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Thellungiella halophila, a salt-tolerant relative of *Arabidopsis thaliana*, possesses effective mechanisms to discriminate between potassium and sodium

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ABSTRACT

Thellungiella halophila **is a salt-tolerant close relative of** ***Arabidopsis thaliana*****. Significant mRNA similarity was confirmed by hybridization of** ***T. halophila*** **mRNA with the** ***A. thaliana*** **GeneChip ATH1. To establish a platform for future molecular comparison of the two species several physiological mechanisms, which may confer high salt tolerance to** ***T. halophila*****, were investigated. Determination of ion content in shoots and roots of** ***A. thaliana*** **and** ***T. halophila*** **indicated different strategies of ion uptake and translocation from root to shoot in the two species. During salt stress** ***T. halophila*** **accumulated less sodium than** ***A. thaliana*****. Tissue concentrations of sodium and potassium showed negative correlation in** ***A. thaliana*** **but not in** ***T. halophila*****. Electrophysiological experiments proved high potassium/sodium selectivity of root plasma membrane channels in** ***T. halophila.*** **In particular, voltage-independent currents were more selective for potassium in** ***T. halophila*** **than in** ***A. thaliana*****. Single cell sampling of** ***T. halophila*** **leaves during salt exposure revealed increased concentrations of sodium and decreased concentrations of potassium in epidermal cells suggesting that this cell type could function to ensure storage of sodium and exchange of potassium with the rest of leaf. Application of salt resulted in a sharp drop of transpiration in** ***A. thaliana*****. By contrast, transpiration in** ***T. halophila*** **responded more slowly and was only slightly inhibited by salt treatment, thus maintaining high water uptake and ion transport.**

Key-words: *Thellungiella halophila*; ion channel selectivity; inductively coupled plasma (ICP); ion transport; microarray; salt tolerance; single cell analysis; transpiration.

INTRODUCTION

Global crop production is now as severely affected by salinity stress as by drought (Ashraf 1994). Large tracts of agricultural land are lost for production due to irrigation-induced soil salinization. All major crops are salt-sensitive. To combat salinity both sophisticated irrigation techniques and breeding of salt-tolerant crops are required. Although salt tolerance is a complex multigene trait, over-expression of ion transporters has recently been proved to be an effective way to increase salt tolerance (Apse *at el.* 1999; Gaxiola *et al.* 2001; Shi *et al.* 2003). For wheat, a single gene was found, which controls K^+/Na^+ discrimination of xylem loading and is beneficial for salt tolerance (Gorham *et al.* 1997). Indeed, many salt stress symptoms arise from the accumulation of toxic levels of Na^+ and Cl^- in the cytoplasm. The ability of plants to exclude toxic ions (e.g. Na^+), while taking up or retaining others (e.g. K^+) is a key determinant of salt tolerance (Maathuis & Amtmann 1999). Therefore, modification of a relatively small number of ion transporters may confer a salt-tolerant phenotype on a salt-sensitive plant. The following parameters are of particular importance: (1) activity and abundance of primary pumps; (2) selectivity, activity and abundance of different cation uptake channels; and (3) selectivity, activity and abundance of export systems that actively remove Na^+ from the cytoplasm. Moreover, tissue-specific requirements have to be taken into account. Hence, transport systems should possess specific characteristics depending on whether they are located in root cells where they absorb nutrients from the soil solution, at the xylem parenchyma/xylem interface where they function in xylem loading, or in leaf cells where ions accumulate via the transpiration stream.

Up to the present, *Arabidopsis thaliana* is the best model organism for dissecting molecular pathways in plants. Recently, several groups have exploited the excellent resources developed for *A. thaliana* to investigate salt tolerance (Zhu 2000; Bressan *et al.* 2001). However, *A. thaliana* is a true glycophyte. It will not undergo a full life cycle even at moderate salinity (~ 100 mM NaCl). Therefore, studies on *A. thaliana* will reveal little information on salt tolerance, but a great deal on stress. *Mesembryanthemum crystallinum*, a halophyte, is now being developed as a model plant for salt tolerance, drought resistance and CAM (Adams *et al.* 1998). However, salt tolerance in *M. crystallinum* is tightly linked to succulence and CAM and it may prove difficult to transfer halotolerance mechanisms

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into crops that show neither of these traits. More recently, another halophyte, *Thellungiella halophila* (salt cress), has been proposed as a model system for studying salt resistance in plants (Bressan *et al*. 2001; Zhu 2001). Few resources have been established for *T. halophila* (approx. 1700 ESTs at NCBI), but *T. halophila* is closely related to *A. thaliana* (90–95% identity at the cDNA level; Bressan *et al*. 2001; Zhu 2001). Therefore the resources of *A. thaliana* (gene and protein databases, mutant lines) will facilitate molecular studies on salt tolerance in *T. halophila*. Furthermore, *T. halophila* shares many of the advantages of *A. thaliana* as an experimental system. It has a small genome (less than twice the size of the *A. thaliana* genome), a short life cycle, abundant seed production and can easily be transformed (Bressan *et al*. 2001; Zhu 2001).

To enable future molecular dissection of salt tolerance mechanisms a detailed analysis of physiological mechanisms underlying salt tolerance in *T. halophila* is required. Particular emphasis should be put on the comparison with *A. thaliana* to facilitate future gene identification. The data presented in this paper point to a crucial role of ion transport properties in determining different salt tolerance in *T. halophila* and *A. thaliana*.

MATERIALS AND METHODS

Plant material

Thellungiella halophila and *A. thaliana* plants were grown in hydroponic conditions on a controlled 14 h/10 h (150 μE m^{-2} s^{-1} light, 24 °C) and 10 h/14 h (200 μE m^{-2} s^{-1}, 24 °C) day/night cycle, respectively. These growth conditions had been optimized to compensate for the differences in the developmental programmes of the two species. The applied conditions resulted in plants that were comparable in growth and development (e.g. development of rosette leaves, delayed flowering). The basic nutrient solution contained 1.25 mM KNO_3, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.625 mM KH_2PO_4 and micronutrients (Arteca & Arteca 2000). Salt treatment was applied by adding NaCl for different periods, from 25 h [for ion analysis by inductively coupled plasma optic emission spectroscopy (ICP-OES)] to several months (to compare plant growth).

Root and shoot ion analysis

Plants of *A. thaliana* and *T. halophila* were grown in hydroponic culture for 4–8 weeks (10 rosette leaves) and then subjected to several experimental treatments for 25 h. The treatments were: 1. ‘Control’ = basic nutrient medium (see above); 2. ‘+ NaCl’ = ‘Control’ medium with 100 mM NaCl added; 3. ‘+ KCl’ = ‘Control’ medium with 10 mM KCl added; 4. ‘+ $CaCl_2$’ = ‘Control’ medium with 5 mM $CaCl_2$ added; 5. ‘+ NaCl + KCl’ = ‘Control’ medium with 100 mM NaCl and 10 mM KCl added; 6. ‘+ NaCl + $CaCl_2$’ = ‘Control’ medium with 100 mM NaCl and 5 mM $CaCl_2$ added. Six to seven plants were pooled for ion extraction. Root and shoot tissue was harvested after 25 h of exposure to treatments and fresh weight and dry weight were determined. 2 M HCl was added to dry plant material, in 1 : 100 (w : v) proportion, the material was incubated overnight, diluted 50 times with distilled water and analysed by (ICP-OES) using Optima 4300 DV optical emission spectrometer (Perkin Elmer Instruments, Wellesley, MA, USA).

Protoplast isolation

Root protoplasts were isolated from 6- to 8-week-old-plants following a procedure developed for *A. thaliana* roots (Demidchik & Tester 2002). About 200 mg of chopped root pieces (1–2 mm long) were digested in 2 mL of enzyme solution containing 1.5% Cellulase Onozuka RS (Yakult Honsha, Tokyo, Japan), 1% cellulysin (CN Biosciences, Nottingham, UK), 0.1% pectolyase Y-23 (Kikkoman Co, Noda City, Japan), 0.1% bovine serum albumin (Sigma, St Louis, MO, USA), 10 mM KCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$ and 2 mM MES (2-(*N*-morpholino)-ethanesulfonic acid). The pH was 5.7 (Tris/MES) and the osmolarity was adjusted with sorbitol to 350 mOsm. Roots were incubated on a shaker (60 r.p.m) for 30–50 min at 28 °C. The digested tissue was gently washed several times with ice-cold ‘storage solution’ containing 10 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 5.7 (2 mM MES/Tris), 350 mOsm (sorbitol). Protoplasts were extracted by squeezing through 20 μm mesh into 2–3 mL of ‘storage solution’.

Electrophysiology

Protoplasts of 20–35 µm diameter with single large vacuoles were patch-clamped using standard techniques (Amtmann *et al*. 1997). The protoplasts originated from the root cortex, as verified by microscopic observation during digestion. Patch clamp pipettes were pulled on a vertical electrode puller (PP-83; Narishige, Tokyo, Japan) from glass capillaries (Kimax 51; Kimble Products, Vineland, NJ, USA). Pipettes were filled with ‘pipette solution’ containing 100 mM KCl, 8–10 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2–2.5 mM EGTA, 2 mM Mg-ATP, 2 mM HEPES, pH 7.0 (Tris). Final pipette resistances were around 10 MΩ. The ‘sealing solution’ was 20 mM $CaCl_2$, 2 mM MES, pH 5.7 (Tris), 350 mOsM (sorbitol). After seal formation ‘sealing solution’ in the bath was replaced by experimental solutions containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM MES, pH 5.7 (Tris), 350 mOsM (sorbitol) and various amounts of KCl or NaCl (see Results and Figures). All solutions were sterile filtered. Most experiments were carried out in whole-cell mode where the bath represents the external solution and the ‘pipette solution’ the cytoplasmic compartment. The reference agar bridge contained 100 mM KCl. Liquid junction potentials (which were not more than 10 mV) were measured and corrected for as described in Amtmann & Sanders (1997). Experiments were carried out at room temperature (22–25 °C) with bath perfusion at a rate of 0.14 mL min^{-1}.

Currents were recorded and processed using a standard patch clamp amplifier L/M-EPC7 (Heka Elektronik, Lam-

brecht/Pfalz, Germany), ITC-18 digitizer (Computer Interface Instrutech Corporation, Port Washington, NY, USA) and PULSE + PULSEFIT software, version 8.53 (Heka Elektronik). Data were online low-pass filtered at 0.5–1 kHz with an eight-pole Butterworth filter (KEMO Ltd, Beckenham, Kent, UK) and sampled at 5–10 kHz. Holding potentials were –70 to –100 mV. Statistical analysis was done using standard software packages (EXCEL 2000 for Windows, Microsoft, Redmond, WA, USA and SIGMAPLOT for Windows; SPSS Science, Chicago, IL, USA]. The K/Na selectivity ratios were determined from reversal potentials in different bath solutions.

Single cell sampling and analysis

Sap from individual epidermal cells was extracted by puncturing cells with a silicon-oil-filled microcapillary. The picolitre-sized cell saps were immediately placed under water-saturated liquid paraffin to avoid evaporation of saps (for details, see Tomos *et al*. 1994; Fricke, Leigh & Tomos 1994). To provide enough sap volume for replicate elemental analysis, three to four epidermal cells were sampled in quick succession. The pooled cell sap was treated as one 'cell'-sap sample. One such sample was analysed for each leaf, and data from five leaves were averaged. Once sap from epidermal cells had been extracted, the leaf blade was placed into custom-built tubular inserts in 1.5 mL microcentrifuge tubes, and bulk sap was extracted as described previously (Fricke & Peters 2002).

Elemental concentrations were determined by energy-dispersive X-ray (EDX) analysis of picolitre droplets, which had been pipetted onto Pioloform-coated copper grids (Pioloform from Agar Scientific, Stansted, UK) and subsequently freeze dried. Details of the method have been described in Tomos *et al*. (1994) and Fricke *et al*. (1994). Two slightly different approaches were used for the analysis of cell and bulk leaf extracts. For cell extracts, a constriction pipette was used to pipette four aliquots, about 5–10 pL in volume, of each cell sample onto the grid. An equal volume of internal standard (300 mM $RbNO_3$ in 0.6 M mannitol) was added to each aliquot. Bulk leaf extracts were much more viscous than the highly vacuolar epidermal cell extracts and would have clogged constriction pipettes easily. Therefore, bulk leaf extracts were mixed at a 1 : 1 ratio with internal standard, and the mixture was placed with a glass microcapillary onto the grid and expelled as picolitre-sized droplets (four replicates each). Grids were analysed with a scanning electron microscope (S-4100; Hitachi, Tokyo, Japan) equipped with a Link X-ray analyser (Link, Oxford, UK).

Transpiration measurements

Transpiration experiments were carried out with 7- to 8-week-old *T. halophila* and 4- to 5-week-old *A. thaliana* plants (10 rosette leaves). Measurements were done for parallel groups of four to six plants during a period of at least 5 d. Plants were mounted with roots in air-tight containers that were filled with either basic nutrient solution, or basic nutrient solution supplemented with 100 mM NaCl. For transpiration measurements, containers with liquid and plants were weighed at different intervals (every 1–2 h during illumination, less frequently during dark periods). Weighing took 1–3 min; during the remaining time the plants were in the growth cabinets under usual growth conditions (70% relative humidity). Total weight loss from a container with four plants was >500 mg h^{-1} during illumination period, and <200 mg h^{-1} during dark period. Assuming that stomata were closed at night, the difference between minimal 'night' values and 'day' values can be attributed to stomatal transpiration. Minimal loss per hour at dark period was taken as 'night' evaporation through leaf cuticle and peristomatal transpiration. Stomatal transpiration rate was calculated as the difference in weight loss during the light period and minimal weight loss during the dark period, divided by the number of plants per container and the time interval.

Microarray experiments

RNA was isolated separately from roots and from shoots of 1 month old *T. halophila* plants. Two groups of plants were analysed, control and salt treatment ('medium 2' for 5 d). Equal amounts of root and shoot RNA were combined for hybridization. Reverse transcription, *in vitro* transcription and labelling were performed according to the protocols supplied with the array (Affymetrix Inc. Santa Clara, CA, USA). Amplified labelled cRNA was hybridized with the Affymetrix ATH1 Arabidopsis GeneChip. In total 22 765 *A. thaliana* probe sets (genes) were analysed using AFFYMETRIX software.

RESULTS

Growth and salinity tolerance

NaCl levels that can be tolerated by *T. halophila* or *A. thaliana* strongly depended on ionic environment and mode of application. *Thellungiella halophila* generally tolerated higher salt concentrations than *A. thaliana* (Fig. 1a & b). In hydroponic 'control' medium supplemented with various amounts of NaCl, *A. thaliana* did not survive NaCl concentrations above 80 mM. Exposure of *A. thaliana* to 100 mM NaCl resulted in bleaching of older leaves within 2–3 d and formation of necrotic spots and death of leaves within 3–5 d of treatment. Younger leaves of *A. thaliana* started to look less turgid within several days after onset of the treatment. After 1 week in 100 mM NaCl only leaves of intermediate age remained visibly turgid and did not show chlorosis. No recovery from stress symptoms was observed when NaCl was removed from the medium after 7 d of treatment. By contrast, *T. halophila* continued growth in hydroponic medium for several weeks after addition of 100 or 200 mM NaCl, though growth inhibition was observed initially. After several weeks of growth on nutrient medium supplied with 100 mM NaCl *T. halophila* plants did not differ visibly from control plants (Fig. 1c). In soil, both plant

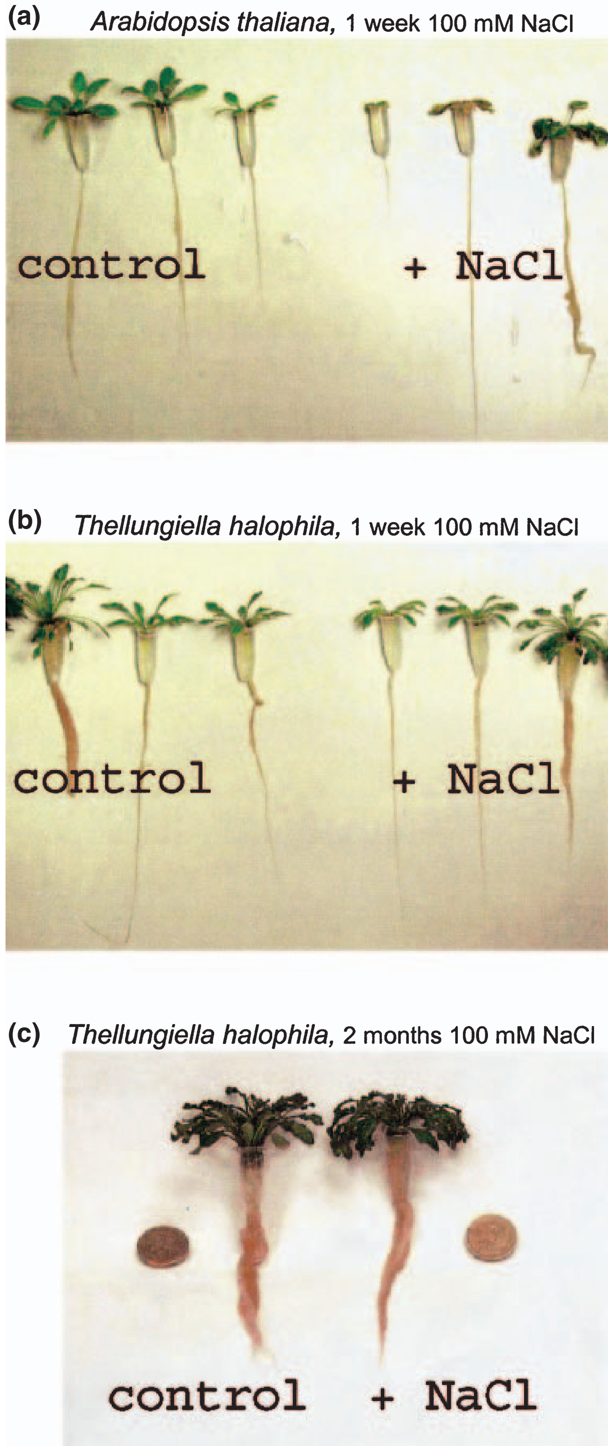


Figure 1. Growth of *A. thaliana* (a) and *T. halophila* (b, c) in saline medium. (a, b) 5–7 weeks old *A. thaliana* and *T. halophila* plants, hydroponically grown in 'control' medium. For salt treatment plants were exposed to an additional 100 mM NaCl for 1 week. (c) Two-month-old *T. halophila* plants grown hydroponically in 'control' medium with or without 100 mM NaCl added. Salt was applied 2 weeks after germination.

species tolerated relatively higher salt concentrations (compare Bressan *et al*. 2001). For further experiments addition of 100 mM NaCl to hydroponic 'control' medium was chosen as treatment since this concentration evoked the strongest difference in the two species (death of *A. thaliana* versus growth of *T. halophila*). For long-term treatments both species were exposed to non-lethal moderate salt concentrations (50 mM for *A. thaliana*, 100 mM for *T. halophila*).

Tissue ion analysis

Use of ICP-OES allowed simultaneous determination of a wide range of minerals in a single tissue sample. We determined root and shoot contents of K, Na, Ca, Mg, Fe, Zn, P and S in *A. thaliana* and *T. halophila* after exposure to various external media. Figure 2 shows typical ion profiles of both species in control medium. The main differences in the profiles relate to shoot Ca and Mg contents (higher in *A. thaliana* than in *T. halophila*) and shoot Na and S (higher in *T. halophila* than in *A. thaliana*). Root profiles were very similar in the two species except for K (higher in *A. thaliana* than in *T. halophila*). Differences in shoot Ca, Mg and S were maintained in all media.

In the following text we focus on K and Na contents of

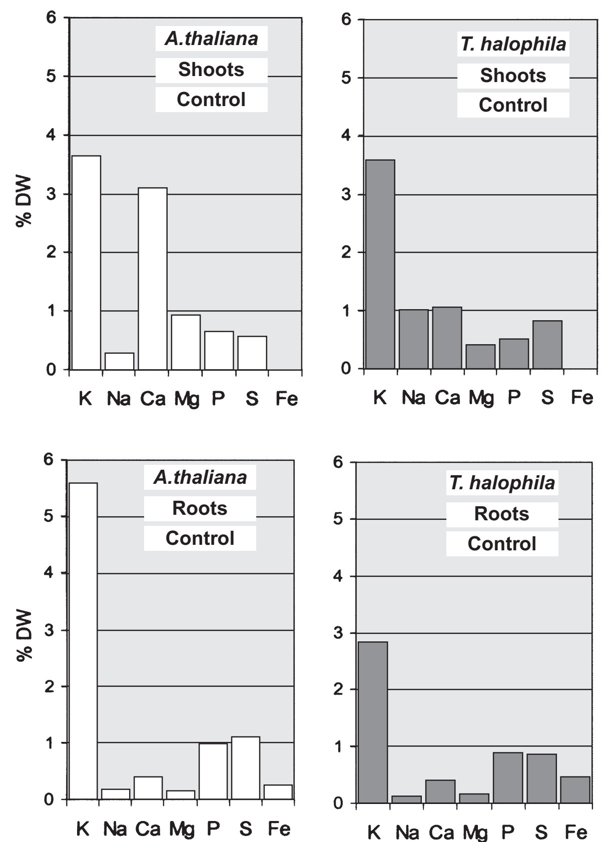
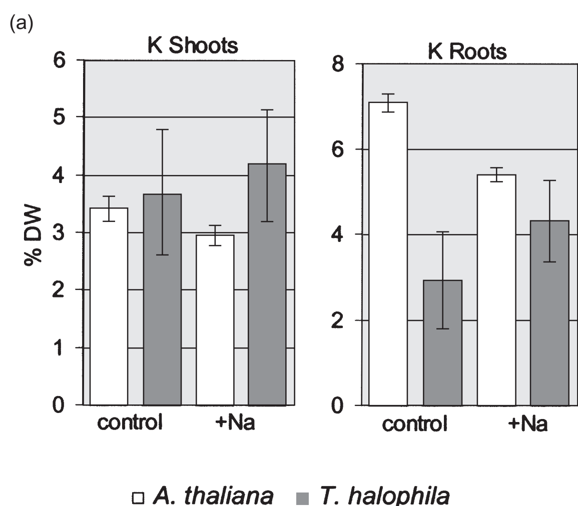


Figure 2. Typical nutrient profiles of *A. thaliana* and *T. halophila* plants (6–7 plants pooled) grown hydroponically in 'control' medium.



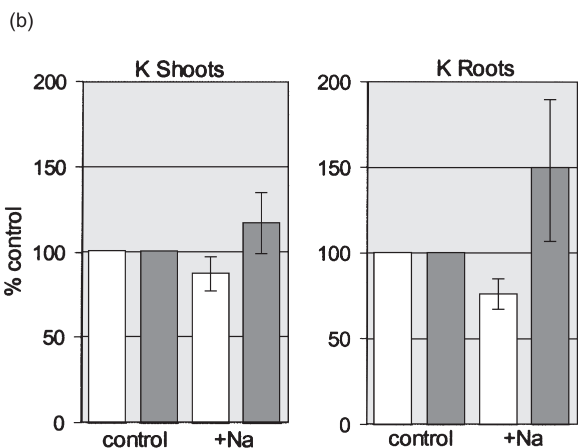
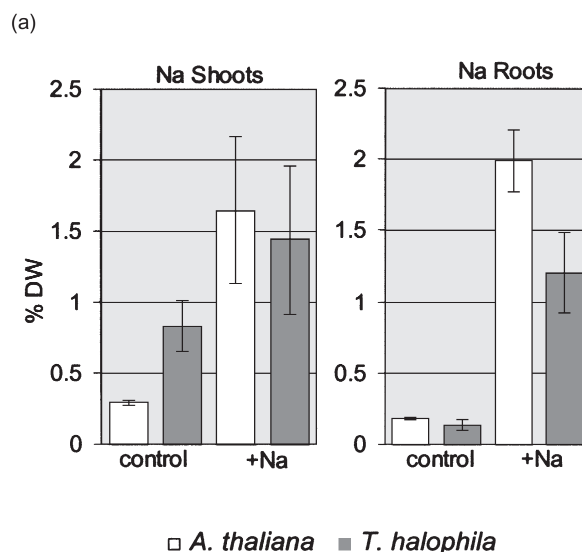


Figure 3. Shoot and root potassium contents of *A. thaliana* and *T. halophila* plants after 25 h exposure to 'control' or '+Na' (100 mM NaCl) medium. Six to seven plants were pooled for each experiment. Averages of three independent experiments are shown. Error bars are SD. (a) Absolute values in percentage dry weight. (b) Relative changes within each experiment.

A. thaliana and *T. halophila* shoots and roots before and after salt treatment (25 h, 100 mM NaCl). Both average absolute values of three independent measurements and relative changes within individual experiments are shown (Figs 3 & 4). The difference between root K in *A. thaliana* and *T. halophila* observed in control medium disappeared after salt treatment. Shoot K content, which did not differ significantly between *A. thaliana* and *T. halophila* in the control medium, was higher in *T. halophila* than in *A. thaliana* after exposure to high NaCl (Fig. 3a). Thus, in both tissues NaCl treatment *de*creased K contents in *A. thaliana* (by 10–20%) and *in*creased K contents in *T. halophila* (by 10–50%; Fig. 3b). Absolute root Na content did not differ significantly between the two species under control conditions but was lower in *T. halophila* than in *A. thaliana* after 25 h NaCl treatment (Fig. 4a). The Na content of the shoot was considerably higher in *T. halophila* than in *A. thaliana* under control conditions (2 mM NaCl). This difference disappeared after exposure to 100 mM NaCl due to a sharp increase in shoot Na in *A. thaliana* (Fig. 4b). In summary, *T. halophila* accumulated less Na and more K than *A. thaliana* during salt exposure.

The combined effect of changes in K and Na contents is shown as K/Na ratios in Fig. 5a. In control medium the K/Na ratios of both root and shoot tissues were higher in *A. thaliana* than in *T. halophila*. In high-salt medium the K/Na ratios dropped in both species, but this effect was considerably stronger in *A. thaliana* resulting in K/Na ratios that were now smaller in *A. thaliana* than in *T. halophila*. Figure 5b shows shoot/root ratios for K and Na in both species. The shoot/root ratio of K was generally higher in *T. halophila* than in *A. thaliana* and this difference was maintained after 25 h NaCl treatment. The shoot/root ratio of Na was higher in *T. halophila* than in *A. thaliana* in control medium but this difference was no longer apparent



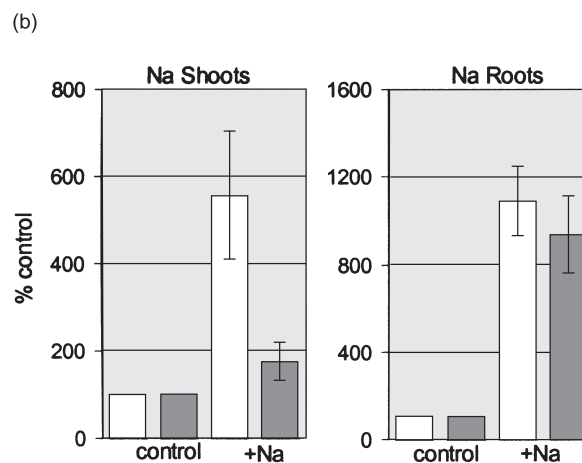


Figure 4. Shoot and root sodium contents of *A. thaliana* and *T. halophila* plants after 25 h exposure to 'control' or '+Na' (100 mM NaCl) medium. Six to seven plants were pooled for each experiment. Averages of three independent experiments are shown. Error bars are SD. (a) Absolute values in percentage dry weight. (b) Relative changes within each experiment.

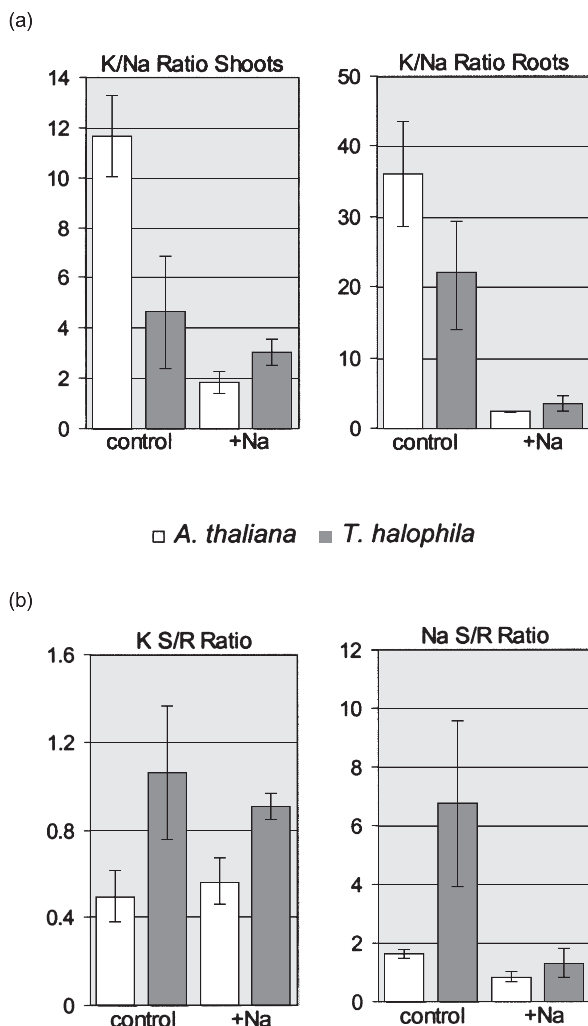


Figure 5. (a) K/Na ratios in shoots and roots of *A. thaliana* and *T. halophila* in control medium and after salt exposure (same experiments as in Figs 3 & 4). (b) Shoot/root ratios of K and Na in *A. thaliana* and *T. halophila* in control medium and after salt exposure (same experiments as in Figs 3 & 4).

after salt treatment, due to a sharp decrease of Na shoot/root ratio in *T. halophila*.

Increased levels of Ca (+5 mM $CaCl_2$) during salt treatment resulted in decreased Na accumulation in both *T. halophila* and *A. thaliana* (data not shown). Root Na contents were reduced to 60 ± 15% in *A. thaliana* and to 52 ± 9% in *T. halophila* after 25 h incubation in 'medium 6' compared with 'medium 2' (see Methods). The addition of 10 mM KCl during salt treatment also decreased root Na contents in *A. thaliana* (to 67 ± 4%, 'medium 5', compared to 'medium 2') but not in *T. halophila*. These effects were mirrored in the shoot, although to a lesser extent. No difference in Na content was found when Ca or K was added to the control medium ('medium 3' or 'medium 4' compared to 'control'). We calculated correlation coefficients for ion interactions from the entire data-set and found that K and Na were negatively correlated in *A. thaliana* ($R^2 = 0.52 \pm 0.15$, *R* negative), whereas there was no significant K–Na correlation in *T. halophila* ($R^2 = 0.21 \pm 0.18$, *R* positive).

Electrophysiology

Several types of ion channels were identified in root protoplasts from *T. halophila*. In symmetric K conditions (pipette solution/bath solution, 100 mM KCl) whole cell time-dependent outward currents were observed in all proto-

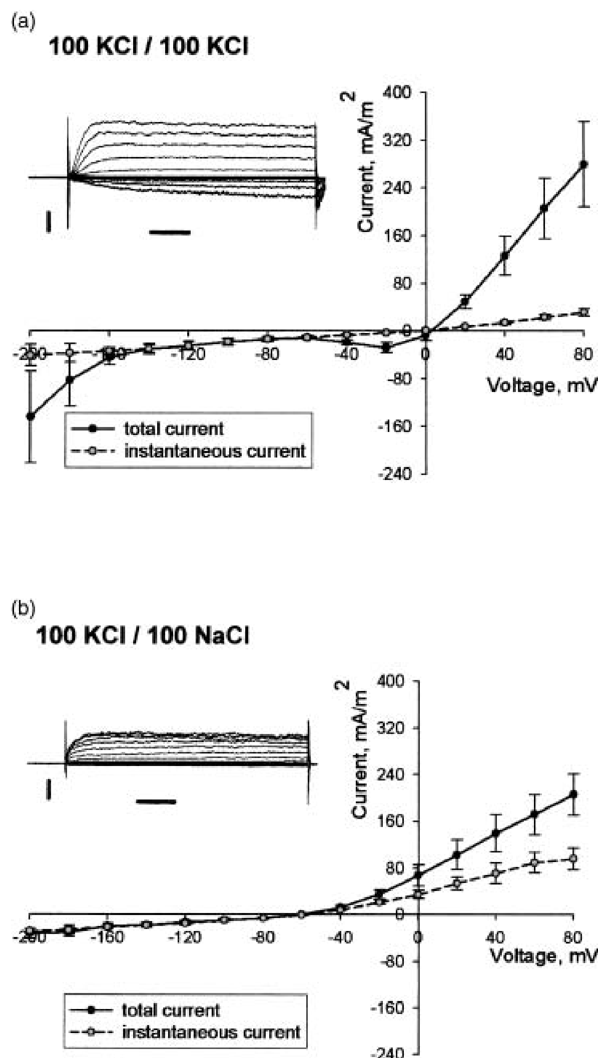


Figure 6. Whole cell current recordings in *T. halophila* root protoplasts and average current density-voltage curves (based on five protoplasts for voltages between −160 mV and +80 mV, based on three protoplasts for −200 and −180 mV). Bars in current recordings are 500 pA (vertical) and 200 ms (horizontal). Holding potential was −100 mV, voltage clamp ranged from −200 mV to +80 mV. (a) Bath solution: 100 mM KCl. Pipette solution: 100 mM KCl. (For other components of solutions see Methods). Note that both total and instantaneous currents reverse near 0 mV. (b) Bath solution: 100 mM NaCl. Pipette solution: 100 mM KCl. (For other components of solutions see Methods). Note that reversal potentials of both currents have shifted negative by about 60 mV (towards E_K).

Table 1. Na/K permeability ratios (P_{Na}/P_K) for cation currents in root protoplasts of *T. halophila* and *A. thaliana*. Data are given as mean ±SE. Values for *A. thaliana* taken from cited references.

	P_{Na}/P_K *T. halophila*	P_{Na}/P_K *A. thaliana*	
Outward rectifying channels	0.07 ± 0.01 (n = 5) [a]	0.29 ± 0.1 (n = 3) [b]	(Maathuis & Sanders 1995)
Inward rectifying channels	<0.1 (n = 4) [c]	0.17 ± 0.03 (n = 3) [b]	(Maathuis & Sanders 1995)
Voltage-independent channels	0.12 ± 0.02 (n = 5) [a]	0.67 (n = 3) [d,e]	(Demidchik & Tester 2002)

[a]Determined from reversal potential of whole cell currents.
[b]Determined from reversal potential of single channel currents.
[c]No current could be detected in 100 mM NaCl at –200 mV.
[d]Determined from conductance at 30 mV below the reversal potential.
[e]Literature value given as K/Na = 1.49 ± 0.15 was converted into Na/K.

plasts explored (n = 16). Time-dependent inward currents were not detected without ATP in the pipette and were observed in nine out of 14 protoplasts when ATP was present. Typical whole cell current recordings and average I–V curves are shown in Fig. 6.

Time-dependent outward currents were selective for K over Cl (according to reversal potential shift by –55 mV in a 100/10 mM KCl, pipette/bath). They resembled those reported for *A. thaliana* root cells (Maathuis & Sanders 1995). In single channel recordings an outward-rectifying channel with a unitary conductance of 35–40 pS was identified in symmetric 100/100 mM KCl solutions (data not shown), which coincides with results for K outward channels from *A. thaliana* root cells (Maathuis & Sanders 1995). Time-dependent outward currents were partially inhibited, when external KCl was substituted by the same concentration of NaCl (Fig. 6). Reversal potentials indicated a high selectivity for K over Na of the time-dependent outward current. The average Na/K permeability ratio was 0.07 ± 0.01 (n = 5). This value is four times smaller than the Na/K permeability ratio of the *A. thaliana* K outward rectifier (Maathuis & Sanders 1995; Table 1). In symmetrical 100 mM KCl activation of the *T. halophila* current was negative of E_K, thus permitting some inward current. This inward current disappeared when NaCl replaced KCl in the bath solution indicating that the activation potential was now close to E_K (i.e. it had not shifted negative with E_K, Fig. 6b).

Time-dependent inward currents of *T. halophila* looked similar to those in *A. thaliana* root protoplasts (Maathuis & Sanders 1995). In symmetrical 100 mM KCl the current activated at voltages below –100 mV and did not show voltage saturation (down to –200 mV). When NaCl replaced KCl in the external medium no time-dependent inward current was detected at voltages positive of –200 mV thus indicating high selectivity for K over Na. Addition of KCl to a background of external NaCl reinstated the current.

Instantaneous currents were measured at 20 ms after application of the voltage pulse. In contrast to voltage-independent currents from *A. thaliana* (Demidchik & Tester 2002), instantaneous currents of *T. halophila* root protoplasts were highly selective for K over Na. After changing the bath solution from 100 mM KCl to 100 mM NaCl the reversal potential shifted negative by 50–60 mV indicating Na/K permeability ratios close to 0.1 (Table 1). Selectivity for K over Na was also visible as a decrease of instantaneous inward current, when changing bath solution from 100 mM KCl to 100 mM NaCl. Instantaneous currents were too small to allow determination of Na/K permeability ratios on the basis of relative currents. Moreover, longer incubation of the protoplasts in NaCl solution (10–15 min) resulted in a further decrease of the instantaneous current. Table 1 summarizes Na/K permeability ratios of different cation currents in *T. halophila* and *A. thaliana* root cells.

Single cell sampling and analysis

Arabidopsis thaliana cannot survive more than a few days in 100 mM NaCl. In contrast, *T. halophila* grows well in the same medium for over a month showing no stress symptoms. Although short-term (25 h) Na accumulation in *T. halophila* was lower than in *A. thaliana* one would expect that *T. halophila* accumulates large amounts of Na in leaves during long-term exposure to salt. We were interested to find out whether under these conditions Na was accumulated in specific leaf tissues. Combining single cell sampling techniques with X-ray analysis (SCS-EDX) we determined tissue-specific ion distribution of K, Na, Ca, Cl, P, and S within leaves of *T. halophila* plants that had been grown in 100 mM NaCl for 5 weeks. Tissue-specific ion contents were also determined in *A. thaliana* plants exposed for a similar length of time to elevated, but sublethal, concentration of NaCl (50 mM). Control plants were grown without salt addition to the medium.

Thellungiella halophila and *A. thaliana* showed different trends in the distribution of ions between the epidermis and bulk tissue both under control conditions and in response to salinity (Fig. 7). Under control conditions Cl and S were equally distributed between epidermis and the rest of the leaf in *A. thaliana*. By contrast, in *T. halophila* S was much higher in the epidermis than in the bulk leaf sample and

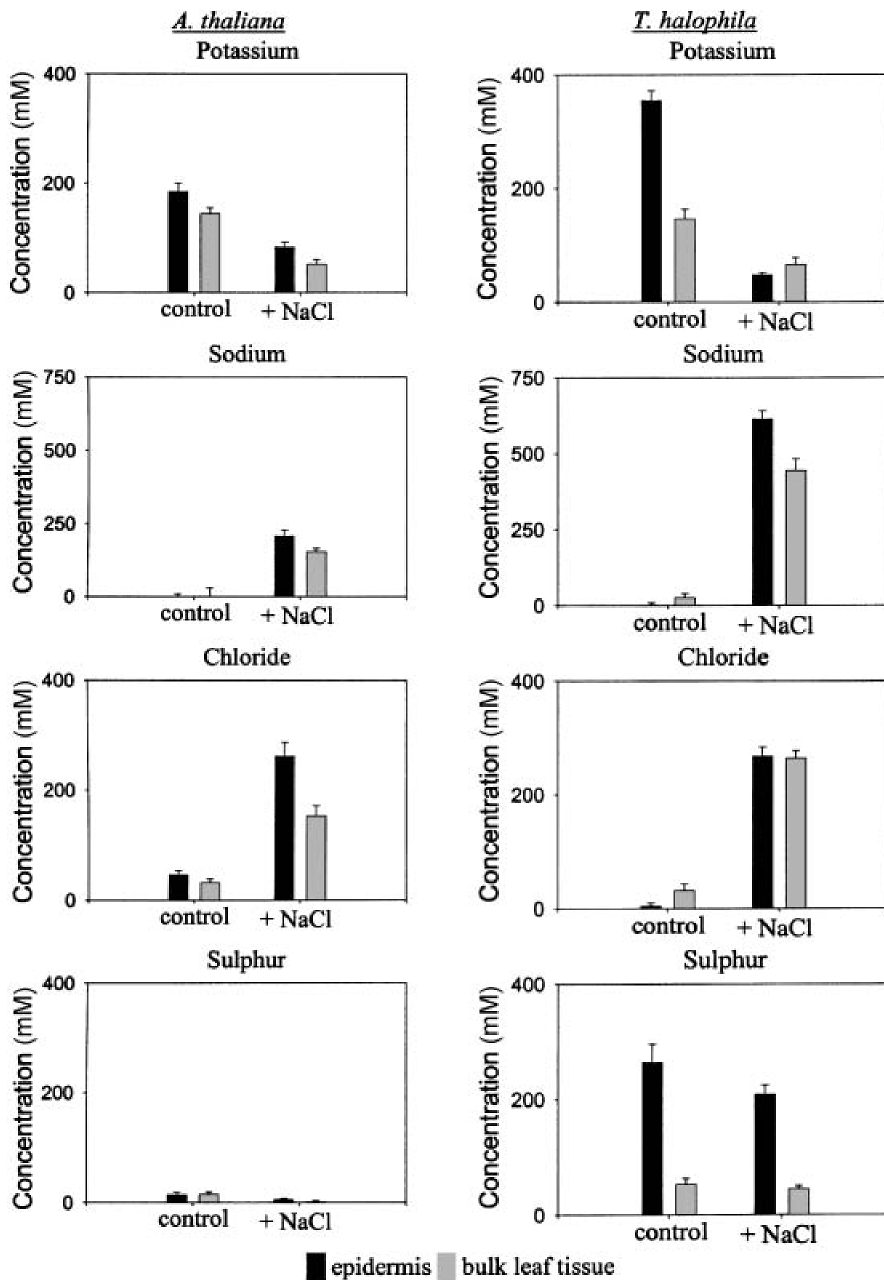


Figure 7. Distribution of potassium, sodium, chloride and sulphur within *A. thaliana* and *T. halophila* leaf tissues after 5 weeks of growth in medium with and without NaCl (50 mM for *A. thaliana* and 100 mM for *T. halophila*). Data from five leaves are averaged. Error bars are SE.

the Cl distribution was the opposite. Epidermal S contents were more than 10 times higher in *T. halophila* compared with *A. thaliana*. K was preferentially accumulated in epidermal cells in both species. Epidermal K concentrations were particularly high in *T. halophila* (350 mM in epidermis, 150 mM in bulk). In both species P accumulated preferentially in the epidermis whereas Ca was higher in the bulk tissue (data not shown). Bulk samples contained a mixture of epidermis and mesophyll. Therefore differences in solute concentrations between epidermis and mesophyll were in all probability larger than differences between epidermal and bulk values.

Long-term salt stress caused a considerable increase in Na and Cl in leaves of both species, whereas K, Ca and P decreased. After 5 weeks of salt exposure bulk leaf Na concentrations in *T. halophila* reached around 450 mM and around 150 mM in *A. thaliana* (note that *A. thaliana* was exposed to lower Na concentrations than *T. halophila*). In both species Na accumulated specifically in the epidermis. Cl was distributed evenly between leaf tissues in *T. halophila*, but not in A. *thaliana*, where it accumulated particularly in the epidermis. Preferential accumulation of K in epidermal tissue of *A. thaliana* was maintained during salt stress. By contrast, in *T. halophila* salt exposure caused an inversion of epidermal/bulk K contents resulting in lower K concentration in the epidermis than in the bulk. As a consequence the Na/K ratio in *T. halophila* epidermal cells was nearly twice the Na/K ratio in the bulk leaf (12.8 and 6.7, respectively). In *A. thaliana* changes in the distribution of K and Na between

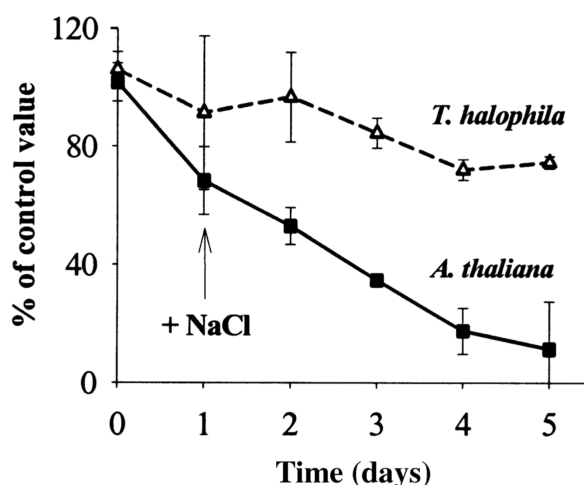


Figure 8. Average daytime transpiration rates in *A. thaliana* and *T. halophila* after addition of 100 mM NaCl, in percentage of transpiration rates in control plants. NaCl was added at midday of day 1.

the epidermis and the rest of the leaf were not observed; the Na/K ratios did not differ significantly between epidermis (2.5) and bulk leaf (2.9).

Various epidermal cell types and both the adaxial and abaxial epidermal layers were analysed. All epidermal cells and layers showed the same distribution of solutes compared with the bulk leaf and the same response to salinity (data not shown).

Transpiration

Transpiration measurements were carried out with *T. halophila* and *A. thaliana* plants of different ages to account for the higher growth rate of *A. thaliana*. Selected plants were similar in transpiration rate in control conditions. However, *T. halophila* had four times lower transpiration rates than *A. thaliana* when transpiration was normalized to dry weight. The addition of 100 mM NaCl to the medium gave rise to more differences between the species (Fig. 8). Salt treatment resulted in a sharp and fast transpiration drop in *A. thaliana* (by about 50% over the first 24 h) whereas *T. halophila* maintained transpiration rates close to control for at least 24 h after salt shock. The detrimental effect of 100 mM NaCl on *A. thaliana* was mirrored by a continuous decrease in transpiration rate over the following days. By contrast, transpiration rates in *T. halophila* dropped only slightly (by 20–30%) within the first 2 d of salt treatment and then remained stable for over 1 week. Removal of salt from the growth medium after 7 d of treatment led to recovery of transpiration in *T. halophila*, but not in *A. thaliana*.

Microarray experiments

A 90–95% nucleotide identity between cDNAs of *A. thaliana* and *T. halophila* has been reported (Zhu 2001). We investigated the similarity between *T. halophila* and *A. thaliana* transcripts using the *A. thaliana* GeneChip ATH1 (Affymetrix). ATH1 contains highly specific 25mer probe sets for approximately 24 000 *A thaliana* genes. One-fifth of the probe sets (4766) produced a significant signal after hybridization with *T. halophila* mRNA. Amongst these were genes for 167 membrane transporters and 180 regulatory proteins (Table 2). In particular, *T. halophila* mRNA for the Na^+/H^+ co-transport systems NHX1, NHE1 as well as the kinase SOS2 were recognized on the GeneChip, but neither of them showed a significant change after salt treatment (see below). Based on hybridization results, we can conclude that at least 21% of *A. thaliana* mRNAs have

Table 2. Numbers of genes encoding transporters or regulatory proteins with high sequence similarity in *A. thaliana* and *T. halophila*[a,b]

Transporters		**Regulatory proteins**	
ABC transporters	24	Serine/threonine protein kinase	35
P-type pumps	22	Receptor protein kinases	27
V-type ATPase subunits	14	MAP kinases	10
Sugar transporters	14	Ca-dependent kinases	8
AA/peptide transporters	19	Other protein kinases	38
Nitrate transporters	5	Protein phosphatases	20
Ammonium transporter	1	Calcineurin/calreticulin/calmodulin	13
Phosphate transporters	2	GTP-binding proteins	23
Sulphate transporters	4	14-3-3 proteins	6
Metal transporters	2	Total	180
Auxin transporters	1		
Ion channels	8	**Known stress-induced proteins**	
Potassium transporters	5	Salt-inducible proteins	10
Antiporters	6	ABA/drought induced proteins	4
Na-cotransporters	2	General stress induced proteins	4
Aquaporins	12	Total	18
Others	26		
Total	167		

[a]Determined by hybridization of *T. halophila* mRNA with ATH1Affymetrix GeneChip.
[b]Accession numbers of individual genes can be obtained from the authors on request.

Table 3. Genes with more than three-fold change of expression level after exposure of *T. halophila* plants to 100 mM NaCl for 5 d. Expression signals were determined by hybridization of *T. halophila* mRNA with *A. thaliana* ATH1Affymetrix GeneChip and therefore accession numbers of the corresponding *A. thaliana* genes are given

Affymetrix ID	AGI ID	Signal ratio (treated/control)	Description (TAIR)
Up-regulated:			
250052_at	At5g17830	11.31	Hypothetical protein
257069_at	At3g28170	11.31	Hypothetical protein
265639_at	At2g27375	8.57	Reverse transcriptase-related
244981_at	AtCg00770	5.28	Chloroplast 30S ribosomal protein S8
246231_at	At4g37080	3.25	Expressed protein
Down-regulated:			
265114_at	At1g62440	0.31	Leucine-rich repeat extensin family
246885_at	At5g26230	0.31	Expressed protein
267384_at	At2g44370	0.29	CHP-rich zinc finger protein
245226_at	At3g29970	0.23	Germination protein-related
259272_at	At3g01290	0.22	Expressed protein
266319_s_at	At2g46720	0.20	Fatty acid elongase 3-ketoacyl-CoA synthase 1 (KCS1)
261256_at	At1g05760	0.20	Jacalin lectin family (RTM1)
253301_at	At4g33720	0.19	Putative pathogenesis-related protein 1 precursor
247024_at	At5g66985	0.19	Expressed protein
257497_at	At1g51430	0.18	Expressed protein
247297_at	At5g64100	0.15	Peroxidase, putative
256922_at	At3g19010	0.14	Flavonol synthase family
256890_at	At3g23830	0.13	RNA-binding protein, putative,
266965_at	At2g39510	0.13	Nodulin MtN21 family protein

highly similar isoforms in *T. halophila*. Only a very small number of these genes (19 genes, most of which unknown) were significantly (more than three-fold) up- or down-regulated after 5 d of salt treatment with 100 mM NaCl (Table 3).

DISCUSSION

Ion profiles in control conditions

In an attempt to understand mechanisms conferring salt tolerance to *T. halophila*, we paid attention to ion transport properties. Before discussing these in detail it should be noted that in a low-salt environment *T. halophila* had slower growth rate and a lower shoot/root fresh-weight ratio than *A. thaliana* (data not shown). Slow growth in low-salt medium is a general feature of halophytic species and a potential problem of transgenic salt-tolerant crops in an agricultural setting. Salt tolerance may require more selective ion acquisition and transport; this selectivity, recognition and exclusion of toxic elements might cost energy and require altered sink–source relations. Thus, specific ion transport mechanisms that have evolved to prevent Na toxicity in salt-tolerant species may be directly linked to a less efficient uptake and/or allocation of important nutrients. We found higher root contents of K and higher shoot contents of Ca and Mg in *A. thaliana* compared with *T. halophila* in low-salt conditions. Future research on individual transporters will reveal whether these differences are related to salt tolerance mechanisms and the observed differences in growth rate. Furthermore, we found that in control medium (containing 2 mM Na) *T. halophila* has considerably higher shoot Na contents than *A. thaliana*. This suggests that *T. halophila* does not exclude Na in low-salt conditions but probably uses Na as well as K for osmotic purposes.

Na and K contents during salt stress

Arabidopsis thaliana and *T. halophila* exhibit important differences in uptake and shoot allocation of Na and K during salt stress. Over the first 25 h after addition of 100 mM NaCl *T. halophila* accumulated more K and less Na than *A. thaliana*. Differences in K accumulation were larger in roots than in shoots whereas differences in Na accumulation were more pronounced in shoots than in roots. This suggests the existence of *two* major barriers for cation transport with different relative K and Na permeabilities in the two species: one at the root/soil boundary and one at the site of xylem loading (root/shoot boundary). Our data suggests that Na repulsion of the root/shoot barrier is particularly strong in *T. halophila* thus translating low Na uptake rates at the root/soil boundary into low Na shoot contents. The importance of a Na barrier in the xylem parenchyma of *T. halophila* is also reflected in shoot/root ratios. Both species had reduced shoot/root Na ratios after salt exposure but this reduction was more dramatic in *T. halophila*.

Further distinctions between ion transport mechanisms in the two species became apparent when applying Na stress in different backgrounds of K and Ca ions. Addition of Ca reduced Na accumulation in both species and this agrees with previous findings that Ca alleviates Na toxicity

(Cramer 2002). High K concentrations are also known to reduce salt stress symptoms (Achilea 2002) and indeed the Na accumulation in *A. thaliana* was smaller in a high K background. However, this was not the case for *T. halophila*. Correlation coefficients calculated for K–Na contents in various ionic backgrounds and tissues confirmed a weak negative correlation between the two ions in *A. thaliana* whereas no correlation or even a weak positive correlation was found for *T. halophila*. Although the exact reasons for this difference in Na–K relations between the two species are not clear at this stage our findings imply that Na and K transport pathways are competitively linked in *A. thaliana* but independent in *T. halophila*. The molecular identity of Na and K transport pathways in different cell types are discussed below.

Role of the leaf epidermis in adaptation to long-term salt stress

Using single-cell sampling techniques we investigated the role of leaf epidermis cells in ion accumulation during long-term salt stress consisting of the application of 50 mM (*A. thaliana*) or 100 mM NaCl (*T. halophila*) for 5 weeks. As found for other plants (Fricke *et al*. 1994; Karley, Leigh & Sanders 2000a) both *T. halophila* and *A. thaliana* accumulate some solutes specifically in the epidermis and others preferentially in the mesophyll. The distribution of Ca and P between epidermis and mesophyll/bulk tissue was the opposite of that observed in barley, and similar to that in *Vicia*, further confirming the idea that the two ions cannot be accumulated at high concentrations in the same tissue (Leigh & Tomos 1993). Epidermal sulphur concentrations were an order of magnitude higher in *T. halophila* compared to *A. thaliana*, and even though it was not tested explicitly, it is quite likely that a high proportion of this sulphur represented glutathione and/or glycosinolates. A direct link to salt tolerance cannot be made at this stage but recently, high concentrations of sulphated flavonoids were found to correlate with salt tolerance in *Lasthenia* (Asteracea) varieties (Rajakaruna *et al*. 2003).

Interestingly, the main difference between cell type specific ion distribution in *A. thaliana* and *T. halophila* during salt stress related to K rather than Na (both species exhibited epidermal Na accumulation after long-term salt exposure). Under control conditions *T. halophila* accumulated K to very high concentrations in the epidermis. During salt stress epidermal K concentrations decreased dramatically whereas bulk K concentrations increased. Such change in K distribution was not observed in *A. thaliana*. As we have not carried out single cell sampling shortly after salt application we do not know whether the observed pattern is the result of increased K efflux from epidermal cells into the mesophyll or of decreased K uptake into epidermal cells, or both. The observed changes in K distribution allow *T. halophila* to maintain relatively high K/Na ratios in metabolically active mesophyll cells. In barley, a moderately salt-tolerant plant, single cell sampling measurements also demonstrated a more pronounced Na increase and K drop in epidermal cells compared with mesophyll cells under high salt treatment (Fricke, Leigh & Tomos 1996). A sharp drop of K in both vacuole and cytoplasm of epidermal cells, but relatively stable concentrations in mesophyll under salt stress were observed in barley using triple-barrelled electrodes (Cuin *et al*. 2003). Since cytoplasmic K concentrations of just 15 mM were recorded in the epidermis, it was proposed that these cells have low metabolic activity (Cuin *et al*. 2003), but they are functional in terms of turgor and osmolality generation (Fricke 1997). In summary, epidermal cells in *T. halophila* can function as an active storage site for K in low-salt conditions and for Na under salt stress. Clearly, this requires regulation of ion transport pathways with differential ion selectivity in specific leaf cell types.

Pathways for Na and K transport

Low-affinity uptake of cations into plant roots occurs mainly through ion channels (Maathuis *et al*. 1997). In patch-clamp experiments we revealed that selectivity for K over Na of both inward and outward rectifying K channels is several times higher in *T. halophila* than in *A. thaliana*. It has been suggested that the major pathway for Na entry into roots of *A. thaliana* and various crop species is through voltage-independent, non-selective cation channels (Amtmann & Sanders 1999; Tyerman & Skerrett 1999; White 1999; Demidchik, Davenport & Tester 2002). In *A. thaliana* this channel type was shown to be gated by cyclic nucleotides (Maathuis & Sanders 2001), extracellular calcium and pH (Demidchik & Tester 2002). Genes encoding voltage-independent non-selective channels remain widely unknown. Homology to glutamate receptor-like or cyclic nucleotide gated channels (CNGC) has been proposed (Davenport 2002; Maathuis & Sanders 2001). Both gene families comprise at least 20 members in *A. thaliana* (Mäser *et al*. 2001; Lacombe *et al*. 2001; Davenport 2002). To date only a few studies have characterized plant CNGC in heterologous expression systems, where either voltage-independent gating and non-selective cation permeability (Balagué *et al*. 2003) or voltage-dependent gating and high K/Na selectivity was revealed (Leng *et al*. 2002).

We studied voltage-independent cation currents in root protoplasts of *T. halophila* and compared our data with published data for corresponding *A. thaliana* channels. In *A. thaliana* voltage-independent channels exhibit very weak selectivity for K over Na (1.5/1, Demidchik & Tester 2002; confirmed in our own laboratory). We found that selectivity for K over Na of voltage-independent cation currents is considerably higher in *T. halophila* root protoplasts (at least 8/1, compare with Na/K permeability ratios, Table 1). To our knowledge this is the first characterization of a voltage-independent K-selective channel. It will be interesting to investigate whether this channel type is also present in other halophytic species. Further experiments are in progress to fully characterize cation channels in *T. halophila*, but we can already conclude that all major cation currents in *T. halophila* root protoplasts exhibit high selectivity for K over Na. These electrophysiological results are

in good agreement with our findings on tissue ion contents (see above) and provide strong evidence that root ion channels impose an important barrier for Na entry into *T. halophila* and thus constitute a major component of salt tolerance in this plant.

Xylem loading, which occurs through ion channels and transporters in the plasma membrane of xylem parenchyma cells, is an important means to control delivery of solutes to the shoot (Wegner & De Boer 1997a, b; Gaymard *et al*. 1998; Shi *et al*. 2002; De Boer & Volkov 2003). For *A. thaliana*, the putative plasma membrane Na^+/H^+ antiporter SOS1was proposed to control long-distance transport of Na to leaves (Shi *et al*. 2002). SOS1 is strongly expressed in cells surrounding xylem vessels and the direction of Na translocation by SOS1 (loading/unloading of Na into/out of the xylem) depended on the external Na concentration and the length of the salt treatment. Since the antiporter uses protons, its activity should be linked to activity of the proton pump and thus be related to the cell membrane potential. The latter provides a possible link with potassium loading of the xylem via voltage-dependent ion channels, such as SKOR (Gaymard *et al*. 1998), and thus explains a negative correlation of Na and K accumulation. Indeed, perfusion experiments with soybean roots (Lacan & Durand 1996), roots of the halophyte *Plantago maritima* (De Boer & Prins 1985) and barley roots (De Boer & Volkov 2003) revealed opposite directions of Na and K transport into/out of the xylem. For example, activation of the xylem parenchyma proton pump by fusicoccin decreased K loading of the xylem (presumably due to membrane hyperpolarization), but stimulated Na loading (presumably via activation of the Na–H antiport). In this context, absence of linkage between Na and K in xylem loading as found for *T. halophila* (see above) is remarkable, but could reflect an effective mechanism to individually control transport of each ion to the shoot.

The distribution pattern of solutes between different leaf tissues points to differences in membrane transport and storage properties of epidermal and mesophyll cells (Karley, Leigh & Sanders 2000b). Karley (1999) characterized ion channels in barley leaf epidermal and mesophyll protoplasts using patch clamp. At least three types of cation channels were identified in the two cell types: K-selective inward and outward rectifying channels and Na-permeable voltage-independent channels. Interestingly, voltage-independent Na currents were larger in epidermal protoplasts than in protoplasts from mesophyll cells and this difference may explain preferential Na accumulation in the barley leaf epidermis. Karley (1999) also suggested that ion channels in the two cell types might be differentially regulated.

Transpiration

Transpiration is the dominant driving force for ion uptake and root to shoot translocation of water and solutes during the light period. Reduced transpiration limits salt loading of the shoot and may be useful in short-term stress adaptation. In the longer term, reduced transpiration will limit nutrient supply and growth of the shoot. Munns (1985) showed for barley that reduced transpirational water loss in response to salt was offset by increased solute concentrations in xylem sap. Whether the same applied to *T. halophila* cannot be said, but maintenance of transpiration rates during salt stress coupled with high K : Na selectivity in *T. halophila* reflects certainly an efficient strategy to guarantee leaf gas exchange and nutrient supply in a saline root environment.

Salt application provoked a fast decrease of transpiration in *A. thaliana*, which at least during the first 24 h can be ascribed to stomatal closure rather than to decreased shoot growth. By contrast, *T. halophila* maintained stable transpiration rates, even days after application of salt. In *A. thaliana* stomatal closure is a known phenomenon under drought and salt stress and is mediated by ABA (Leung & Giraudat 1998; Zhu 2002). It appears that ABA signalling under the same conditions is either absent or modified in *T. halophila.*

In a halophytic *Aster* species (*A. tripolium*) external NaCl induced stomatal closure in epidermal strips (Perera, Mansfield & Malloch 1994) and this response was associated with inhibition of guard cell inward-rectifying K channels by Na (Véry *et al*. 1998). The authors proposed Na-induced stomatal closure as a general strategy of halophytes to restrict salt delivery to the shoot (Robinson *et al*. 1997). Our results from *T. halophila* and *A. thaliana* do not support this idea. First, the effect of salt treatment on stomatal transpiration was weaker in the halophyte than in the glycophyte. Second, the partial reduction of transpiration observed in *T. halophila* was not specific for Na but also occurred in elevated K concentrations (data not shown). This is in disagreement with the findings from *A. tripolium* where stomatal closure was specific for Na (Perera *et al*. 1994).

Genetic similarity between *A. thaliana* and *T. halophila*

Our microarray experiments showed that at least 20% of *A. thaliana* transcripts have highly homologous isoforms in *T. halophila*. Due to the short probe length of the employed array (ten 25mers per gene) only transcripts with 100% identity in the majority of the probe sequences were detected. Out of these less than 20 genes were differentially expressed in plants exposed to control medium or 100 mM NaCl for 5 d. The small number of salt-regulated transcripts detected in our experiment might be due to the relatively long salt exposure and/or to the fact that the hybridization signal was biased towards non-regulated genes because of the necessity for high homology to *Arabidopsis* isoforms. Previous analysis of random sequencing of *T. halophila* mRNAs showed 90–95% overall sequence identity between *T. halophila* and *A. thaliana* transcripts (Bressan *et al*. 2001; Zhu 2001). On the basis of these results one can assume that *A. thaliana* microarrays with longer, less specific probes could be used to compare *A. thaliana* and *T. halophila* transcripts in more detail and to monitor gene

expression of *T. halophila* in response to salt stress. Such studies may provide a clue as to whether differential salinity tolerance of *A. thaliana* and *T. halophila* is the result of mutations within homologous isoforms (e.g. mutations in selectivity filters of ion channels or regulatory units) or whether it is due to a small number of completely novel genes in *T. halophila*. Considering the phenotypic similarity of the two plant species the former explanation seems more likely. In this context it is interesting that among the highly homologous genes detected in our GeneArray hybridization were several genes that are known to strongly influence salinity tolerance in *A. thaliana* (i.e. genes for plasma membrane and vacuolar Na/H antiporters). This knowledge provides an excellent opportunity for future investigation into the effects of silencing or over-expression of these well-known systems in a halophytic plant environment.

SUMMARY AND OUTLOOK

Salt tolerance in *T. halophila* is associated with specific features of ion transport, which are missing in its glycophytic relative *A. thaliana*. These are high K/Na selectivity of ion uptake into root cells and root-shoot translocation, and K/Na exchange between leaf epidermal and mesophyll cells.

Our results have created a platform for future identification of genes responsible for salt tolerance in *T. halophila*. Full genome information of *A. thaliana* will be extremely useful to allow molecular dissection of salt tolerance in comparative studies. *Thellungiella halophila* mutant lines are under construction at the moment and the *T. halophila* EST collection is growing fast (Ray Bressan, Purdue University, West Lafayette, IN, USA; personal communication). Amenability of both species to transformation protocols will allow transfer of genes between the *A. thaliana* and *T. halophila* and thus enable to study their effects on ion allocation in both species. The present study shows that *T. halophila* is a suitable system for electrophysiological investigation. Future electrophysiological experiments should aim to characterize ion transport pathways in specific cell types, namely root cells, xylem parenchyma cells and leaf epidermal cells. The possibility of using molecular and electrophysiological techniques as well as single cell solute analysis to study ion transport processes in *T. halophila,* together with our findings that ion transport is an important component of salinity tolerance in this plant, underpins the suitability of *T. halophila* as a model organism for plant salt tolerance.

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