and the osmolarity (mOsmol/kg) was measured as described in "Materials and Methods." Data represent the means ± SE of four experiments. Each replicate experiment was performed using independent plants.

Treatment	Young Leaves		Old Leaves		Taproot		Lateral Roots	
	Week 1	Week 2	Week 1	Week 2	Week 1	Week 2	Week 1	Week 2
Control	118 ± 3	122 ± 3	98 ± 4	110 ± 5	120 ± 10	156 ± 11	98 ± 12	96 ± 10
Salt	162 ± 6	385 ± 5	218 ± 6	474 ± 9	269 ± 12	657 ± 15	114 ± 10	196 ± 3



**Figure 4.** Purity of membrane fractions separated by Suc density gradients from leaf tissue of salt cress plants. Western-blot analysis was used to confirm the purity of the TP and PM fractions by immunodetection using antibodies directed against a PM P-ATPase (AHA3; 100 kD), and the subunit E of the TP V-ATPase (VHA-E; 31 kD). Immunodetection was carried out as described in "Materials and Methods." Individual blots (eight lanes/blot) were digitally photographed (Kodak DC-120; Eastman-Kodak) and then images were aligned and joined using the imaging software Photo Impact SE 3.01 (Ulead Systems) in order to enable visualization of all representative fractions. Top, Suc concentrations in collected fractions. Middle, Immunological detection in the respective fractions of AHA3 and VHA-E. Bottom, Mean intensity of the protein bands detected, V-ATPase (■) and P-ATPase (▲) in microsomal fractions. Blots are representative of at least four independent experiments.

#### Western-Blot Analysis of TP and PM Transporters

The observed increase in activity of the proton pumps at the PM and TP suggested induction of these enzymes at the protein level. Expression of one PM  $H^+$ -ATPase isoform, AHA3, was examined in PM isolated from control and salt-treated plants. In leaves and roots, western-blot analysis using an Arabidopsis anti-AHA3 antibody directed against the C terminus of the 100-kD protein (Parets-Soler et al., 1990) cross-reacted with a polypeptide of expected molecular mass in the PM fractions from all the treatments (Fig. 5, A and B). However, no consistent changes in the intensity of band recognition could be observed between the control membranes and those isolated from

the salt-treated plants, indicating similar amounts of this P-ATPase isoform were present under all conditions.

Possible changes in the levels of expression of the salt cress V-ATPase holoenzyme were studied using polyclonal antibodies directed against the subunits VHA-A and VHA-B from mung bean (*Vigna radiata*; Matsuura-Endo et al., 1992) and a *Hordeum vulgare* VHA-E subunit antibody (VHA-E; Dietz and Arbing, 1996). Western-blot analysis indicated that VHA-A and -B subunits showed no major changes in protein expression with salt treatment in both leaves and roots (Fig. 5A; data not shown). In contrast, VHA-E showed an increased expression when the plants were treated with either 200 or 400 mM NaCl in leaves, but not in roots (Fig. 5, A and B).

Changes in the levels of expression of the salt cress V-PPase enzyme were studied using polyclonal antibodies (PBA-HK) directed against a peptide sequence located in a cytoplasmic loop between transmembrane domains 12 and 13 of the AVP1 protein (Sarafian et al., 1992; Kim et al., 1994). Increased protein expression was detected in TP from leaves when plants were treated with either 200 or 400 mM NaCl (Fig. 5A); however, no major changes in V-PPase protein expression were observed in salt-treated root tissue (Fig. 5B).

The observed increase in TP  $Na^+/H^+$  exchange activity in membrane vesicles isolated from plants treated with 200 and 400 mM NaCl suggested an increased expression of one of the NHX family members that are localized to the TP (Apse et al., 1999; Gaxiola et al., 1999; Zhang and Blumwald, 2001; Zhang et al., 2001; Ohta et al., 2002; Fukuda et al., 2004). To test this, we used an antibody raised against the C-terminal deduced 122 amino acids of *AtNHX1* (Fig. 6; Qiu et al., 2004). Sequence analysis indicates that this antibody would possibly recognize *AtNHX1*, *AtNHX2*, and *AtNHX3* (data not shown) based on their high degree of homology within this region. No detection of a salt cress protein was observed in either the TP or other membrane fractions of leaf tissue (Fig. 6A; data not shown), indicating that the protein responsible for the NaCl-inducible  $Na^+/H^+$  exchange activity at the TP in salt cress leaves was not closely related to the Arabidopsis *AtNHX1* protein. In roots, a salt-induced increase in expression of an NHX protein was observed

**Table IV.** Effect of NaCl on vanadate-sensitive, nitrate-resistant, and azide-resistant ATP hydrolysis in PM (P-ATPase), and nitrate-sensitive, azide-resistant, and vanadate-resistant ATP hydrolysis (V-ATPase), and V-PPase inorganic pyrophosphate hydrolysis in TP of salt cress leaf and root tissue

Membrane vesicles were isolated by discontinuous Suc gradients from leaves or roots of salt cress treated for 2 weeks with either 200 or 400 mM NaCl. ATPase activity was assayed as described in "Materials and Methods." Units for ATP or inorganic pyrophosphate hydrolysis are  $\mu\text{mol inorganic phosphate mg}^{-1} \text{ protein h}^{-1}$ . Data represent means  $\pm$  SE of four replicate experiments. Each replicate experiment was performed using independent membrane preparations. ND, No data available.

Treatment	P-ATPase		V-ATPase		V-PPase	
	Leaves	Roots	Leaves	Roots	Leaves	Roots
Control	10.4 $\pm$ 0.5	11.2 $\pm$ 1.2	13.8 $\pm$ 0.5	10.1 $\pm$ 0.6	12.1 $\pm$ 0.3	13.9 $\pm$ 1.8
200 mM NaCl	13.7 $\pm$ 0.3	14.5 $\pm$ 0.1	20.5 $\pm$ 0.8	14.7 $\pm$ 0.4	15.9 $\pm$ 0.5	15.3 $\pm$ 1.0
400 mM NaCl	18.1 $\pm$ 1.7	ND	26.8 $\pm$ 1.2	ND	16.7 $\pm$ 0.5	ND

**Table V.** Effect of NaCl on the V-ATPase H<sup>+</sup> transport activity in TP vesicles from salt cress leaf and root tissue

Membrane vesicles were isolated by discontinuous Suc density gradients from leaves of salt cress treated for 2 weeks with either 200 or 400 mM NaCl. H<sup>+</sup> transport activity was assayed as described in "Materials and Methods." Units for initial rates of ATP-dependent H<sup>+</sup> transport are % *F* mg<sup>-1</sup> protein min<sup>-1</sup>, where *F* is relative fluorescence intensity. Data represent means ± SE of four replicate experiments. Each replicate experiment was performed using independent membrane preparations. ND, No data available.

Treatment	V-ATPase H <sup>+</sup> Transport Activity	
	Leaves	Roots
Control	256 ± 8.1	381 ± 38
200 mM NaCl	387 ± 6.2	469 ± 21
400 mM NaCl	627 ± 28	ND

in the PM fraction (Fig. 6B), but no protein was detected in the TP fraction (data not shown).

The Na<sup>+</sup> transporter *AtHKT1* and the Na<sup>+</sup>/H<sup>+</sup> exchanger *AtSOS1* are PM Na<sup>+</sup> transporters also known to be regulated by cellular Na<sup>+</sup> homeostasis in *Arabidopsis* (Rus et al., 2001; Shi et al., 2003). The expression of these transporters was therefore investigated under conditions of salt stress in salt cress. Using an ice plant anti-HKT1 antibody directed against the C terminus of the 56-kD polypeptide (Su et al., 2003), a protein of the expected molecular mass was identified in PM fractions from leaves and roots (Fig. 6, A and B); however, no changes in the intensity of band recognition could be observed between the control membrane fractions and those isolated from either 200 or 400 mM NaCl-treated plants (Fig. 6, A and B). Antibodies raised against the C terminus of the 130-kD *AtSOS1* protein (Qiu et al., 2003) cross-reacted with a polypeptide of the expected molecular mass in PM fractions from all treatments, although no measurable Na<sup>+</sup>/H<sup>+</sup> exchange was detected in PM vesicles isolated from control or salt-treated plants. Furthermore, an increase in protein expression of *AtSOS1* was detected in plants treated with either 200 or 400 mM NaCl from leaves and roots (Fig. 6, A and B).

## DISCUSSION

With the recent interest in adopting the extremophile salt cress as an *Arabidopsis* relative model system to investigate salinity tolerance (Amtmann et al., 2005), it is important to study the physiological mechanisms responsible for this plant's adaptation. The ability to effectively manage and control tissue and cellular Na<sup>+</sup> levels through the activity and expression of transporters involved in the movement of Na<sup>+</sup> is essential for plant salinity tolerance. Our aim was to investigate the distribution of Na<sup>+</sup> within salt cress and begin to define the role of Na<sup>+</sup> transporters by studying their activity and expression under salt stress conditions.

To reproduce the stress conditions that are reported to be tolerated by true halophytes, our studies were

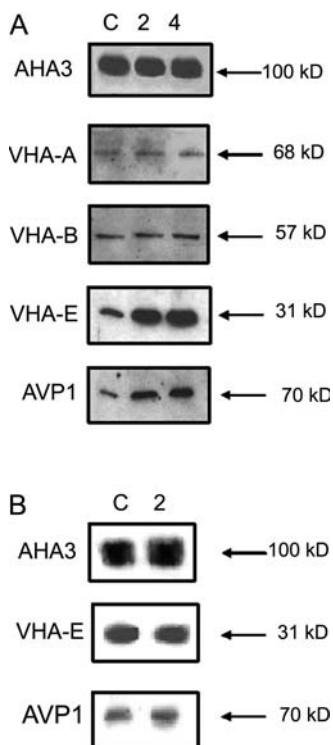
carried out under conditions of immediate high salinity for long periods, in the absence of preconditioning, and plants were grown in soil medium. Long-term exposure to 200 or 400 mM NaCl resulted in diminished plant growth and a decrease in survival, measured as a significant reduction in chlorophyll content in salt cress leaf tissue (Fig. 1; Table I). Taji et al. (2004) have reported that salt cress treated with 500 mM NaCl continues to grow for a period of 3 weeks with no deleterious effects; however, plants were grown in a perlite-vermiculite mix, which may substantially reduce the exposure to salt due to high drainage of the medium. This is supported by the ability of *Arabidopsis* to survive the same conditions, albeit with notable, but not complete, chlorosis for a similar 3-week period (Taji et al., 2004). More similar to our findings are the results shown in the same paper in which plants were grown in hydroponics in the presence of 250 mM NaCl. Under these conditions, only 50% of the salt cress plants survived the 96-h treatment (Taji et al., 2004). Other studies have also reported the ability of salt cress to survive long-term high salinity stress; however, plants are grown in turf (high drainage capacity) and subjected to gradual increases in Na<sup>+</sup> to precondition the plants over a period of 26 d, and these plants are then able to survive for several months (Inan et al., 2004). It appears that choice of growth medium and preconditioning play important roles in the ability of salt cress to survive high salinity.

Following long-term exposure, the major site for accumulation of Na<sup>+</sup> in salt cress occurred in old leaves, with the least accumulation of Na<sup>+</sup> in the lateral roots (Table II). The use of older leaves as sinks for Na<sup>+</sup> ions, restricting ion deposition in meristematic tissues and actively growing and photosynthesizing cells, is common behavior observed in glycophytes (Munns, 2002). The large, well-defined taproot appears to be the main site for Na<sup>+</sup> accumulation within the salt cress root system. Following 2 weeks of exposure to NaCl, a 3-fold higher level of Na<sup>+</sup> was detected in the taproot as compared to the lateral roots (Table II). This root morphology distinguishes salt cress from *Arabidopsis*

**Table VI.** Effect of NaCl on the PM P-ATPase H<sup>+</sup> transport activity of salt cress leaf and root tissue

Membrane vesicles were isolated by two-phase partitioning of microsomal membranes from leaves and roots of salt cress treated for 2 weeks with either 200 or 400 mM NaCl. H<sup>+</sup> transport activity was assayed as described in "Materials and Methods." Units for initial rates of ATP-dependent H<sup>+</sup> transport are %*F* mg<sup>-1</sup> protein min<sup>-1</sup>, where *F* is relative fluorescence intensity. Data represent means ± SE of four replicate experiments. Each replicate experiment was performed using independent membrane preparations. ND, No data available.

Treatment	P-ATPase H <sup>+</sup> Transport Activity	
	Leaves	Roots
Control	238 ± 13	285 ± 25
200 mM NaCl	341 ± 3.4	376 ± 9.4
400 mM NaCl	389 ± 4.8	ND



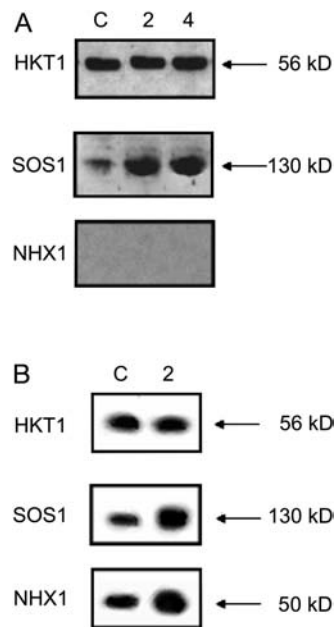
**Figure 5.** Expression of P-ATPase, V-ATPase, and V-PPase in salt cress under salt treatment. Plants were incubated for 2 weeks in the absence (C) or presence of 200 mM (2) or 400 mM (4) NaCl before isolation of TP and PM from leaves (A) or roots (B) on discontinuous Suc density gradients at the 0% to 22% (w/v) and the 34% to 38% (w/v) Suc interface, respectively. Western-blot analysis was carried out as described in "Materials and Methods" using antibodies directed against P-ATPase (AHA3), V-ATPase subunits (VHA-A, -B, and -E), and V-PPase (AVP1). Blots are representative of five independent experiments.

because the primary root of *Arabidopsis* is much smaller and less defined. Most plants with taproots have roots distributed deep within the soil, which primarily serve for increasing water availability and, in the case of salt cress, as a potential site for  $\text{Na}^+$  sequestration. The total  $\text{Na}^+$  accumulated in salt cress leaf cell sap in this study (300 mM) is much higher than that reported by Inan et al. (2004), who showed an accumulation of approximately 30 mM  $\text{Na}^+$  over a 28-d period with preconditioning. However, as stated above, these authors grow the plants in surface, which may lower the  $\text{Na}^+$  concentration that the plants are actually exposed to due to the high drainage capacity of this medium, thus reducing the accumulation of  $\text{Na}^+$  and increasing the viability of the plants.

Measurements of  $\text{K}^+$  in the different tissues showed that, over a wide concentration range of  $\text{Na}^+$ , salt cress plants were able to maintain stable concentrations of  $\text{K}^+$ . Similar findings were reported by Volkov et al. (2003) in salt cress plants treated for shorter periods with lower  $\text{Na}^+$  concentrations. This suggests that  $\text{K}^+$  homeostasis is unaffected; however,  $\text{K}^+$  to  $\text{Na}^+$  ratios, a measure of the plant's ability to discriminate between the two ions, reflecting its salinity tolerance (Flowers, 2004), will decrease as  $\text{Na}^+$  accumulates.

Parallel to the increased  $\text{Na}^+$  content in salt-treated salt cress plants, cell sap osmolarity was observed to increase as a factor of time and  $\text{Na}^+$  concentration (Table III). Presumably,  $\text{Na}^+$  ions are sequestered into the vacuole and cytoplasmic osmotic potential is maintained by the synthesis of compatible solutes. In salt cress, the synthesis and accumulation of Pro in the cytoplasm of leaf tissue has been reported upon salt stress (Inan et al., 2004); however, there are no reports on compatible solutes present in root tissue.

The transporters involved in increasing  $\text{Na}^+$  efflux across the PM and via  $\text{Na}^+$  compartmentalization across the TP into the vacuole are widely considered important determinants of salt tolerance (Zhu, 2001); however, little is known on their expression or activity in salt cress. Negligible  $\text{Na}^+/\text{H}^+$  exchange activity was measured in the control plants grown in the absence of NaCl. Similarly, low exchange rates were observed in vacuoles isolated from leaves of control *Arabidopsis* plants (Apse et al., 1999), although *Arabidopsis* cell suspension cultures showed relatively high constitutive  $\text{Na}^+/\text{H}^+$  exchange activity when grown in the absence of NaCl (Qiu et al., 2004). Leaf and root TP  $\text{Na}^+/\text{H}^+$  exchange was highly induced by growth of the plants in NaCl (Table VII). This inducible activity could not be attributed to a salt cress homolog of *AtNHX1* because an anti-*AtNHX1* antibody (Qiu et al., 2004) failed to recognize a protein in the TP of either



**Figure 6.** Expression of HKT1, SOS1, and NHX1 in salt cress under salt treatment. Plants were incubated for 2 weeks in the absence (C) or presence of 200 (2) or 400 mM NaCl (4) before isolation of TP and PM fractions from leaves (A) and roots (B) on a discontinuous Suc density gradient at the 0% to 22% and the 34% to 38% Suc interface, respectively. Western-blot analysis was carried out as described in "Materials and Methods" using antibodies directed against the  $\text{Na}^+$  transporter HKT1, the PM  $\text{Na}^+/\text{H}^+$  exchanger SOS1, and the TP  $\text{Na}^+/\text{H}^+$  exchanger NHX1. Blots are representative of five independent experiments.

**Table VII.** Effect of NaCl on the Na<sup>+</sup>/H<sup>+</sup> exchange activity in TP vesicles isolated from salt cress leaf and root tissue

Membrane vesicles were isolated by discontinuous Suc density gradient methods from leaves and roots of salt cress treated for 2 weeks with either 200 or 400 mM NaCl. Na<sup>+</sup>/H<sup>+</sup> exchange activity was assayed as described in "Materials and Methods." Units for initial rates of Na<sup>+</sup>-dependent H<sup>+</sup> transport are %F mg<sup>-1</sup> protein min<sup>-1</sup>, where F is relative fluorescence intensity. Data represent means ± SE of four replicate experiments. Each replicate experiment was performed using independent membrane preparations. ND, No data available.

Treatment	Na <sup>+</sup> /H <sup>+</sup> Exchange Activity	
	Leaves	Roots
Control	9.4 ± 0.7	2.3 ± 0.1
200 mM NaCl	120 ± 2.4	138 ± 14
400 mM NaCl	178 ± 5.5	ND

control or salt-treated salt cress root or leaf tissue despite the high sequence similarity between the Arabidopsis and salt cress *NHX1* genes (Volkov et al., 2003). The AtNHX1 antibody did recognize a 50-kD protein in root tissue that was salt inducible (Fig. 6B); however, this protein was present only in the PM and not in the TP, suggesting that the antibody is detecting a closely related Na<sup>+</sup>/H<sup>+</sup> exchanger isoform that is localized to the PM, but distinct from SOS1, which has a molecular mass of 130 kD and is very divergent from NHX1 in the C-terminal region (Qiu et al., 2003; Fig. 6, A and B). Similar findings were also observed in the ice plant, where the AtNHX1 antibody only recognized a salt-inducible protein in the root PM fraction (B.J. Barkla, R. Vera-Estrella, and O. Pantoja, unpublished data). The SOS1 protein was present in untreated tissue and protein levels were up-regulated by salinity in the PM of root and leaf salt cress tissue (Fig. 6, A and B). Previously, SOS1 RNA was shown to be constitutively expressed in salt cress plants; however, no induction of transcript was observed with a 2-h short-term exposure to salinity (Taji et al., 2004), suggesting that longer treatment periods are required to regulate SOS1.

However, despite the presence of two Na<sup>+</sup>/H<sup>+</sup> exchanger isoforms in the PM of root tissue, and one in leaf tissue, no PM Na<sup>+</sup>/H<sup>+</sup> exchange was detected. This indicates that the PM Na<sup>+</sup>/H<sup>+</sup> exchange activity may require activation by regulatory molecules that are not present in our in vitro transport assays with isolated membrane vesicles, suggesting a role for a similar pathway to the Arabidopsis SOS signaling pathway in salt cress (for review, see Zhu, 2000).

Na<sup>+</sup>/H<sup>+</sup> exchange is driven by the primary H<sup>+</sup> pumps at the PM and TP, including TP V-ATPase, V-PPase, and the PM P-ATPase. It is therefore not surprising that increases in both the hydrolytic and/or H<sup>+</sup> transport activity of these transporters were observed in salt-treated salt cress plants (Tables IV–VI). Similar salt-induced increases have been observed in a number of plant species, including halophytes, as well as glycophytes (Barkla and Pantoja, 1996). Changes in pump activity were in some cases mirrored by changes in levels of expression of the protein(s).

V-PPase protein levels increased in TP from leaves and roots, reflecting the moderate 1.3- and 1.1-fold increases in hydrolytic activity, respectively (Fig. 5, A and B). The V-PPase is presumed to play a minor role in energizing the TP under the increasing demands imposed by high levels of Na<sup>+</sup>/H<sup>+</sup> exchange as a factor of salinity. In the ice plant and *Suaeda salsa* halophytes, V-PPase expression and/or activity were unaffected by growth of the plants in NaCl (Wang et al., 2001; Barkla et al., 2002). Protein-blot analysis of TP proteins using antibodies directed against three subunits of the multimeric V-ATPase (VHA-A, VHA-B, and VHA-E; Fig. 5) indicated that, in leaf tissue, solely subunit VHA-E showed increased expression upon salt stress, while none of these subunits were regulated at the protein level in roots (Fig. 5; VHA-E). In the ice plant, transcript levels for subunit E were also seen to preferentially increase in leaves, but not in roots, when plants were salt stressed (Golldack and Dietz, 2001). Subunit E of the V-ATPase is located in the peripheral stalk connecting the V<sub>1</sub> and V<sub>0</sub> sectors; its function has not been well characterized in plants, but evidence from yeast (*Saccharomyces cerevisiae*) suggested this subunit is involved in the assembly of the V<sub>1</sub> and V<sub>0</sub> sectors of the V-ATPase (Owegi et al., 2005). This could imply that changes in the amount of VHA-E regulate the amount of assembled V-ATPase complexes. However, the role of other subunits in the salt-induced increase in V-ATPase hydrolytic and H<sup>+</sup> pumping activity cannot be ruled out, especially because in roots there is no salt-induced up-regulation of VHA-E (Fig. 6B). At the PM, it appears that the salt-induced increases in H<sup>+</sup>-ATPase hydrolytic and H<sup>+</sup> pumping activity cannot be attributed to the AHA3 isoform because levels of this protein in the PM from both leaf and root tissue are not affected by NaCl (Fig. 5, A and B). Isoforms of AHA are encoded by a large multigene family with members showing distinct expression profiles and biochemical characteristics (Palmgren, 2001). While there is evidence that AHA3 is induced by salinity in *Medicago citrina* (Sibole et al., 2005), it appears that the activity measured in this study is due to one or more of the other AHA family members that are expressed in the leaves and/or roots. Sequence alignment of the AHA3 isoform with other AHA members indicates that, in the region of the C terminus, there is high sequence diversity (Harper et al., 1990), suggesting that the antibody used in this study would not recognize other isoforms.

Studies have shown that AtHKT1, a Na<sup>+</sup> influx transporter from Arabidopsis (Uozumi et al., 2000), involved in Na<sup>+</sup> recirculation from shoots to roots via the phloem, is crucial for plant salt tolerance. Mutations in AtHKT1 resulted in overaccumulation of Na<sup>+</sup> in Arabidopsis shoot tissue (Berthomieu et al., 2003). In this study, a salt cress HKT homolog was detected in both leaves and roots; however, protein expression did not change upon salt treatment. Whether or not this transporter is important for salt cress salinity tolerance requires further investigation.

In general, there appeared to be little or no correlation between activity and expression (determined by use of homologous antibodies), of the transport proteins investigated in this study. This lack of induction of proteins recognized by the antibodies used in this study may suggest the presence of divergent salt cress proteins that are responsible for the transport activities measured. These results may help to explain previous work with microarrays, which appeared to show few changes in transcription of salt cress genes in response to salt stress (Inan et al., 2004; Taji et al., 2004), but alternatively may reflect that important salt-inducible genes in salt cress are novel or divergent and do not hybridize with *Arabidopsis* microarrays.

This work provides some detailed analyses of physiological mechanisms that underlie salinity tolerance in salt cress and provides important supporting information for the future molecular dissection of salt tolerance mechanisms in this *Arabidopsis* relative model system. Transport proteins involved in the sequestration of  $\text{Na}^+$  into the vacuole, or the removal of  $\text{Na}^+$  across the PM, including the TP V-ATPase, the  $\text{Na}^+/\text{H}^+$  exchanger, and the PM P-ATPase, appear to be key mechanisms for salinity tolerance in salt cress as they have been shown to be in other halophytes, including the ice plant, *S. bigelovii*, and *A. gmelini* (Ayala et al., 1995; Hamada et al., 2001; Barkla et al., 2002).

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Salt cress (*Thellungiella halophila*) plants were grown from seeds (derived from material originally collected from the Shandong Province in China and kindly supplied by Dr. Hans Bohnert and Dr. Ray Bressan) in soil (Metro mix 500; Scotts) in a propagation tray. Four weeks following germination, individual seedlings were transferred either to pots containing soil with two plants per 15-cm-diameter pot or, for experiments using root tissue, a single plant was transferred to 1-L opaque tubs containing 800 mL of one-half-strength Hoagland medium (Hoagland and Arnon, 1938). All plants were watered daily with tap water (unless under treatment), and one-half-strength Hoagland solution was applied weekly. Salt treatment (200 or 400 mM NaCl) was initiated 7 weeks after germination (approximately 4 weeks following transfer to soil or hydroponics conditions) by inclusion in the Hoagland medium or in the water. Plants were grown in a glasshouse under natural irradiation and photoperiod. Minimum temperatures ranged from 20°C to 24°C and maximum temperature was maintained at 25°C.

### Membrane Isolation and Purification

Membrane vesicles used for hydrolytic assays, TP  $\text{H}^+$  transport assays, and western-blot analysis were isolated by Suc density gradient centrifugation. Leaves and roots of salt cress plants were harvested and sliced into small pieces. Leaf and root material (30 g fresh weight) was placed directly into 300 mL of ice-cold homogenization medium (400 mM mannitol, 10% [w/v] glycerol, 5% [w/v] PVP-10, 0.5% [w/v] bovine serum albumin [BSA], 1 mM phenylmethylsulfonyl fluoride [PMSF], 30 mM Tris, 2 mM dithiothreitol [DTT], 5 mM EGTA, 5 mM  $\text{MgSO}_4$ , 0.5 mM butylated hydroxytoluene, 0.25 mM dibucaine, 1 mM benzamide, and 26 mM  $\text{K}^+$ -metabisulfite, adjusted to pH 8.0 with  $\text{H}_2\text{SO}_4$ ), and all subsequent operations were carried out at 4°C. Leaf and root tissue was homogenized in a commercial blender, filtered through four layers of cheesecloth, and centrifuged at 10,000g (20 min at 4°C) using a JA20 rotor (Beckman) in a superspeed centrifuge (model J2-HS; Beckman). Pellets were discarded and the supernatants were centrifuged at 80,000g (50 min at 4°C) using a fixed-angle rotor (model 40 Ti; Beckman) in an

ultracentrifuge (model L8-M; Beckman). The supernatant was aspirated and the microsomal pellet was resuspended in suspension medium (consisting of 400 mM mannitol, 10% [w/v] glycerol, 6 mM Tris/MES, pH 8.0, and 2 mM DTT). The microsomal suspension was then layered onto either continuous (5%–46% [w/v] Suc) or discontinuous Suc gradients (consisting of a top layer of 9 mL of 20% [w/v] Suc over 9 mL of 34% [w/v] Suc, on a cushion of 9 mL of 38% [w/v] Suc). Gradients were centrifuged at 100,000g (3 h at 4°C) using a SW 28 swinging-bucket rotor in a Beckman L8-M ultracentrifuge. On a discontinuous Suc gradient, TP from salt cress separates at the 0% to 20% Suc interface, while PM is collected from the 34% to 38% Suc interface. Bands from the discontinuous gradient were collected, diluted in suspension medium, and centrifuged at 100,000g in 60 Ti rotor (Beckman) in an ultracentrifuge (model L8-M; Beckman). Resuspended pellets or fractions (0.5 mL) from the continuous Suc gradient were collected, frozen in liquid  $\text{N}_2$ , and stored at  $-80^\circ\text{C}$ . The Suc concentration of fractions from continuous gradients was measured using a Zeiss refractometer (Zeiss).

PM vesicles used for transport assays were isolated using two-phase partitioning according to Qiu et al. (2002) with modifications. Leaf or root tissues (30 g) were placed in 300 mL of ice-cold homogenization medium consisting of 330 mM Suc, 10% (w/v) glycerol, 0.2% (w/v) BSA, 5 mM EDTA, 5 mM DTT, 5 mM ascorbic acid, 1 mM PMSF, 0.6% (w/v) PVP-10, 0.25 mM dibucaine, 1 mM benzamide, 0.5 mM butylated hydroxytoluene,  $\text{K}^+$ -metabisulfite, 1  $\mu\text{g}/\text{mL}$  leupeptin, and 50 mM HEPES-KOH (pH 7.5). The tissue was homogenized in a commercial blender filtered through four layers of cheesecloth, and centrifuged at 10,000g (20 min at 4°C) using a JA20 rotor (Beckman) in a superspeed centrifuge (model J2-HS; Beckman). Pellets were discarded and the supernatants were centrifuged at 80,000g (50 min at 4°C) using a fixed-angle rotor (model 40 Ti; Beckman) in an ultracentrifuge (model L8-M; Beckman). The supernatant was aspirated, and the microsomal pellet was resuspended in a buffer containing 330 mM Suc, 3 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PSMF 1  $\mu\text{g}/\text{mL}$  leupeptin, and 5 mM potassium phosphate (pH 7.8). The microsomal suspension was added to an aqueous phase system (36 g final weight) composed of 6.2% (w/w) dextran T-500, 6.2% (w/w) polyethylene glycol 3350, 5 mM potassium phosphate (pH 7.8), 330 mM Suc, and 3 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 1 mg/mL leupeptin. The phase system was then centrifuged at 1,000g for 10 min in an HB4 rotor (Beckman) in a superspeed centrifuge (model J2-HS; Beckman). The final upper phase was collected, diluted in suspension buffer (330 mM Suc, 10% [w/v] glycerol, 0.1% [w/v] BSA, 0.1 mM EDTA, 2 mM DTT) and centrifuged at 100,000g using a 60 Ti rotor (Beckman) in an ultracentrifuge (model L8-M; Beckman). Pellets were resuspended and frozen in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$ .

### Protein Determination

Protein content of purified TP and PM fractions 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 addition of the dye reagent concentrate (Bio-Rad). BSA was employed as the protein standard.

### $\text{H}^+$ Transport Assays and Hydrolytic Activity

Hydrolytic activities of the PM P-ATPase and TP V-ATPase and V-Pase were measured by the release of inorganic phosphate, according to the method of Ames (1966), as described previously (Vera-Estrella et al., 1994), using isolated membrane fractions. The values are presented as micromoles of inorganic phosphate released per milligram of membrane protein per hour.

The fluorescence quenching of quinacrine (6-chloro-9-[[4-(diethylamino)-1-methylbutyl] amino]-2-methoxyacridine dihydrochloride) was used to monitor the formation of inside acid pH gradients across TP and PM vesicles, as described previously (Barkla et al., 1995; Qiu et al., 2003), with modifications. Purified TP vesicles (30  $\mu\text{g}$  of protein for TP vesicles and 50  $\mu\text{g}$  for PM vesicles) were added to 500  $\mu\text{L}$  of buffer consisting of 250 mM mannitol, 10 mM Tris/MES (pH 8.0), 50 mM TMA-Cl, 6 mM  $\text{MgSO}_4$ , and 3 mM ATP for TP assays, and 250 mM mannitol, 10 mM Tris/MES (pH 6.5), 40 mM TMA-Cl, 10 mM KCl, 6 mM  $\text{MgSO}_4$ , and 3 mM ATP for PM assays. Fluorescence quenching was monitored in a thermostated cell at 25°C using a fluorescence spectrometer (model LS-50; Perkin-Elmer) at excitation and emission wavelengths of 427 and 495 nm, respectively, both with a slit width of 5 nm. For measurements of  $\text{Na}^+$ -dependent dissipation of a preformed, inside acid pH gradient, the ATP-dependent  $\text{H}^+$  transport activity was inhibited by the addition of 200 nM bafilomycin  $\text{A}_1$  (Bowman et al., 1988) in 0.001% (v/v) dimethyl sulfoxide, 250 mM mannitol, and 10 mM Tris/MES (pH 8.0). After a constant level of

fluorescence was obtained, aliquots of  $\text{Na}^+$  (as indicated) were added to the cells and the initial rate of  $\text{Na}^+$ -dependent fluorescence recovery was determined. As shown by Bennett and Spanswick (1983), the rate of fluorescence quench recovery is directly proportional to proton flux. Thus, the initial rate of fluorescence quenching or recovery represents initial rates of proton transport.

### SDS-PAGE and Protein Immunoblotting

Protein was precipitated by dilution of the samples 50-fold in ethanol: acetone (1:1 [v/v]) and incubated overnight at  $-30^\circ\text{C}$  according to the method of Parry et al. (1989). Samples were then centrifuged at 13,000g for 20 min at  $4^\circ\text{C}$  using an F2402 rotor in a GS-15R tabletop centrifuge (Beckman). Pellets were air dried, resuspended with Laemmli (1970) sample buffer (2.5% [w/v] SDS final concentration), and heated at  $60^\circ\text{C}$  for 2 min before loading onto 12.5% (w/v) linear acrylamide minigels. After electrophoresis, the gels were prepared for immunoblotting. SDS-PAGE-separated proteins were electrophoretically transferred onto nitrocellulose membranes (ECL; Amersham) as previously described (Vera-Estrella et al., 1999). Following transfer, membranes were blocked with Tris-buffered saline (100 mM Tris, 150 mM NaCl) containing 0.02% (w/v) Na-azide, and 5% (w/v) fat-free milk powder for 2 h at room temperature. Blocked membranes were incubated for a minimum of 3 h at room temperature with the appropriate primary antibodies, followed by the addition of a 1:5,000 dilution of secondary antibodies (goat anti-rabbit or -mouse) conjugated to horseradish peroxidase. Immunodetection was carried out using the chemiluminescent ECL western-blotting analysis system (Amersham). Mean intensity of the immunodetected protein bands was calculated using ECL  $M_1$  markers as loading control standards (Amersham). Images were captured using Kodak 1D image analysis software (Eastman-Kodak).

### Osmolarity, Sodium, and Potassium Measurements

Leaves and roots were collected and washed twice with deionized water. Tissue (2 g), cut in small pieces, was loaded into a 5-mL syringe containing a Whatman number 1 filter disk. The material was frozen at  $-30^\circ\text{C}$ , and the cell sap was obtained in thawed samples by centrifugation at 1,200g for 15 min using a S4180 rotor in a GS-15R tabletop centrifuge (Beckman). The osmolarity of the cell sap was measured in 50- $\mu\text{L}$  samples with a cryoscopic osmometer (Osmomat 030; Genotec). The concentration of sodium and potassium in the collected cell sap was determined by flame photometry (model 943; Instrumentation Laboratory).

### Chlorophyll Measurement

Chlorophyll was measured according to Porra et al. (1989). Leaves from control and salt-treated salt cress plants were collected, washed, cut in small pieces, and ground in a mortar with 1 mL of 80% (v/v) acetone. Samples were centrifuged at 12,000g for 10 min and the supernatant collected. Chlorophyll was measured using a diode array spectrophotometer (Hewlett-Packard). Absorbance of the chlorophyll *a* extract was detected at 645 nm; chlorophyll *b* at 663 nm. The values were calculated using the molar extinction coefficients 20.2 and 8.02 for chlorophyll *a* and *b*, respectively.

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