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Low unidirectional sodium influx into root cells restricts net sodium accumulation in *Thellungiella halophila*, a salt-tolerant relative of *Arabidopsis thaliana*

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Abstract

Thellungiella halophila **is a useful model species for research into plant salt tolerance. It is closely related to *Arabidopsis thaliana*, but shows considerably higher salt tolerance. Comparative analysis of ion homeostasis in the two species allows the identification of ion transport pathways that are critical for salt tolerance and provides the basis for future studies into their molecular features. Previous studies indicated that salt tolerance in *T. halophila* is accompanied by low accumulation of Na in the leaves. Kinetic analysis of net ion uptake over three days confirmed lower Na uptake and K loss in *T. halophila* compared with *A. thaliana*. Differential net Na uptake rates were still apparent after 6 weeks of salt treatment. To assess the contribution of unidirectional Na fluxes to net Na uptake, kinetic studies of ^{22}Na fluxes were carried out in both species. The results show that unidirectional root Na influx is significantly lower in salt-grown *T. halophila* plants than in *A. thaliana* exposed to the same level of salinity (100 mM). Quantitative comparison of unidirectional influx and net Na accumulation suggests that both species operate efficient Na efflux, which partly compensates for Na influx. Kinetic analysis of ^{22}Na efflux indicated higher root Na efflux in *A. thaliana* than in *T. halophila*. Thus *A. thaliana* appears to spend more energy on Na export while nevertheless accumulating more Na than *T. halophila*. It is proposed that limitation of Na influx is the main mechanism by which *T. halophila* secures low net Na accumulation in saline conditions. This strategy provides the basis for a positive balance between growth and net Na uptake rates, which is essential for survival in high salt.**

Key words: *Arabidopsis thaliana*, potassium, root, salt tolerance, sodium, *Thellungiella halophila*.

Introduction

A diverse range of plant species, halophytes, can thrive on high salt concentrations, but most plants including all major crops are sensitive to NaCl concentrations above 50–100 mM (glycophytes). Different scientific approaches have been taken to understand plant responses to salt stress with the common aim of enhancing salt tolerance in food crops. These include the study of halophytic species (Flowers *et al.*, 1977; Véry *et al.*, 1998; Chauhan *et al.*, 2000), comparative analysis of crop varieties differing in salt tolerance (Munns *et al.*, 2002; Davenport *et al.*, 2005), and screening for mutants in *A. thaliana* (Zhu, 2000). Each of these approaches has its advantages and limitations. Halophytic species can reveal how high levels of salt tolerance have been achieved by natural selection, but tolerance may be linked to complex morphological and metabolic adaptations which will be difficult to transfer to crops. Working with crop species has direct implications for agriculture, but variations in salt tolerance are relatively small. *A. thaliana* is the best species for genetic and molecular experiments but is salt-sensitive and therefore may generate more information on plant stress management than on stress tolerance.

Over recent years *Thellungiella halophila* ('salt cress' synonymous to *T. salsuginea*; Al-Shebaz *et al.*, 1999; Wong *et al.*, 2005) has attracted growing interest as a model for research into plant abiotic stress tolerance (Bressan *et al.*, 2001; Amtmann *et al.*, 2005). *T. halophila* is a close

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relative of *A. thaliana* and possesses many of its experimental advantages but, in contrast to *A. thaliana*, it is an extremophile exhibiting high tolerance to salinity, drought, and cold. Genotypic and phenotypic similarity between the two species (Inan *et al.*, 2004) facilitates gene identification in *T. halophila* and provides an unprecedented opportunity for functional analysis of putative tolerance genes in a highly similar glycophytic background. Several molecular tools have been created for *T. halophila* including collections of ESTs, T-DNA insertion mutants and ecotypes, as well as cDNA libraries and microarrays (Amtmann *et al.*, 2005, http://thellungiella.org/). Initial studies into salt tolerance of *T. halophila* have shown that, during salt exposure, *T. halophila* accumulates less Na in its shoots than *A. thaliana* (Inan *et al.*, 2004;Volkov *et al.*, 2004). Interestingly, a negative correlation between Na and K accumulation as reported for *A. thaliana* and other species was not found in *T. halophila*, suggesting that K uptake in *T. halophila* occurs independently of Na uptake (Volkov *et al.*, 2004). Maintenance of high K/Na ratios is a key feature of salt tolerance as Na toxicity is largely due to Na out-competing K (e.g. at enzyme binding sites; Serrano, 1996; Amtmann *et al.*, 2004). Electrophysiological analysis of root cation channels showed that all major K uptake channels exhibit higher K/Na selectivity in *T. halophila* than in *A. thaliana* (Volkov *et al.*, 2004), thus providing a possible explanation for the observed differences in tissue Na/K ratios.

In this study, Na uptake characteristics in *T. halophila* were explored further. In particular, this study set out to identify the respective contributions of unidirectional influx and efflux in restricting net Na uptake into root cells of *T. halophila*. This issue was addressed using ^{22}Na tracer flux experiments, which were designed to measure unidirectional fluxes of Na under steady-state conditions of 100 mM extracellular Na. Na fluxes were measured in mature transpiring plants thus taking into account *in vivo* mass flow of Na from the root periphery towards the stele. These results show that restriction of Na influx into roots of *T. halophila* is an important mechanism to control tissue Na levels in this plant.

Materials and methods

Plant growth

Arabidopsis thaliana (ecotype Columbia 0) and *Thellungiella halophila* (ecotype Shandong) plants were germinated on agar-filled Eppendorf tubes. After 10–14 d the tips of the tubes were cut off and the tubes were fitted into the lids of 1.0 l black plastic containers (12 plants per container) containing a Minimum Nutrient Solution (MNS) with 1.25 mM KNO_3, 2 mM NaCl, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.625 mM KH_2PO_4, and micronutrients (modified from Arteca and Arteca, 2000; Volkov *et al.*, 2004). Plants were grown at 24 °C and 60–70% humidity in two light regimes (10 h light at 200 μE m^{-2} s^{-1} for *A. thaliana*, 14 h light at 250 μE m^{-2} s^{-1} for *T. halophila*) to obtain plants that were similar in growth and development.

Determination of tissue ion content

For short-term salt-stress experiments, 4-week-old plants were treated with 100 mM NaCl in MNS medium for 25 h. For long-term salt treatments, plants were exposed to 50 mM NaCl (*A. thaliana*) or 100 mM NaCl (*T. halophila*) in MNS for 6 weeks. Six plants were pooled for each sample and all experiments were repeated four times. To study the kinetics of net K and Na accumulation, 4-week-old plants were treated with 100 mM NaCl in MNS. Nine plants were harvested individually at 0, 6, 24, and 72 h after salt treatment. Roots and shoots were separated and dried to determine dry weights before ion content analysis. Dried tissues were incubated overnight in 2 M HCl (1:100 w:v), diluted 50 times with double-distilled H_2O, and analysed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) using an Optima 4300 DV (Perkin Elmer Instruments, Wellesley, MA, USA).

^{22}Na influx

Four-week-old plants were transferred to MNS supplemented with 100 mM NaCl 1 week before the experiment, to achieve steady-state Na uptake during the tracer flux experiments. The experimental procedure comprised pretreatment with non-labelled loading solution for 5 min (3 min for various blockers to minimize toxicity), labelling with ^{22}Na for various times, and subsequent washing with ice-cold rinse solution (100 mM NaCl, 10 mM $CaCl_2$, 2 mM $LaCl_3$, and 5 mM MES at pH 4.1) in two 1.5 min steps. Intact roots were excised, blot-dried, and weighed. Roots were then transferred to plastic vials with 4 ml scintillation cocktail (OptiPhase HiSafe3, PerkinElmer) to be counted in a liquid scintillation counter (Beckman Instruments, Fullerton, CA). Four plants were measured separately for each sample. To determine the time-course of ^{22}Na influx, the loading solution was MNS with $NaNO_3$ replacing $Ca(NO_3)_2$ and 100 mM NaCl and 0.1 mM $CaCl_2$ added ('−Ca MNS + Na'). Plants were labelled for 0.5–60 min. For the Ca-inhibition experiment, the loading solution was '−Ca MNS + Na' with Ca activities adjusted to 0.03, 0.1, 0.6, 1, and 3 mM (Ca activities were calculated using GEOCHEM-PC; Parker *et al.*, 1995). Plants were labelled for 15 min in ^{22}Na labelled loading solution. To determine the Na dependence of ^{22}Na influx the loading solution was '−Ca MNS' with 1, 25, 50, 100, 200, or 400 mM NaCl added and additional $CaCl_2$ to achieve a Ca activity of 3 mM. For blocker experiments, the loading solution was '−Ca MNS + Na' with 5 mM CsCl or 20 mM tetra-ethyl-ammonium (TEA)-Cl added. Plants were labelled for 0.5 min and 5 min to determine the influx.

^{22}Na efflux

Four-week-old plants were loaded with ^{22}Na labelled '−Ca MNS + Na' solution overnight. Intact roots were excised and quickly dipped into ice-cold de-ionized water to rinse off the surface ^{22}Na, then transferred to unlabelled '−Ca MNS + Na' solution (efflux solution). The roots were then progressively transferred to aliquots of 5 ml efflux solution to determine ^{22}Na released from the roots for 20 time points between 0.5 min and 250 min. At the end of the experiment, the roots were blotted and weighed. All aliquots and the root samples were mixed with 10 ml scintillation solution and counted. The specific activity of ^{22}Na in the loading solution was determined. Flux data are expressed as total Na flux based on the assumption that the proportion of ^{22}Na in the flux equals its proportion in the loading solution (note that this is only true during the early stages of the experiment).

Data analysis

Statistical analysis of Na and K contents in *A. thaliana* and *T. halophila* was carried out using a paired *t* test in which batches of *A. thaliana* and *T. halophila* that were grown in parallel were

treated as pairs. If appropriate, experimental data were fitted with informative models using Sigma Plot. The respective equations and fitted parameters are listed in Table 1.

Results

T. halophila *and* A. thaliana *differ in tissue cation content*

Ion profiling of roots and shoots from *A. thaliana* and *T. halophila* plants grown in low or high-salt conditions revealed both constitutive and salt-induced differences in cation contents, in particular, for Ca, Mg, K, and Na (Volkov *et al.*, 2004). Statistical evaluation of an extended data set for K and Na contents is shown in Fig. 1. Under low-salt conditions, *T. halophila* contained a significantly lower concentration of K in its roots and a higher concentration of Na in its shoots than *A. thaliana*. Both differences disappeared after a 25 h treatment with 100 mM NaCl. This was due to a considerable decrease of K concentration in *A. thaliana* roots during salt treatment, which did not occur in *T. halophila*, and an increase in shoot Na concentrations, which was much more drastic in *A. thaliana* than in *T. halophila*. Data for shoot K and root Na, albeit statistically less significant, indicated similar species-specific changes of K and Na contents in these tissues.

T. halophila *and* A. thaliana *differ in the kinetics of ion accumulation during salt stress*

Time-courses (over 72 h) of salt-induced changes in tissue K and Na concentrations were analysed in both species (Fig. 2). Plants were treated with 100 mM NaCl at time 0. In *A. thaliana* roots, K concentrations increased slightly over the first 6 h of treatment but then decreased steadily during extended salt treatment. In *T. halophila*, root K concentrations were lower than in *A. thaliana* at the beginning of the treatment, but decreased only slightly over the remaining period of time (Fig. 2, top panel). The two curves intersect at approximately 26 h, which accounts for similar

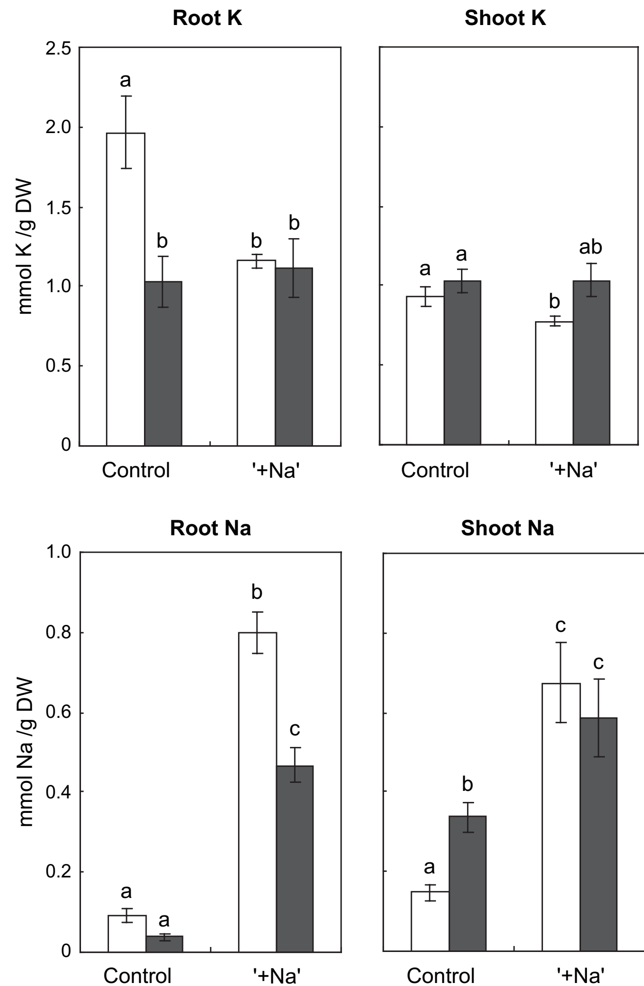

Fig. 1. Tissue concentrations of K and Na in *A. thaliana* (white bars) and *T. halophila* (black bars) under control conditions and after 25 h salt treatment (100 mM NaCl, '+Na') in hydroponic growth solution. Cation contents were determined with ICP-OES. Data shown are averages of four experimental replicates. Six plants were pooled in each replicate. Error bars are SE. Bars with different letters are significantly different at P <0.05 (paired t test).

Table 1. *Kinetic analysis of ion accumulation and fluxes: fitted equations and parameters*

Parameters extracted	Fitted equation	R^2	Fig.
Rates of net K loss (mmol g^{-1} DW h^{-1})	*At*: $f(t)$=1.3–0.0083 t	0.94	2
	Th: $f(t)$=1.1–0.0026 t	0.9	
Time constants of net Na uptake into roots (h)	*At:* $f(t)=0.42\ (1-e^{-0.23t}) + 0.47\ (1-e^{-0.03t})$	0.99	2
	Th: $f(t)=0.34\ (1-e^{-0.45t}) + 0.09\ (1-e^{-0.065t})$	0.99	
Time constants of net Na uptake into shoots (h)	*At*: $f(t)=0.12 + 1.25\ (1-e^{-0.015t})$	0.99	2
	Th: $f(t)=0.23 + 0.22\ (1-e^{-0.058t})$	0.99	
Na influx (μmol g^{-1} FW min^{-1})	*At*: $f(t)=0.95 + 0.66\ t$	0.94	4B
	Th: $f(t)=0.62 + 0.31\ t$	0.99	
Na affinity of influx	*At*: $f(c)=0.79\ c/(102 + c)$	0.99	5
	Th: $f(c)=0.66\ c/(671 + c)$	0.99	
Ca inhibition of influx K_i (mM)	$f(c)=1.4 + 4.9\ e^{-3.7\ c}$	0.97	6
	inset: $f(c)=100\ c/(0.16 + c)$	0.98	
Time constants of Na efflux (min)	*At*: $f(t)=41\ e^{-36586t} + 7.9\ e^{-0.45t} + 7.8\ e^{-0.03t} + 0.77$	0.99	7A
	Th: $f(t)=29\ e^{-3898t} + 13\ e^{-1.67t} + 6.1\ e^{-0.02t} + 1.5$	0.99	

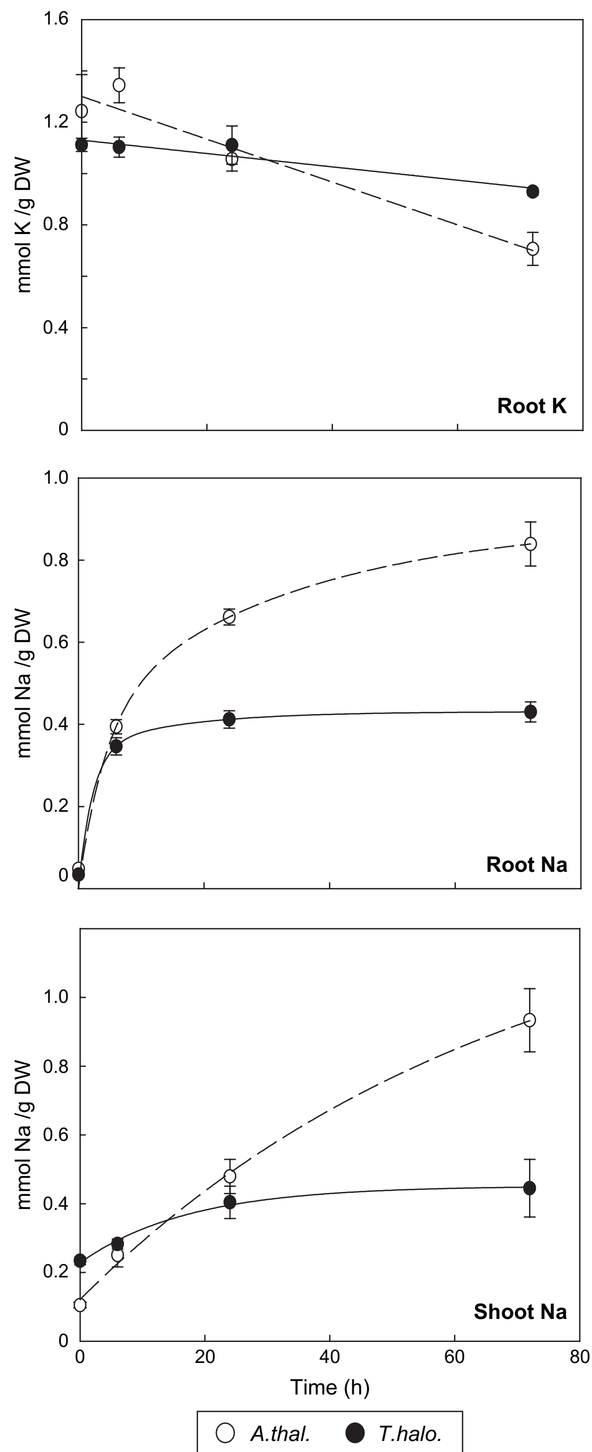

Fig. 2. Kinetics of net accumulation of K and Na in roots and/or shoots of *A. thaliana* (open circles) and *T. halophila* (closed circles) during a 72 h treatment with 100 mM NaCl in hydroponic growth solution. Ion concentrations were determined by ICP-OES from single plants (n=9). Error bars are SE.

concentrations of K determined in the 25 h experiment (Fig. 1). Fitting linear regression revealed rates of K loss of 8.3 and 2.8 μmol g^{-1} DW h^{-1} in *A. thaliana* and *T. halophila*, respectively (Table 1). Shoot K concentration was always higher in *T. halophila* than in *A. thaliana*, but did not show significant changes over the 72 h period in either species (data not shown).

An increase in root and shoot Na concentrations was observed in both species, but was much more pronounced in *A. thaliana* (Fig. 2). Na uptake into roots (Fig. 2, central panel) was best described by the sum of two kinetic components. Interestingly, both species showed very similar kinetics of Na uptake into roots during the first few hours after the addition of salt. However, a second, slower component of the uptake had a considerably lower amplitude in *T. halophila* than in *A. thaliana* (Table 1) resulting in the difference of Na concentrations determined after 25 h. *T. halophila* had higher shoot Na concentrations than *A. thaliana* at the beginning of the treatment, but after 72 h salt exposure its shoot Na concentration was significantly lower than that of *A. thaliana* (Fig. 2, bottom panel). The curves intersect at approximately 16 h of salt treatment accounting for similar Na concentrations determined in the 25 h experiment (Fig. 1). The kinetics of shoot Na accumulation in response to a change in the external medium could be fitted with a simple model assuming constant influx and a first order kinetic efflux, $f(t)=a+b(1-e^{-kt})$, with time constants $1/k$ of 67 and 17 h for *A. thaliana* and *T. halophila*, respectively (Table 1). It is clear that such a simple model does not fully reflect the complexity of the pathways determining Na accumulation in the shoot. This became apparent in the observation that, after longer periods of salt exposure, shoot Na concentrations of *T. halophila* had increased much more than predicted from the final rate of the 72 h experiment (see below).

Low accumulation of Na in shoots of T. halophila *is maintained during long-term growth on high salt*

To determine whether the observed differences in Na accumulation persist over longer periods of salt treatment *A. thaliana* and *T. halophila* plants were compared after 6 weeks of growth in elevated salt. Due to the differences in salt sensitivity these experiments had to be carried out with different NaCl concentrations for the two species; i.e. 50 mM for *A. thaliana* and 100 mM for *T. halophila*. It is remarkable that *T. halophila* plants, although subjected to twice the external Na concentration, exhibited only half the shoot Na concentration of *A. thaliana* after 6 weeks growth on salt (Fig. 3). The Na concentration in roots of *T. halophila* after 6 weeks in 100 mM NaCl was higher than in *A. thaliana*, but the difference was relatively small taking into account the higher external Na concentration. These data clearly show that Na accumulation in *T. halophila* is much lower than in *A. thaliana* both in the short and in the long term.

Net Na accumulation in whole plants reflects the difference between unidirectional Na influx into plant roots and unidirectional Na efflux from the roots. In the following experiments, the question of how these two

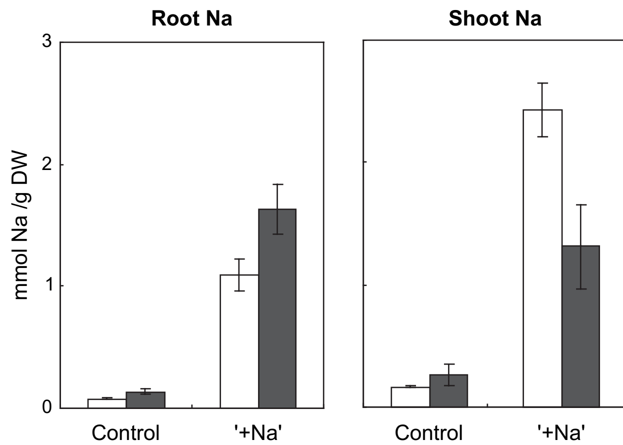

Fig. 3. Na concentrations in shoots and roots of *A. thaliana* (white bars) and *T. halophila* (black bars) after 6 weeks with and without salt treatment (+Na) consisting of the addition of 50 mM NaCl (*A. thaliana*) or 100 mM NaCl (*T. halophila*) to the hydroponic growth solution. Na contents were determined by ICP-OES. Data shown are averages of four experimental replicates. Six plants were pooled in each replicate. Error bars are SE.

parameters differed in the two species was addressed in order to account for the overall differences in net Na uptake during salt treatment. For this purpose, influx and efflux kinetics of radioactive ^{22}Na in roots were determined. Plants were grown in 100 mM NaCl for 1 week prior to the experiments to ensure that the measured fluxes reflected steady-state conditions (compare Fig. 2). Under these conditions the ^{22}Na flux kinetics are only affected by changes in the relative amounts of radioactive tracer in the external medium and the plant. During the early time points of such experiments these changes are negligible and therefore uptake and loss of ^{22}Na are directly proportional to steady-state unidirectional Na fluxes between the external medium and the root cell cytoplasm.

Unidirectional Na influx into roots is lower in T. halophila *than in* A. thaliana

Figure 4A shows a 60 min time-course of Na influx into *T. halophila* roots as determined by the accumulation of ^{22}Na. The plot of ^{22}Na accumulation against time reflects a typical influx curve with an initial linear rise and subsequent attenuation due to increasing ^{22}Na efflux from the root cells back into the external medium. Since whole plants were used for these experiments, the kinetics of ^{22}Na accumulation in the roots are very complex and therefore no attempt was made to obtain a quantitative model of this curve, but it was merely used to determine the time period of the initial linear phase. The slope of this phase was compared between *T. halophila* and *A. thaliana* (Fig. 4B). Linear regression fits of these data revealed rates of 0.66 and 0.31 μmol Na g^{-1} FW min^{-1} for unidirectional Na influx into *A. thaliana* and *T. halophila* roots, respectively (Table 1). Thus unidirectional Na influx in *A. thaliana* is more than twice that in *T. halophila*.

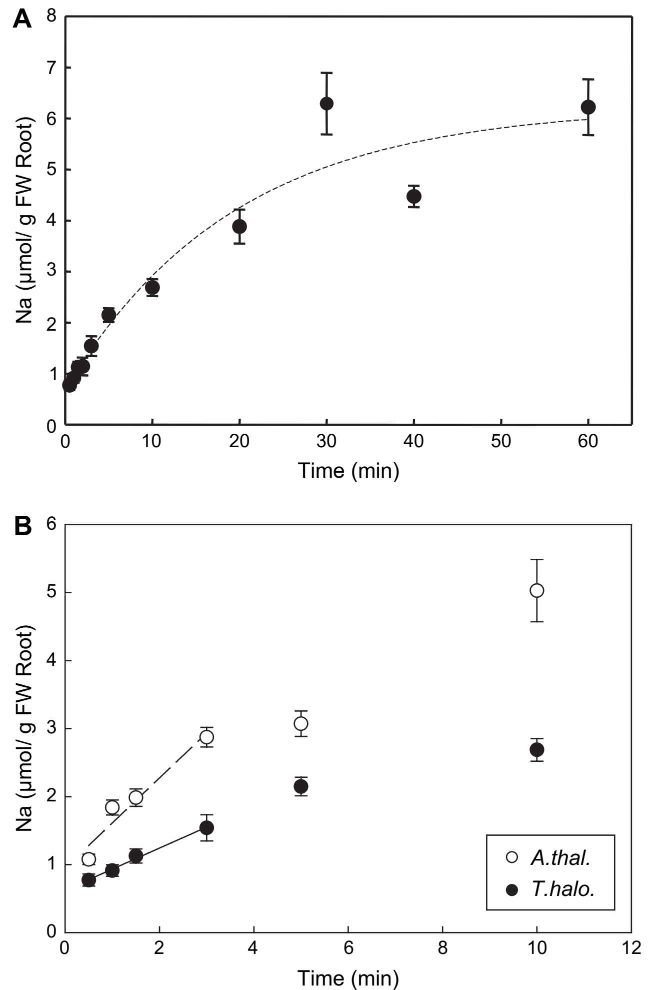

Fig. 4. Kinetics of steady-state root Na influx in *A. thaliana* (open circles) and *T. halophila* (closed circles) as determined from ^{22}Na accumulation of individual plants from ^{22}Na labelled nutrient solution with 100 mM NaCl and 0.1 mM $CaCl_2$. Error bars are SE (n=4). (A) Time-course of ^{22}Na influx to roots of *T. halophila*. (B) Initial unidirectional Na influx into roots of *T. halophila* and *A. thaliana*.

Dependence of Na influx on external Na is less steep in T. halophila *than in* A. thaliana

Dose–response curves for Na influx were determined by plotting Na influx against external Na concentrations applied to both species (Fig. 5). Again, plants were grown for 1 week in the respective external solution to achieve steady-state. Since *A. thaliana* cannot tolerate Na concentrations above 100 mM for extended periods of time, higher Na levels could only be applied to *T. halophila*. Both curves were tentatively fitted with Michaelis–Menten kinetics (note that saturation was not yet reached in *A. thaliana*) revealing K_m values of 102 mM and 671 mM and V_{max} values of 0.79 and 0.66 μmol g^{-1} FW min^{-1} for *A. thaliana* and *T. halophila*, respectively (Table 1). This suggests that Na influx pathways exhibit lower affinity for Na in *T. halophila* than in *A. thaliana*.

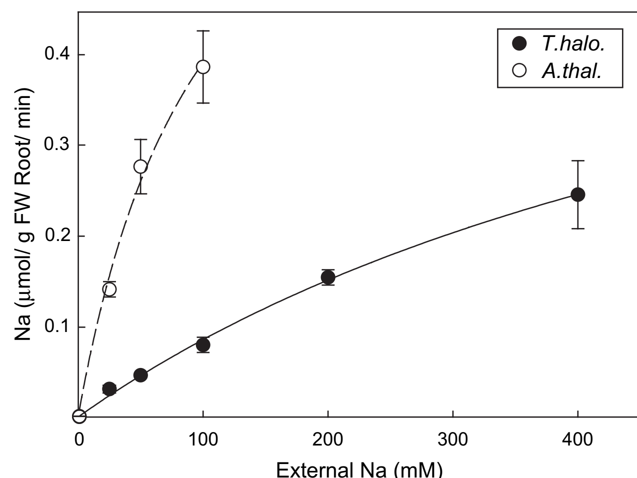

Fig. 5. Dependence of Na influx to roots of *A. thaliana* (open circles) and *T. halophila* (closed circles) on external Na concentration. Na content was determined by measuring tracer ^{22}Na level in roots of individual plants after 18 min labelling in ^{22}Na-labelled growth solution containing 0.1 mM $CaCl_2$ and the indicated amount of NaCl. Error bars are SE (n=4).

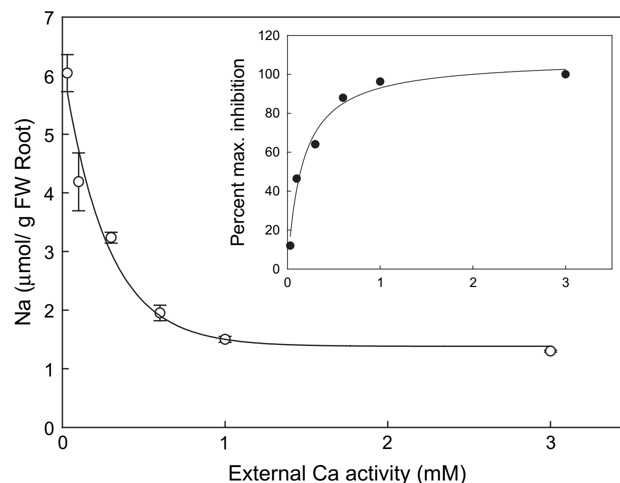

Fig. 6. Effect of external Ca on Na influx into roots of *T. halophila*. Na content was determined by measuring tracer ^{22}Na level in roots of individual plants after 15 min labelling in ^{22}Na-labelled growth solution with 100 mM NaCl and various Ca activities. Error bars are SE (n=4).

Na influx into roots is not inhibited by Cs and TEA

Voltage-dependent K channels are inhibited by Cs and TEA (Véry and Sentenac, 2003) whereas voltage-independent cation channels are not affected by these blockers (Maathuis and Sanders, 2001; Demidchik and Tester, 2002; V Volkov and A Amtmann, unpublished results). To assess the contribution of these channel types to unidirectional Na uptake, the effect of Cs and TEA on root Na influx (initial 5 min) was measured in the two species. In both species Na influx *increased* rather than decreased when Cs or TEA was applied. Influx after the addition of 5 mM CsCl was 135.4±2.7% and 161.0±3.6% of the control influx in *A. thaliana* and *T. halophila*, respectively. After the addition of 20 mM TEACl, influx increased to 192.1±2.2% of the control influx in *A. thaliana* and to 156.6±4.5% in *T. halophila*.

T. halophila *Na uptake is inhibited by increasing external Ca concentrations*

Inhibition of Na uptake by external Ca has been shown for *A. thaliana*, wheat, and maize (Zidan *et al.*, 1991; Davenport *et al.*, 1997; Essah *et al.*, 2003) and patch clamp experiments with root protoplasts from these species indicated that this is partly due to Ca inhibition of Na-permeable voltage-independent channels (Roberts and Tester 1997; Tyerman *et al.*, 1997; Demidchik and Tester, 2002). The effect of external Ca on Na influx in *T. halophila* was determined by measuring ^{22}Na levels in roots after 15 min incubation in ^{22}Na-labelled medium with 100 mM NaCl and various Ca activities. A dose–response curve is shown in Fig. 6. Similarly to other species, Na influx into *T. halophila* roots was strongly inhibited by external Ca with a K_i of 160 μM (see inset in Fig. 6; Table 1). Inhibition by Ca was not complete with approximately 20% of the maximum influx remaining at 3 mM external Ca.

Differences in Na efflux between A. thaliana *and* T. halophila *are small*

To determine Na efflux kinetics, *A. thaliana* and *T. halophila* plants were loaded with ^{22}Na for 24 h and excised roots were subsequently transferred to unlabelled solution containing 100 mM Na. The time-course of ^{22}Na release into the external medium was measured over 250 min by transferring the roots into fresh solution at given time points and measuring the radioactivity in the medium samples. Assuming that in both species root ^{22}Na was equilibrated with the external medium at the beginning of the efflux measurements, total Na efflux can be determined from the amount of ^{22}Na released into the medium. Figure 7A shows the time-courses of Na efflux for both species. Best fits were achieved with equations for an exponential decay described by the sum of three exponentials plus a constant offset, thus reflecting the contribution of at least four different types of Na pools within the roots (Table 1). Due to the complex arrangements of cellular compartments within a whole root, assignment of the kinetic components to distinct compartments is difficult. The first extremely fast component is likely to reflect removal of loosely bound Na from the root surface. The fact that this parameter differs between the two species was also apparent in the influx curves (Fig. 4B at time 0) and could be due to different physical and chemical cell wall properties. It was assumed that the second kinetic component with time constants of 2.2 min and 0.6 min in *A. thaliana* and *T. halophila*, respectively, combines apoplastic and cytoplasmic efflux components, whereas the third component with time constants of 33 min and 50 min, respectively, embraces

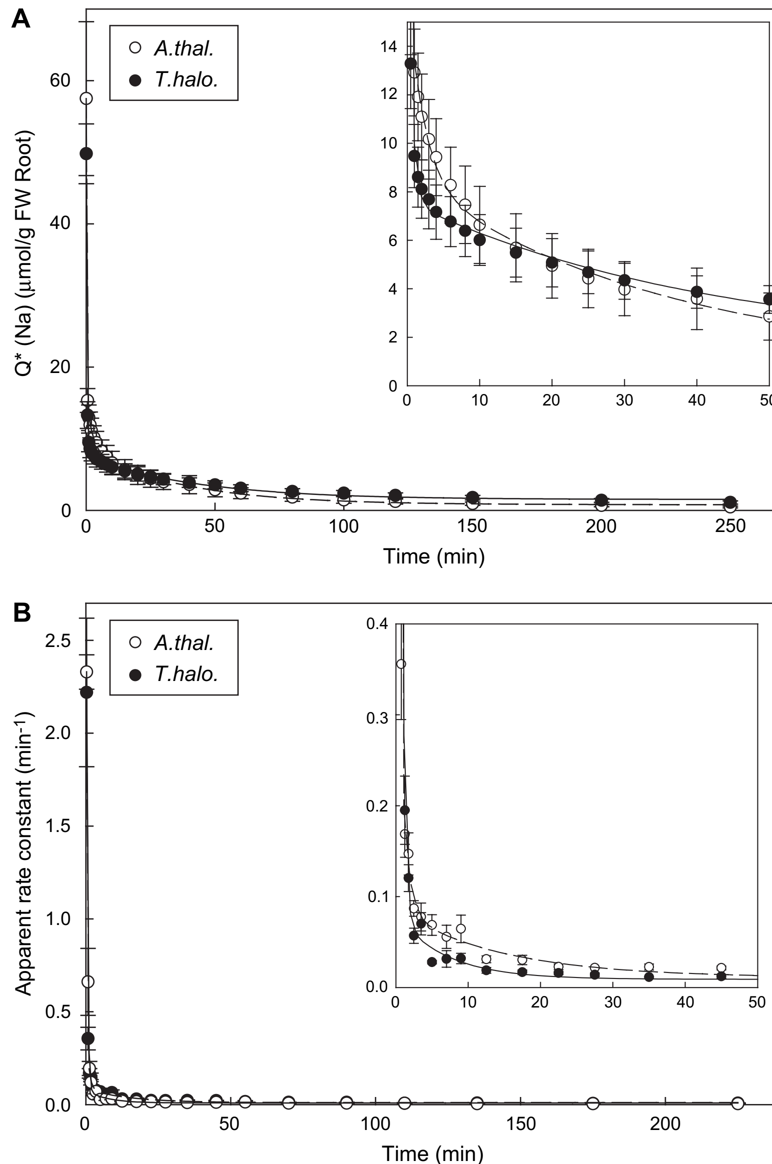

Fig. 7. Kinetics of Na efflux from roots of *A. thaliana* (open circles) and *T. halophila* (closed circles). (A) Na contents of individual plants were determined by measuring tracer ^{22}Na level in root samples and aliquots of the efflux solution at a series of time points. Error bars are SE (n=3). (B) Apparent rate constants were calculated according to MacRobbie (1981).

cytoplasmic and vacuolar contributions. A pool of Na that did not exchange with the external medium over the assessed period of time, apparent as a constant offset in the fitted equation, might reflect Na trapped in the root xylem.

To decrease the complexity of the efflux kinetics, apparent rate constants were calculated according to MacRobbie (1981). This analysis takes into account the amount of ^{22}Na remaining in the tissue at any moment of the efflux time-course. Thus ^{22}Na release over a given period of time (i.e. between two adjacent time points) is related to the mean ^{22}Na concentration present in the tissue over this period of time. A plot of apparent efflux rate constants against time is shown in Fig. 7B. The resulting curve shows that, apart from the first minute of the experiment (reflecting exchange of apoplastic Na), efflux rate constants were always higher (i.e. efflux was faster) in *A. thaliana* than in *T. halophila*.

Discussion

Comparative analysis of ion transport in *A. thaliana* and *T. halophila* plants provides an exciting opportunity to study the molecular and regulatory features of ion transporters as they have evolved through natural selection in saline conditions. The availability of molecular tools and

transformation protocols for both species will allow for gene transfer between *T. halophila* and *A. thaliana*, thus enabling the transfer of isolated components of halophytic strategies into a glycophyte and the assessment of their implications at the organismal level. The current study has been carried out bearing in mind this diagnostic potential of *T. halophila*.

Comparison of Na and K tissue levels between *T. halophila* and *A. thaliana* after salt application revealed two main differences that could explain differential salt tolerance of the two species. Firstly, *T. halophila* maintains tissue K levels when exposed to high salt whereas *A. thaliana* loses K under these conditions. The observed net loss of K from *A. thaliana* roots is likely to be due to salt-induced membrane depolarization of root cells. Recent experiments from this laboratory showed that a positive shift of the membrane potential of root cells after the addition of NaCl to the medium was indeed more pronounced in *A. thaliana* than in *T. halophila*, thus favouring K efflux in this species (V Volkov and A Amtmann, unpublished results). Secondly, *T. halophila* accumulates less Na than *A. thaliana* both in the short term and over extended periods of salt stress. Interestingly, net Na uptake rates into roots were very similar during the first hours after the addition of NaCl, but strongly differed at later time points (Fig. 2). It is suggested that the initial rise of root Na provides a measure for the steady-state Na uptake capacity established under low-salt conditions, whereas later kinetic components reflect an adjustment of this parameter to high-salt conditions (e.g. through transcriptional regulation of ion transporters). To test whether low net Na accumulation in salt-acclimated *T. halophila* was due to reduced uptake or enhanced export of Na into/from root cells, unidirectional fluxes of ^{22}Na were measured under steady-state conditions of 100 mM external NaCl. The unidirectional Na influx determined for *A. thaliana* was smaller than previously measured by Essah *et al.* (2003) using comparable Na and Ca concentrations in the medium. The most likely explanation for this difference is the fact that, for this study, plants were acclimated to the salt treatment prior to the experiments and therefore steady-state fluxes were determined, whereas Essah and colleagues applied NaCl together with ^{22}Na and therefore measured non-steady-state fluxes. It was found that unidirectional Na influx is significantly lower in *T. halophila* than in *A. thaliana* (Fig. 4B). Assessment of Na dependency of Na influx suggested that the affinity and the maximal rate of the root Na uptake were lower than in *A. thaliana*. It therefore appears that the low rate of net Na accumulation observed for *T. halophila* is at least in part due to reduced influx of Na into the roots of this species.

These findings are in agreement with results from this laboratory's previous analysis of cation currents across the plasma membrane of root cells using patch clamp (Volkov *et al.*, 2004). Inward current through voltage-independent channels, which provide the main pathway for Na uptake in *A. thaliana* (Maathuis and Sanders, 2001; Demidchik and Tester, 2002), was smaller in *T. halophila* and more selective for K over Na than in *A. thaliana*. A recent more detailed analysis of this channel type in *T. halophila* revealed that it is inhibited by external Ca but not by Cs or TEA although the latter two ions block voltage-dependent inward and outward rectifying channels (V Volkov and A Amtmann, unpublished results). The observation that unidirectional Na influx in *T. halophila* roots is inhibited by external Ca, but not by Cs or TEA, suggests that voltage-independent channels are the main pathways responsible for Na uptake in *T. halophila*. Enhancement of Na influx in the presence of Cs and TEA was found for both *A. thaliana* and *T. halophila*. The data can be explained with a hyperpolarization of the root plasma membrane due to *decreased* K permeability, which in turn drives increased uptake of Na through channels that are *not* blocked by these compounds. It therefore appears that voltage-independent channels comprise the main uptake pathway for Na in both species. More detailed analysis of the effects of Cs and TEA on membrane potential and K conductance in the two species is required to explain these data fully.

The observed inhibitory profiles of Na influx and voltage-independent channels in *T. halophila* are qualitatively very similar to those determined for *A. thaliana* (Maathuis and Sanders, 2001; Demidchik and Tester, 2002), wheat (Tyerman *et al.*, 1997; Davenport and Tester, 2000), and maize (Roberts and Tester, 1997) suggesting that pathways for Na uptake are of similar nature in all of these species. However, quantitative data for conductance and ion selectivity of the voltage-independent cation channel type differ between *T. halophila* and the glycophytic species, which suggests that the respective channel proteins have species-specific structural features. No genes for Na uptake channels have been identified so far, although members of the cyclic-nucleotide gated channel (CNGC) gene family have emerged as likely candidates (Maathuis and Sanders, 2001). Identification of the genes underlying root Na uptake in *T. halophila* and *A. thaliana* is one of the major challenges for the future and will allow their role in salt tolerance to be studied further. In particular, structure–function analysis is likely to reveal important differences between ion channel proteins of salt-sensitive and salt-tolerant species with respect to their K/Na selectivity.

It is interesting to compare the measured rates of unidirectional uptake with those of net Na uptake into the plants. Quantitative comparison can be based on previous determination of shoot/root dry matter ratios (S:R 7:1 in *A. thaliana* and 3.5:1 in *T. halophila*) and fresh weight to dry weight ratios (FW/DW equal to 20 and 12 in roots of *A. thaliana* and *T. halophila*, respectively). Thus whole plant net Na accumulation in 100 mM salt was 4.35 mmol Na g^{-1}

root DW in *A. thaliana* and 1.31 mmol Na g^{-1} root DW in *T. halophila* over 25 h (from Fig. 1). Measured unidirectional influx of Na into the roots of 0.66 μmol Na g^{-1} FW min^{-1} in *A. thaliana*, and 0.31 μmol Na g^{-1} FW min^{-1} in *T. halophila* (Table 1) would lead to a net uptake of 19.8 mmol Na g^{-1} root DW in *A. thaliana* and 5.6 mmol Na g^{-1} root DW in *T. halophila* over 25 h if not counteracted by Na efflux. These values are 4.6 and 4.3 times higher than the respective values for net Na accumulation. This suggests that, in both species, a large proportion (77–78%) of the Na taken up into the plant is immediately exported back into the external medium and does not therefore appear as net Na accumulation. Even if the different Ca levels in the media used for determining net accumulation (0.5 mM Ca) and unidirectional influx (0.1 mM Ca) and their inhibitory effect on Na influx are taken into account, as well as the fact that low transpiration during the dark period will reduce the average influx over the 25 h period, values for unidirectional Na influx that are twice the net Na uptake are still obtained. This value will rise again if it is considered that net Na accumulation was determined in non-steady-state conditions and therefore includes an initial period of high uptake rates. Within the range of these assumptions *absolute* rates of unidirectional efflux are calculated between 0.44 and 0.51 μmol Na g^{-1} FW min^{-1} for *A. thaliana* and between 0.09 and 0.24 μmol Na g^{-1} FW min^{-1} for *T. halophila* roots. Efflux of this magnitude was indeed observed within the first 10 min of the efflux time-course (excluding the first 2 min, Fig. 7B), suggesting that the cytoplasmic compartment is the main contributor to ^{22}Na efflux over this period of time.

The main conclusions from this exercise are the following: (i) lower net accumulation of Na in *T. halophila* compared with *A. thaliana* is due to lower Na influx rather than higher efflux; (ii) in both species a large proportion (more than half) of unidirectional Na influx is counterbalanced by Na efflux, and (iii) considering the Na influx in *A. thaliana* is twice that in *T. halophila*, absolute Na efflux has to be larger in the glycophytic species than in the halophytic species to account for the observed rates of net Na uptake. It is concluded that the glycophytic species spends more energy on Na efflux and is still not capable of reducing its net Na uptake to the low rate observed for the halophyte.

Only small differences were found between *T. halophila* and *A. thaliana* in Na efflux curves and the variation of the data did not allow statistically significant separation. As discussed by Davenport *et al.* (2005), this is an inherent problem when using efflux curves for quantitative comparative analysis due to the fact that large differences in efflux result in only very small deviations of the curves. Quantitative analysis of the measured efflux kinetics revealed a complex arrangement of exchangeable Na pools. This was expected as the experiments were carried out on whole roots and reflected the transport between different cellular compartments and tissues. However, determination of apparent efflux rate constants showed that Na efflux is generally higher in *A. thaliana* than in *T. halophila*. This finding agrees with the above calculations. It might also explain why *A. thaliana* accumulates less Na under low-salt conditions although influx under these conditions appeared to be similar in the two species (see Fig. 1 and initial slope of root Na uptake in Fig. 2). Active Na export in higher plants is coupled to proton import (Amtmann and Sanders, 1999). Molecular candidates for Na/H antiport are genes of the NHX family such as SOS1 (Shi *et al.*, 2000), which, when over-expressed, increases salt tolerance in *A. thaliana* (Shi *et al.*, 2003). Using an *A. thaliana* full-length cDNA microarray, Taji *et al.* (2004) found that SOS1 paralogues showed constitutively higher expression in *T. halophila* than in *A. thaliana*. However, recent studies with *A. thaliana* oligomer arrays did not confirm this observation (Gong *et al.*, 2005; B Wang and A Amtmann, unpublished results). In the light of the results presented here SOS1 is considered an unlikely candidate for explaining salt tolerance in *T. halophila*.

The results from this study stress the importance of reduced unidirectional Na influx in *T. halophila* and suggest that salt tolerance in *A. thaliana* is limited by the capability to compensate Na influx with energy-consuming Na efflux. Voltage-independent ion channels emerge as prime candidates for molecular entities underlying differential salt tolerance in the two species. It is proposed that low conductance and high K/Na selectivity of this channel type (Volkov *et al.*, 2004) allows *T. halophila* to maintain a positive balance between growth and Na accumulation in saline conditions. Further studies are required to understand the contributions of root–shoot barriers and tissue-specific ion allocation to Na homeostasis in *T. halophila*.

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