Variation in salinity tolerance and shoot sodium accumulation in *Arabidopsis* ecotypes linked to differences in the natural expression levels of transporters involved in sodium transport

D. JHA, N. SHIRLEY, M. TESTER & S. J. ROY

*The Australian Centre for Plant Functional Genomics and the University of Adelaide, PMB1, Glen Osmond, SA 5064, Australia*

**ABSTRACT**

Salinity tolerance can be attributed to three different mechanisms: Na\(^+\) exclusion from the shoot, Na\(^+\) tissue tolerance and osmotic tolerance. Although several key ion channels and transporters involved in these processes are known, the variation in expression profiles and the effects of these proteins on Na\(^+\) transport in different accessions of the same species are unknown. Here, expression profiles of the genes *AtHKT1;1*, *AtSOS1*, *AtNHX1* and *AtAVP1* are determined in four ecotypes of *Arabidopsis thaliana*. Not only are these genes differentially regulated between ecotypes, the expression levels of the genes can be linked to the concentration of Na\(^+\) in the plant. An inverse relationship was found between *AtSOS1* expression in the root and total plant Na\(^+\) accumulation, supporting a role for *AtSOS1* in Na\(^+\) efflux from the plant. Similarly, ecotypes with high expression levels of *AtHKT1;1* in the root had lower shoot Na\(^+\) concentrations, due to the hypothesized role of *AtHKT1;1* in retrieval of Na\(^+\) from the transpiration stream. The inverse relationship between shoot Na\(^+\) concentration and salinity tolerance typical of most cereal crop plants was not demonstrated, but a positive relationship was found between salt tolerance and levels of *AtAVP1* expression, which may be related to tissue tolerance.

**Key-words:** *AtSOS1*, *AtHKT1;1*, *AtAVP1*, *AtNHX1*; sodium transport.

**INTRODUCTION**

Soil salinity is a major abiotic stress affecting agricultural land which results in significant losses of crop yield. A large contributor to salinity stress is the build-up of the sodium ion (Na\(^+\)) in the cytoplasm of leaf cells. High cytoplasmic Na\(^+\) interferes with processes that require the binding of potassium (K\(^+\)), protein synthesis and the activation of key metabolic enzymes (Bhandal & Malik 1988; Blaha et al. 2000; Tester & Davenport 2003; Munns, James & Lauchli 2006; Munns & Tester 2008). One key mechanism of salinity tolerance is the ability of a plant to control Na\(^+\) transport at both the tissue and cellular level, either by secreting Na\(^+\) into tissues, cells and organelles where it can do little damage, or by minimizing the amount of Na\(^+\) entering the plant through its roots (Tester & Davenport 2003; Apse & Blumwald 2007; Munns & Tester 2008). Exclusion of Na\(^+\) from the shoot has frequently been observed as a central mechanism in salinity tolerance for cereal crops such as durum wheat (Gorham 1990; Munns & James 2003), bread wheat (Gorham 1990; Schachtman & Munns 1992; Pouštini & Siosomardeh 2004), barley (Forster 2001; Wei et al. 2003; Pritchard et al. 2004; Garthwaite, von Bothmer & Colmer 2005) and rice (Zhu, Kinet & Lutts 2001); however, it has become apparent in some crop varieties that tissue tolerance mechanisms are also important (El-hendawy, Hu & Schmidhalter 2005; Gené, McDonald & Tester 2007). Therefore, an understanding of the mechanisms and control of movement of Na\(^+\) through a plant and the genes involved is crucial if the ability of our current crop varieties to grow in saline soil is to be improved.

Na\(^+\) exclusion from the shoot has often been cited as an important mechanism in plant salinity tolerance (Munns et al. 2000; Tester & Davenport 2003; Garthwaite et al. 2005; Colmer, Flowers & Munns 2006). In *Arabidopsis* and other species, genes belonging to the *HKT* or *SOS1* families have been implicated in controlling Na\(^+\) movement throughout the plant. The *HKT* gene family encodes proteins that are responsible for the influx of Na\(^+\) into a cell (Uozumi et al. 2000). Members of the family can be divided into two subgroups depending on their function, as either a Na\(^+\) uniporter or a Na\(^+\) and K\(^+\) symporter (Platten et al. 2006). Members of subgroup 1, the Na\(^+\) uniporters (Platten et al. 2006), have been shown to be important in salinity tolerance. In *Arabidopsis*, *Athkt1;1* mutants and the ecotypes Ts1 and Tsu1, which have no root *AtHKT1;1* expression, hyperaccumulate Na\(^+\) in the shoot while showing reduced Na\(^+\) accumulation in the root (Maser et al. 2002; Berthomieu et al. 2003; Rus et al. 2004; Rus et al. 2006). Further characterization has revealed that...
AtHKT1;1 is important in retrieving Na\(^+\) from the xylem in the root, thereby minimizing the amount of Na\(^+\) entering the shoot through the transpiration stream (Davenport et al. 2007). A role of AtHKT1;1 in the recirculation of Na\(^+\) from the shoot, through removal of Na\(^+\) from the xylem and the facilitation of Na\(^+\) loading into the phloem, has also been proposed by Sunarpi et al. (2005). Anti-

HKT1;1 antibodies were found to bind to the plasma membrane of shoot xylem parenchyma cells and Athkt1-3 knockouts had higher concentrations of Na\(^+\) in the shoot xylem and lower concentrations in the phloem than wild-type plants. Similar functions have been identified or proposed for members of HKT subgroup 1 in rice (Ren et al. 2000, 2002) and wheat (James, Davenport & Munns 2006; Byrt et al. 2007).

Members of the plasma membrane Na\(^+\)/H\(^+\) antipporter SOS1 family have also been implicated in reducing the amount of Na\(^+\) transported to the shoot in the transpiration stream; however, unlike members of the HKT family, SOS1 proteins have been shown to efflux Na\(^+\) from cells back into the external medium (Qiu et al. 2002; Shi et al. 2002). In Arabidopsis, AtSOS1 mutants have been shown to be hypersensitive to Na\(^+\) stress, showing severe growth retardation and high shoot and xylem Na\(^+\) concentrations which ultimately lead to the death of the plant (Wu, Ding & Zhu 1996; Shi et al. 2000, 2002). While initial studies localized AtSOS1 gene expression to epidermal cells at the root tip and in parenchyma cells at the xylem/symplast boundary, therefore suggesting a role in regulating xylem loading (Shi et al. 2002), more recent results suggest a role in Na\(^+\) efflux from the root (Shabala et al. 2005). Similar results for other SOS1 proteins have also been observed in poplar (Wu et al. 2007). Thellungiella halophila (Vera-Estrella et al. 2005), wheat (Mullan, Colmer & Francki 2007) and rice (Martinez-Atienza et al. 2007). Interestingly, the salt sensitivity of AtSOS1 mutants can be reduced by mutation of ATHKT1;1 (Rus et al. 2004).

A key group of transporters involved in Na\(^+\) tissue tolerance mechanisms are the vacuolar Na\(^+\)/H\(^+\) antiporters, such as AtNHX1, which are responsible for detoxifying the cytoplasm by pumping Na\(^+\) into the vacuole (Gaxiola et al. 1999; Pardo et al. 2006). There are numerous studies showing that overexpression of AtNHX1, or its homologs from other plant species, results in salt-tolerant plants (Apse et al. 1999; Zhang & Blumwald 2001; Xue et al. 2004; He et al. 2005; Chen et al. 2007). Similarly, overexpression of AtAVP1, or its homologs, leads to increased salinity tolerance by enhancing the accumulation of Na\(^+\) in the vacuole (Gaxiola et al. 2001; Zhao et al. 2006). AtAVP1 encodes a vacuolar H\(^+\) translocating pyrophosphatase which increases the difference in the electrochemical potential of H\(^+\) between the vacuole and cytoplasm, thereby energizing the movement of Na\(^+\) into the vacuole through Na\(^+\)/H\(^+\) antiporters such as AtNHX1 (Gaxiola et al. 2001; Tester & Davenport 2003; Munns & Tester 2008).

While much is known about the mechanism of action of individual plant transporters under conditions of salt stress, little work has been carried out on the characterization and interaction of the genes encoding these transporters. Furthermore, it is not known how widespread particular mechanisms are to deal with salt stress, or whether different plants regulate different genes to achieve a particular level of tolerance.

The aim of this study was to investigate the salinity tolerance of four Arabidopsis ecotypes, Columbia (Col), Landsberg erecta (Ler), Wassilewskija (Ws) and C24, commonly used in salinity studies (Zhu, 2000; Quesada et al. 2002; Gao et al. 2003; Sunarpi et al. 2005; Kim et al. 2007; Kant et al. 2008; Möller et al. 2009), to determine whether the tolerance of these ecotypes is correlated with the expression of four important transporters, AtHKT1;1, AtSOS1, AtNHX1 and AtAVP1, which are involved in regulating the movement of Na\(^+\). While the results reveal no inverse relationship between shoot Na\(^+\) accumulation and salinity tolerance in these Arabidopsis ecotypes, there is a positive relationship between tolerance and levels of AtAVP1 expression, which may be related to tissue tolerance that could be conferred by this vacuolar H\(^+\) pump. Inverse relationships were demonstrated between the expression levels of AtSOS1 in the root and total plant Na\(^+\) accumulation and between the expression levels of AtHKT1;1 in the root and shoot Na\(^+\) concentration. These can be explained by the removal of Na\(^+\) from the root by AtSOS1 and by retrieval of Na\(^+\) from the transpiration stream in the roots by AtHKT1;1.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

Seeds of Arabidopsis thaliana ecotypes Col, Ws, Ler and C24 were obtained from the European Arabidopsis Stock Centre (Nottingham, UK). Seeds were first surface sterilized, by soaking in 70% ethanol for 2 min followed by 3–4 rinses in sterile milli-Q water, before individual seeds were planted in 1.5 mL microfuge tubes filled with half-strength Arabidopsis nutrient solution (Artica & Artica 2000) and 0.8% Bacto agar. The seeds were vernalized for 2 d at 4 °C and then transferred to a growth room with a 10 h light/14 h dark photoperiod, an irradiance of 70 mmol m\(^{-2}\) s\(^{-1}\) and a constant temperature of 21 °C. The bottom 0.5–0.7 cm of the microfuge tubes were removed after emergence of the cotyledon and the roots of the seedling had grown approximately two-thirds down the length of the tube. Upon emergence of the root from the agar, the plants were transferred to a constantly aerated hydroponics tank containing full-strength Arabidopsis nutrient solution. The pH of the hydroponic solution was monitored and maintained at pH 5.7. Salt stress was applied 5 weeks after germination by the addition of 100 mM NaCl in 12 hourly increments of 25 mM. Calcium activity in the growth medium was maintained at 0.3 mM at each salt application by addition of the correct amount of calcium, as calculated using Visual Minteq Version 2.3 (KTH, Department of Land and Water Resources Engineering, Stockholm, Sweden).
Phenotyping of ecotypes

Plants were harvested after 5 d of salt treatment. Whole roots and shoots of control and salt-treated plants were excised separately and fresh weights were recorded. Plant material was dried at 65 °C for 2 d and salinity tolerance was determined by comparing differences between the dry weights of control and salt-treated plants. Samples were digested in 70% nitric acid and 30% hydrogen peroxide for 2.5 h at 120 °C in a Hot Block (Environmental Express, Mt. Pleasant, SC, USA). Na⁺ and K⁺ concentrations were measured using inductively coupled plasma spectrometry–atomic emission spectra. The results presented are the average and standard error of the mean (SEM) for five biological replicates.

Expression analysis of Na⁺ related transporters

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the protocol described by Chomczynski 1993. Genomic DNA contamination was removed using Ambion’s DNA-free kit (Promega, Madison, WI, USA) and 200 ng of total RNA was used to synthesize cDNA using Superscript III (Invitrogen). Quantitative real-time PCR (Q-PCR) was performed on the cDNA for the transporters AtHK1;1 (At4g10310), AtSOS1 (At2g01980), AtAVP1 (At1g78920) and AtNHX1 (At5g27150), following the protocol outlined in Burton et al. 2008 using a RG6000 Rotor-Gene Real Time Thermal Cycler (Corbett Research, Sydney, Australia). For primer sequences, see Supporting Information Table S1.

A two-round normalization of Q-PCR data, by geometric averaging of multiple control genes, was carried out as described by Vandesompele et al. 2002. Cyclophilin (At2g36130), tubulin alpha-2 chain (TUA2, At1g50010), actin 2 (ACT2, At3g18780) and glyceraldehyde 3-phosphate dehydrogenase A (GAPA, At3g26650) were used in this study. Briefly, the expression data for each of the genes are normalized with respect to the biological replicates from the same treatment, using the three control genes which show the lowest variation between samples. The geometric mean of expression for all genes is then calculated from the normalized replicates and SE determined. The second round of normalization standardizes the mean of gene expression from each individual treatment to each other (Vandesompele et al. 2002). The SE derived from the first round of normalization was scaled using the normalization factors for the second round. The results presented are the average ± SEM for three biological replicates.

22Na⁺ influx and translocation assays

Unidirectional sodium influx, root to shoot Na⁺ translocation and the amount of sodium retained in the upper part of the root was measured using 22Na⁺ (GE Healthcare, Rydalmere, Australia), following previously published protocols (Essah, Davenport & Tester 2003; Davenport et al. 2007). Unidirectional 22Na⁺ influx was determined in roots of 4-week-old seedlings grown in square Petri dishes on half-strength Murashige and Skoog medium (pH 5.7) (Sigma-Aldrich, St. Louis, MO, USA), 1% w/v sucrose and 0.3% phytagel (Sigma-Aldrich). Excised roots, pooled from three to four individual plants, were pre-treated for 10 min in unlabelled influx solution, containing 50 mM NaCl and 0.5 mM CaCl₂. Roots were blotted gently and transferred to 15 mL of labelled influx solution, containing 50 mM NaCl, 0.5 mM CaCl₂ and 5.5 μCi 22Na⁺, and gently incubated at room temperature (RT) on a rotating shaker for 2 min. The roots were rinsed twice in ice-cold rinsing solution (50 mM NaCl and 10 mM CaCl₂) for 2 and 3 min, respectively. Once blotted dry, the roots were weighed, mixed with 4 mL of EcoLume liquid scintillation fluid (MP Biomedicals, Sydney, Australia) and measured using a liquid scintillation counter (Beckman Coulter LS6500, Gladesville, Australia).

For measurements of root to shoot sodium translocation, as well as the amount of 22Na⁺ retained in the upper root, plants were grown in hydroponics for 5 weeks, followed by pre-treatment with 50 mM NaCl for 5 d, as described earlier. Individual plants were removed from the hydroponics and the lower half of their roots suspended in 50 mL polypropylene tubes, containing 10 mL of labelled influx solution (50 mM NaCl, 0.5 mM CaCl₂ and 5 μCi 22Na⁺). The plants were gently incubated at RT on a gently rotating shaker for 1 h before being separated into upper unlabelled root, lower labelled root and shoot. The samples were then rinsed, blotted, weighed, mixed with scintillation fluid and measured as previously described.

RESULTS

Arabidopsis salinity tolerance not related to shoot Na⁺ concentration

The Arabidopsis ecotypes Col, Ler, Ws and C24 were grown in hydroponics for 5 weeks before being incubated in either 0 or 100 mM NaCl for a further 5 d. As expected, salt-stressed plants showed a significant reduction in the amount of dry mass produced over the 5 d period; however, the level of reduction varied between ecotypes (Fig. 1 and Table 1). Ler and Ws are the more tolerant ecotypes, showing a minimal reduction of dry matter production and a high plant tolerance index when grown in 100 mM NaCl, while the growth rate of the two salt-sensitive ecotypes, Col and C24, was significantly reduced. Although there are large variations in dry weight production between ecotypes under salt stress, there appears to be little variation in terms of percentage water content of their roots and shoots, or in their root dry weight ratio under salt stress (Table 1).

Variation can also be observed in the concentrations of Na⁺ and K⁺ accumulating in the shoots and roots of the four different ecotypes (Table 2). Col and Ws accumulate low concentrations of Na⁺ in the shoot and higher concentrations in the root when compared with C24 and Ler. C24, however, appears better able to maintain higher concentrations of K⁺ in its roots when compared with the other.

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Figure 1. Representative photographs of Arabidopsis ecotypes Columbia (Col), Wassilewskija (Ws), Landsberg erecta (Ler) and C24 grown for 5 weeks in hydroponics before treatment with either 0 or 100 mM NaCl.

Table 1. Differences in salinity tolerance of four ecotypes of Arabidopsis

<table>
<thead>
<tr>
<th>Ecotype</th>
<th>Treatment (NaCl)</th>
<th>Total dry mass (mg)</th>
<th>Plant tolerance index (Total DW&lt;sub&gt;sal&lt;/sub&gt;/Total DW&lt;sub&gt;control&lt;/sub&gt;)</th>
<th>Root DW ratio (root&lt;sub&gt;DW&lt;/sub&gt;/root + shoot&lt;sub&gt;DW&lt;/sub&gt;)</th>
<th>% Water content (FW-DW)/FW</th>
</tr>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col</td>
<td>0 mM NaCl</td>
<td>59.9 ± 5.9</td>
<td>0.59</td>
<td>0.137</td>
<td>95.2</td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl</td>
<td>35.1 ± 2.9</td>
<td></td>
<td>0.175</td>
<td>94.6</td>
</tr>
<tr>
<td>Ws</td>
<td>0 mM NaCl</td>
<td>38.6 ± 1.6</td>
<td>0.84</td>
<td>0.134</td>
<td>95.4</td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl</td>
<td>32.6 ± 1.7</td>
<td></td>
<td>0.155</td>
<td>95.2</td>
</tr>
<tr>
<td>Ler</td>
<td>0 mM NaCl</td>
<td>22.4 ± 0.8</td>
<td>0.93</td>
<td>0.094</td>
<td>93.9</td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl</td>
<td>20.8 ± 2.0</td>
<td></td>
<td>0.091</td>
<td>95.4</td>
</tr>
<tr>
<td>C24</td>
<td>0 mM NaCl</td>
<td>42.5 ± 4.6</td>
<td>0.65</td>
<td>0.085</td>
<td>94.5</td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl</td>
<td>27.4 ± 1.7</td>
<td></td>
<td>0.072</td>
<td>94.9</td>
</tr>
</tbody>
</table>

Plants were grown in hydroponics for 5 weeks before treated with either 0 or 100 mM NaCl for 5 d. Fresh (FW) and dry weights (DW) of both roots and shoots were measured and used to calculate total plant dry mass, root DW ratio, % tissue water content and plant salinity tolerance. Results for total dry mass are the mean ± SEM of five biological replicates.

Table 2. Na<sup>+</sup> and K<sup>+</sup> concentrations in the roots and shoots of four ecotypes of Arabidopsis grown hydroponically for 5 weeks before treated to either 0 or 100 mM NaCl for 5 d

<table>
<thead>
<tr>
<th>Ecotype</th>
<th>Treatment</th>
<th>Root</th>
<th>Shoot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[Na&lt;sup&gt;+&lt;/sup&gt;] (mm)</td>
<td>[K&lt;sup&gt;+&lt;/sup&gt;] (mm)</td>
</tr>
<tr>
<td>Col</td>
<td>0 mM NaCl</td>
<td>1.5 ± 0.1</td>
<td>77 ± 2</td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl</td>
<td>57 ± 3</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>Ws</td>
<td>0 mM NaCl</td>
<td>2 ± 0.4</td>
<td>74 ± 3</td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl</td>
<td>55 ± 9</td>
<td>50 ± 6</td>
</tr>
<tr>
<td>Ler</td>
<td>0 mM NaCl</td>
<td>2 ± 0.2</td>
<td>93 ± 5</td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl</td>
<td>35 ± 1</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>C24</td>
<td>0 mM NaCl</td>
<td>1.5 ± 0.1</td>
<td>80 ± 2</td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl</td>
<td>36 ± 2</td>
<td>71 ± 3</td>
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</tbody>
</table>

Results are the mean ± SEM for five biological replicates.

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ecotypes. All ecotypes show a reduction in shoot K⁺ concentrations under salt stress. Interestingly, the plant salinity tolerance index is not inversely related to the amount of shoot Na⁺. Col, the ecotype accumulating the lowest shoot Na⁺, exhibits the lowest salinity tolerance, whereas Ler accumulates significantly higher concentrations of shoot Na⁺ while still maintaining near-normal levels of growth in 100 mM NaCl (Tables 1 & 2, Fig. 2a). Indeed, it appears that salinity tolerance in *Arabidopsis* may be related to a tissue tolerance mechanism (Fig. 2a), or to plant growth rate, with those ecotypes exhibiting slower growth and biomass accumulation in control conditions being more salt-tolerant plants (Fig. 2b). C24 is an exception to this observation, having high shoot Na⁺ accumulation and low tolerance. Indeed, C24 may not detect the salinity stress or might be missing a vital signalling mechanism as there is no change in the expression of the transporters studied in either the root or the shoot of C24, while the other ecotypes show considerable changes (Table 3). Using gas chromatography-mass spectrometry, and protocols previously established in the laboratory (Jacobs et al. 2007), we have also observed that there are no changes in the profile of 93 metabolites (including sugars, organic acids, amino acids and fatty acids) in the roots of salt-stressed C24 when, under similar circumstances, there are changes in the metabolite profile in the roots of Col-0 (data not shown).

### Expression profiling of Na⁺-related transporters

The variation observed between the growth and cation accumulation characteristics of the four ecotypes is also seen at the expression level. In the roots, Col, Ws and Ler show an up-regulation of the genes *AtSOS1*, *AtAVP1* and *AtNHX1* under salt-stressed conditions which is not observed in C24, while only Col and Ws show an increase in *AtHKT1;1* expression (Table 3). Similar observations are apparent in the shoot of salt-stressed Col, Ws and Ler, where significant increases in *AtAVP1* are observed which are not seen in C24; it appears that *AtNHX1* is up-regulated under saline conditions only in the shoot of Ler (Table 3). In the shoots, *AtSOS1* is up-regulated only in salt-treated Col and Ler (Table 3).

### Plant tissue tolerance related to *AtAVP1* expression

In both root and shoot, plant salinity tolerance is positively correlated with *AtAVP1* expression (Fig. 3a,b). Ler and Ws, which exhibit the highest expression levels of *AtAVP1* in both roots and shoots, demonstrate the highest salinity tolerance when compared with ecotypes Col and C24, which have low levels of both *AtAVP1* expression and salinity tolerance. The expression of *AtAVP1* is also correlated with the levels of *AtNHX1* expression under salt stress, in both roots and shoots, with the salt-tolerant Ler showing significantly higher levels of both *AtAVP1* and *AtNHX1* than the salt-sensitive C24 (Fig. 3c,d). Interestingly, this relationship between the genes is not observed in non-stressed conditions. Overall, though, there is no strong relationship between root or shoot *AtNHX1* expression and salinity tolerance (data not shown). There was also no correlation observed between tissue Na⁺ concentrations or amounts and expression of either *AtAVP1* or *AtNHX1*.

### Plant Na⁺ content related to *AtSOS1* and *AtHKT1;1* expression in roots

The amount of total plant Na⁺ was negatively correlated with root *AtSOS1* expression. Those ecotypes with high root *AtSOS1* expression under salt-stressed conditions, such as Ler and Col, showed significantly lower total plant Na⁺ than C24 which has low root *AtSOS1* expression and high
higher shoot Na+ than in Col. Indeed, Col is able to retain more Na+ in the upper half of the root than C24 suggesting the presence of a mechanism for removing Na+ from the transpiration stream that is present in Col but not in C24 (Table 4).

**DISCUSSION**

*Arabidopsis*, a good model for understanding salinity tolerance?

For many crop species, salinity tolerance is linked to shoot Na+ exclusion (Gorham 1990; Schachtman & Munns 1992; Forster 2001; Zhu et al. 2001; Munns & James 2003; Pouštini & Siosemardeh 2004; Garthwaite et al. 2005); however, in the four ecotypes of *Arabidopsis* used in this study, no such relationship was found. Instead, with the exception of C24, a positive relationship was found between Na+ accumulation and plant salinity tolerance, suggesting that *Arabidopsis* may use mechanisms involved with Na+ tissue tolerance, such as intracellular compartmentation and increased accumulation of compatible solutes (Munns & Tester 2008), more than Na+ exclusion. Interestingly, there appears to be an inverse correlation between growth rate and tolerance, with those ecotypes that are slow growing under control conditions appearing to be more salt tolerant under stressed conditions (Table 1 and Fig. 2b). Both Ler and Ws grew significantly slower than Col and C24 but showed greater salinity tolerance, perhaps because the slower growth rate results in slower water uptake, thereby enabling these plants to better partition Na+ entering the shoot from the transpiration stream. Rus et al. (2006) also observed no relationship between elevated shoot Na+ concentrations resulting in increased salt sensitivity in the ecotype Tsu1 which exhibited reduced *AtHKT1;1* expression, increased shoot Na+ and a greater salt tolerance when compared to the low shoot Na+ accumulating Col-0. This suggests that other mechanisms of tolerance, such as osmotic tolerance or tissue tolerance, may be more important in enabling *Arabidopsis* plants to grow in saline conditions than Na+ exclusion.

Salinity tolerance in wheat has been shown to rely on Na+ exclusion from the shoot (Munns & James 2003). Our results suggest that Na+ exclusion in *Arabidopsis* is not linked to salinity tolerance as strongly as it is in the cereals, consistent with previous concerns that *Arabidopsis* is not a good model system for studying overall salinity tolerance in cereals (Møller & Tester 2007); the use of other more relevant model species, such as rice, may be preferable. However, there is increasing evidence that shoot Na+ exclusion is not the only mechanism of salinity tolerance in

Table 3. Expression profile of *AtSOS1*, *AtHKT1;1*, *AtAVP1* and *AtNHX1* in the roots and in the shoots of Col, Ws, Ler and C24 grown for 5 weeks in hydroponics before treatment with 0 or 100 mm NaCl for 5 d
cereals; some studies, such as that of Australian bread wheat varieties (Genc et al. 2007) and of near-wild relatives of wheat, such as *Triticum monococcum* (Rajendran, Tester & Roy 2009), reveal a lack of correlation between shoot Na$^+$ and tolerance. *Arabidopsis* may serve as a model for studying the other salinity tolerance mechanisms which may be present in cereals, although further work will be required to establish whether or not this is the case.

*Arabidopsis* is nevertheless a good system for understanding Na$^+$ transport in to and through a plant and for investigating the effects of manipulation of the expression of transporters on shoot Na$^+$ concentrations. Indeed, many of the genes identified as important in the transport of Na$^+$, including some of those used in this study, were first discovered in *Arabidopsis*. Expression of these *Arabidopsis* genes in other species have been shown to increase the salt tolerance of these species (Zhang & Blumwald 2001; Xue et al. 2004; He et al. 2005; Zhao et al. 2006; Bao et al. 2009). In addition, the ability to complement knockout mutants of Na$^+$ transporters in *Arabidopsis*, using orthologous genes from cereals, provides important information regarding the function of these cereal genes, such as the complementation of *Atsos1* knockouts with *OsSOS1* (Martinez-Atienza et al. 2007). Furthermore, *Arabidopsis* has been used for the development of systems for manipulating the temporal and spatial expression of genes to control the transport of ions through a plant. Cell type-specific expression of *AtHKT1;1* in root stelar cells resulted in reduced shoot Na$^+$ and increased salinity tolerance (Møller et al. 2009). Such knowledge is invaluable to research in crops.

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**Figure 3.** (a,b) Relationship between root or shoot *AtAVP* expression, respectively, and plant salinity tolerance in four ecotypes of *Arabidopsis*. (c,d) Relationship between root or shoot *AtAVP* and *AtNHX1* expression, respectively, for Col (○), Ws (△), Ler (▼) and C24 (□). Plants were grown for 5 weeks in hydroponics before treatment with either 0 (open symbols) or 100 mM (closed symbols) NaCl for 5 d. The solid lines are linear regressions plotted between *AtAVP* and *AtNHX1* gene expression at 100 mM NaCl. Results are the mean ± SEM of three biological replicates. Col, Columbia; DW, dry weight; Ws, Wassilewskija; Ler, Landsberg erecta.
The roles of some key genes involved in Na⁺ transport

**AtHKT1;1**

Previous work has highlighted the importance of *AtHKT1;1* in reducing the amount of shoot Na⁺ accumulation through the retrieval of Na⁺ from root xylem cells (Davenport et al. 2007) as well as recirculation from shoot to root (Berthomieu et al. 2003; Sunarpi et al. 2005), rather than through reducing the initial entry of Na⁺ into the roots (Berthomieu et al. 2003; Essah et al. 2003). Studies using *AtHKT1;1* knockouts (Maser et al. 2002; Berthomieu et al. 2003; Rus et al. 2004) and root stellar-specific *AtHKT1;1* overexpression (Møller et al. 2009) confirm that the gene is important in reducing the amount of Na⁺ translocated to the shoot. In this study of wild-type ecotypes, an inverse relationship between levels of root *AtHKT1;1* expression and shoot Na⁺ accumulation (Fig. 4b) was also found, as well as a positive relationship between root Na⁺ accumulation and root expression (Tables 2 & 3, respectively). Ws and Col, which both significantly up-regulate *AtHKT1;1* under salt stress conditions, show lower shoot and higher root Na⁺ concentrations than the high shoot and low root accumulators, Ler and C24, which show no increase in gene expression. Similar observations have been reported in other *Arabidopsis* populations, such as the ecotypes Ts-1 and Tsu-1 which show low *AtHKT1;1* expression and high shoot Na⁺ accumulation (Rus et al. 2006). Interestingly, it was also found in this study that low shoot Na⁺ accumulation was not linked to salinity tolerance, with the Tsu1 plants having low root *AtHKT1;1* expression, high shoot Na⁺ accumulation and lower leaf death observed than Col-0. By selection of high and low *AtHKT1;1* expressing ecotypes (Col and C24, respectively), it can be demonstrated using ²²Na⁺ that this difference in gene expression is related to the amount of Na⁺ being translocated to the shoot and not the initial influx of Na⁺ from the growth medium (Table 4). It is hypothesized that Col uses *AtHKT1;1* to remove Na⁺ from the transpiration stream for compartmentation in the root.

**AtSOS1**

While increased root expression of *AtHKT1;1* leads to lower shoot Na⁺ accumulation, increased expression of *AtSOS1* in roots results in lower total plant Na⁺ (Fig. 4a), consistent with a role for *AtSOS1* in the efflux of Na⁺ from the plant roots back to the growth solution. While the results of this study reveal differences in *AtSOS1* root expression in natural populations leading to reduced plant Na⁺, similar observations have been made in transgenic *Arabidopsis*. In high-salt conditions, constitutive overexpression of *AtSOS1* in *Arabidopsis* has been shown to reduce plant Na⁺ accumulation by over 50%, with transgenic plants showing greater survival and growth rates than wild-type controls (Shi et al. 2003). Furthermore, *Atsos1* knockout plants hyperaccumulate Na⁺ and show severe reductions in plant survival (Shi et al. 2002). *AtSOS1* has been shown to be a plasma membrane-bound Na⁺/H⁺ antiporter which transports Na⁺ out of a cell (Shi et al. 2000, 2002; Qiu et al. 2003; Mahajan, Pandey & Tuteja 2008); this would suggest that the primary function of *AtSOS1* involves the movement of Na⁺ from the root back into the growth medium.

Such an observation would seem to be at odds with gene::GUS and gene::GFP fusions which suggest that *AtSOS1* is expressed predominantly around vascular tissue, the only epidermal tissue expression being at the root tip (Shi et al. 2002). In this case, the expectation is that salt would be pumped into the transpiration stream, resulting in an increase in shoot Na⁺, unless the protein were specifically targeted towards the plasma membrane on the opposite
At NHX1 activity.

Tolerance or a greater role for post-translational control of the greater importance of AtAVP1 in determining salinity

studies have shown that when Apse described to increase the salinity tolerance of Arabidopsis et al. constitutively overexpressed, plants such as tobacco (Gao vacuole (Gaxiola et al. greater salinity tolerance (Fig. 3a,b). that those ecotypes with high expression levels in ecotypes of the same species. It is shown but are a consequence of the natural variation in gene here are similar to those from the overexpression studies significantly higher salinity tolerance. The results presented which is consistent with the results presented here.

Results for unidirectional influx are presented as the mean ± SEM of 9 to 39 biological replicates.

Col, Columbia; Ws, Wassilewskija; Ler, Landsberg erecta.

side of the cell from the xylem conducting elements. Clearly, further work is required to investigate this discrepancy. However, Shabala et al. have demonstrated more recently that epidermal AtSOS1 activity could be found along the whole root and not just at the root tip (Shabala et al. 2005), which is consistent with the results presented here.

AtAVP1

Ecotypes which have increased salinity tolerance were found to have higher AtAVP1 expression in either or both the root and shoot (Fig. 3a,b). While AtAVP1 is not directly involved in Na⁺ transport, it is involved in establishing and maintaining an electrochemical potential difference for protons between the cytosol and the vacuole, to enable Na⁺/H⁺ antiporters, such as AtNHX1, to pump Na⁺ into the vacuole (Gaxiola et al. 2001). Previous overexpression studies have shown that when AtAVP1, or its homologs, are constitutively overexpressed, plants such as tobacco (Gao et al. 2006; Duan et al. 2007), rice (Zhao et al. 2006) and Arabidopsis (Gaxiola et al. 2001; Brini et al. 2007) have significantly higher salinity tolerance. The results presented here are similar to those from the overexpression studies but are a consequence of the natural variation in gene expression levels in ecotypes of the same species. It is shown that those ecotypes with high AtAVP1 expression also have greater salinity tolerance (Fig. 3a,b).

As might be expected, the level of expression of AtAVP1 was positively correlated with the expression of AtNHX1 in all tissues, but this was seen only in salt-stressed conditions (Fig. 3c,d). Overexpression of NHX genes has often been described to increase the salinity tolerance of Arabidopsis (Apse et al. 1999; Brini et al. 2007), rice (Chen et al. 2007), cotton (He et al. 2005), wheat (Xue et al. 2004) and tomato (Zhang & Blumwald 2001); nevertheless, the activity of the transporter depends upon a difference in electrochemical potential for protons, such as could be established and maintained by AtAVP1 ( Munns & Tester 2008). However, it is interesting to note that in the present work, there was no relationship observed between tolerance or tissue Na⁺ concentrations and AtNHX1 expression. This may reflect either the greater importance of AtAVP1 in determining salinity tolerance or a greater role for post-translational control of AtNHX1 activity.

Table 4. Measurements of unidirectional 22Na⁺ influx in excised roots, % translocation of 22Na⁺ to shoot and % 22Na⁺ retained in the unlabelled upper root, in the ecotypes Col and C24

<table>
<thead>
<tr>
<th>Ecotype</th>
<th>Unidirectional 22Na⁺ influx</th>
<th>Retrieval of 22Na⁺ from xylem</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>22Na⁺ influx (µmoles g⁻¹ root FW min⁻¹)</td>
<td>22Na⁺ translocated to shoot (% relative to total in plant)</td>
</tr>
<tr>
<td>Col</td>
<td>1.92 ± 0.14 (n = 23)</td>
<td>47.5 (n = 11)</td>
</tr>
<tr>
<td>C24</td>
<td>1.55 ± 0.09 (n = 39)</td>
<td>72.5 (n = 9)</td>
</tr>
</tbody>
</table>

Limitations of study

It should be noted, however, that changes in gene expression are not necessary linked with changes in protein abundance, nor can changes in gene expression elucidate the activity of the individual transporters. It may well be the case that one ecotype possesses a more effective version of a Na⁺ transporting protein or there are differences between the ecotypes in the effectiveness of the regulators of these proteins, such as differences within the CBL/CIPK Ca²⁺ signalling pathways. This study has only established correlations between the expression of commonly studied genes involved in Na⁺ transport through a plant and is not intended to establish causal relationships.

Does C24 lack a Na⁺ sensor?

Of particular interest in this study is the lack of response of C24 to salinity. While it is clear that the plant is salt stressed (Table 2, Fig. 1), there is no substantial increase in expression of AtHKT1;1, AtSOS1, AtNHX1 or AtAVP1 in either root or shoot tissue (Table 3). Similar observations have been made in C24 of a lack of increase in expression of other salt-responsive genes (S.J. Roy unpublished data) and in the metabolic profile of roots (D. Jha et al. unpublished data). By contrast, Col, which is similarly a salt-sensitive ecotype, responds to salinity stress by up-regulating genes in the root and the shoot. Indeed, all the genes studied were up-regulated in the root of Col plants (Table 3), and both AtSOS1 and AtAVP1 were up-regulated in the shoot (Table 3). The results here suggest either that C24 up-regulates other, perhaps novel, transporters not examined in this study, or that the ecotype lacks a key protein involved in the detection of salt stress or of a component at a high level in the pathway signalling salt stress to the cell. If the latter is the case, identification of this detection and/or signalling mechanism could provide insights into how a plant perceives salinity stress.

CONCLUSIONS

While it is demonstrated that there is no inverse relationship between shoot Na⁺ accumulation and salinity tolerance in Arabidopsis, shoot Na⁺ accumulation was found to vary
significantly between *Arabidopsis* ecotypes and this appears to be affected by the expression levels of *AtHKT1;1*, *AtAVP1* and *AtSOS1* (and not *AtNHX1*). An inverse relationship is observed between the expression levels of *AtSOS1* in the root and whole plant Na⁺ accumulation, as well as between the expression levels of *AtHKT1;1* in the root and Na⁺ concentration in the shoot. The effects of increased *AtSOS1* expression are proposed to be due to increased Na⁺ extrusion from the root; the effects of *AtHKT1;1* expression are likely to be due to enhanced retrieval of Na⁺ from the transpiration stream in the roots. The results also indicate that the expression of *AtAVP1* and *AtNHX1* is co-regulated, and that increased levels of *AtAVP1* expression are correlated with increased plant salinity tolerance.

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**REFERENCES**


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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Sequences of gene-specific primer pairs used in QRT-PCR experiments, including Temperature of acquisition (Taq) for Q-PCR.

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