Functional analysis of *AtHKT1* in *Arabidopsis* shows that Na\(^+\) recirculation by the phloem is crucial for salt tolerance

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Two allelic recessive mutations of *Arabidopsis*, *sas2-1* and *sas2-2*, were identified as inducing sodium over-accumulation in shoots. The *sas2* locus was found (by positional cloning) to correspond to the *AtHKT1* gene. Expression in *Xenopus* oocytes revealed that the *sas2-1* mutation did not affect the ionic selectivity of the transporter but strongly reduced the macroscopic (whole oocyte current) transport activity. In *Arabidopsis*, expression of *AtHKT1* was shown to be restricted to the phloem tissues in all organs. The *sas2-1* mutation strongly decreased Na\(^+\) concentration in the phloem sap. It led to Na\(^+\) overaccumulation in every aerial organ (except the stem), but to Na\(^+\) under-accumulation in roots. The *sas2* plants displayed increased sensitivity to NaCl, with reduced growth and even death under moderate salinity. The whole set of data indicates that *AtHKT1* is involved in Na\(^+\) recirculation from shoots to roots, probably by mediating Na\(^+\) loading into the phloem sap in shoots and unloading in roots, this recirculation removing large amounts of Na\(^+\) from the shoot and playing a crucial role in plant tolerance to salt.

*Keywords*: Na\(^+\) exclusion/Na\(^+\) transport/phloem/positional cloning/salt tolerance

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**Results**

**Identification of the *sas2* locus**

The screening procedure previously described for the isolation of the *sas1* mutant (Nublat *et al.*, 2001), applied to M\(_2\) seed derived from *Arabidopsis thaliana* ecotype Landsberg erecta after mutagenesis with ethyl methanesulfonate (EMS), led to the identification of a second mutant line, *sas2*, which also displayed the Sas1 phenotype, i.e. sodium overaccumulation in shoot. Another mutant line overaccumulating Na\(^+\) in shoot, 444B, was isolated later from the screening of a M\(_2\) population issued from EMS mutagenesis of *A. thaliana* ecotype Wassilewskija. Unlike *sas1* plants, which displayed a severely reduced size when compared with wild-type plants, *sas2* and 444B plants displayed no apparent phenotype in the absence of salt stress. When compared with wild-type plants, *sas2* and 444B plants overaccumulated Na\(^+\) whatever the growth conditions: *in vitro*, in
hydroponic conditions, or in the greenhouse (data not specifically illustrated but see Figures 1, 3 and 8). When grown in the greenhouse on compost watered with tap water supplemented with 50 mM NaCl, sas2 and 444B plants accumulated 2 and 7.5 times more Na⁺ in shoots, respectively, than wild-type plants (Figure 1).

The genetic cause of the Ssburg phenotype was analyzed in both the sas2 and the 444B mutant lines by measuring the leaf Na⁺ concentration of 171 and 160 plants, respectively, of selfed F₂ populations issued from crosses between the mutant lines and the wild type of the corresponding ecotypes. For both sas2 and 444B, the Szburg phenotype was caused by a single recessive nuclear mutation since the wild type:mutant ratios (131:40 and 115:45, respectively) fitted a 3:1 segregation ratio (χ² = 0.24, P > 0.05 and χ² = 0.83, P > 0.05, respectively). Allelism of the sas2 and 444B mutations was established from the observation that the 45 F₁ plants obtained from reciprocal crosses between sas2 and 444B plants all overaccumulated Na⁺ in leaves. sas2 and 444B were renamed sas2-1 and sas2-2, respectively.

sas2 was then shown to correspond to a different locus from sas1, since none of the 163 F₁ plants obtained from reciprocal crosses between sas2 and sas1 plants displayed the Sasburg phenotype.

The sas2 locus was mapped by genetically analyzing 43 plants of the Sasburg phenotype resulting from the screening of a selfed F₂ population issuing from the cross between a homozygous sas2-1 plant and a wild-type plant of ecotype Columbia. Linkage between sas2 and the markers nga8 and AG anchored the sas2 mutation to the top of chromosome IV (Figure 2A). The above characteristics defined sas2-1 and sas2-2 as new mutant lines.

Positional cloning of the sas2 locus
The sas2 locus was mapped with greater accuracy by analyzing 107 plants of the Sasburg phenotype recovered from the cross between a homozygous sas2-1 plant and a wild-type plant of ecotype Columbia (Figure 2A). The sas2 locus was mapped between the markers F17A8M and F24G24SE. 1.4 cM apart on the genetic map and 273 kb on the physical map. This region was found to contain a candidate for the sas2 locus, the AtHKT1 gene, known to encode a Na⁺ transporter (Uozumi et al., 2000). A transgenic sas2-1 plant complemented with the complete wild-type AtHKT1 gene was obtained and its self progeny (T₃) was analyzed. Among the 89 T₂ plants tested, 63 displayed a wild-type phenotype and all of them were found to contain the transgene. None of the remaining 26 T₂ plants, which displayed the Sasburg phenotype, contained the transgene. Thus, the presence of the wild-type AtHKT1 transgene in the sas2-1 plants reverted the Sasburg phenotype. Finally, sequencing the sas2-1 and sas2-2 alleles of the AtHKT1 gene and the corresponding Landsberg erecta and Wassilewskija wild-type alleles revealed two different single point mutations, each of them characterizing one of the two alleles. The whole set of data demonstrated that AtHKT1 corresponds to the sas2 locus.

The sas2-1 mutation corresponds to a C→T transition at position +845 of the coding sequence, which changed the serine at position 282 of the protein for a leucine (Figure 2B). This amino acid substitution lies just upstream of the fifth transmembrane segment, according to the available AtHKT1 protein structure prediction (Durell and Guy, 1999; Kato et al., 2001; Figure 2D). This region is relatively weakly conserved among the known plant members of the TRK-HKT gene family (Figure 2B) and has never been mentioned as being of any importance for Na⁺ or K⁺ transport in the mutagenesis studies performed on the wheat or Arabidopsis members of the HKT family (Rubio et al., 1995, 1999; Dietloff et al., 1998; Liu et al., 2000; Kato et al., 2001; Mäser et al., 2002a).

The sas2-2 mutation corresponded to a G→A transition at position +974 of the coding sequence, which changed the glycine at position 325 of the protein for a glutamic acid (Figure 2C). This amino acid substitution lies in the loop connecting the fifth and sixth transmembrane segments (Figure 2D). This region contributes to the structure of the transporter’s pore, according to the structural model computed for TaHKT1/AtHKT1 and the non-plant members of the TRK/HKT family (Durell and Guy, 1999). This model indicates that the three-dimensional protein structure is conserved between the wheat TaHKT1 and Arabidopsis AtHKT1 in the region encompassing the sas2-2 mutation. Interestingly, according to this model, the AtHKT1 Gly325 would correspond to Asn358 in TaHKT1, which is located exactly two α-helix turns closer to the entry of the pore than Asn365, a residue shown to be essential for Na⁺ transport by TaHKT1 (Rubio et al., 1999). However, the current knowledge does not allow prediction of the consequences of the sas2 mutations on the structure of AtHKT1.

The sas2 mutation specifically increased Na⁺ accumulation
To check whether the overaccumulation phenotype of sas2 plants was specific for Na⁺, wild-type and sas2-1 plants were grown in medium supplemented with either K⁺, Na⁺, Li⁺, Mg²⁺ or Ca²⁺ before their shoot content in the corresponding cation was determined (Figure 3). The sas2-1 plants overaccumulated Na⁺, when compared with the wild-type plants, but not K⁺, Mg²⁺, Ca²⁺ nor Li⁺,
indicating that the sas2-1 mutation specifically affected Na⁺ accumulation. This result is reminiscent of the high ionic selectivity of the AtHK1 transporter for Na⁺ against divalent cations, K⁺ and even the toxic Na⁺ analog, Li⁺ (Uozumi et al., 2000), and is consistent with the hypothesis that altered AtHK1 transport activity underlies Na⁺ overaccumulation in sas2-1 plants.

The influence of the counter anion on Na⁺ accumulation was checked in another set of experiments. Plants were cultivated in vitro on standard medium supplemented with various Na⁺ salts (NaCl, Na₂SO₄, or NaNO₃) at a final Na⁺ concentration of 35 mmol/l. The Na⁺ content in sas2-1 shoots was in the same range and always 35–40% higher than in wild-type shoots, regardless of the accompanying anion (data not shown).

**Functional expression of wild-type AtHK1 and sas2-1 cDNAs in Xenopus oocytes**

The effect of the sas2-1 mutation on functional properties of the AtHK1 transporter was investigated by expression in Xenopus oocytes. Experiments were performed in parallel, on the same batch of oocytes. Similar results were independently obtained in two laboratories. Voltage clamp experiments were carried out 2 days after injection, in bath solutions differing in K⁺ and Na⁺ concentrations. Expression of AtHK1 in oocytes (Figure 4A and C)

![Image](image-url)
resulted in the appearance of a slightly inwardly rectifying highly Na⁺-selective conductance, in agreement with previous reports (Uozumi et al., 2000; Mäser et al., 2002a). Expression of SAS2-1 also resulted in the appearance of a highly Na⁺-selective conductance (Figure 4B and C). Indeed, the current intensity and the zero-current potentials in SAS2-1-expressing oocytes, like in AtHK1-expressing oocytes, were poorly sensitive to changes in external K⁺ concentration. Furthermore, the relative shifts in the current–voltage relationship upon changing the external Na⁺ concentration were the same in the mutant and wild-type transporters (Figure 4A and B), indicating that the sas2-1 mutation did not significantly alter the transporter’s Na⁺/K⁺ selectivity. In contrast, the sas2-1 mutation had a major impact on the apparent activity of the AtHK1 transporter; the current intensity recorded in oocytes injected with sas2-1 cRNA was strongly reduced compared with that recorded in oocytes injected with wild-type AtHK1 cRNA (e.g. 5–6 times lower at −140 mV in all ionic conditions, Figure 4D; note also the difference in scale of the y-axis between panels A and B). This reduction in macroscopic current can be ascribed to a decrease in the individual protein activity and/or in the number of proteins active at the oocyte cell membrane due to impaired expression, targeting to the membrane, or stability.

The sas2-1 mutation also resulted in an apparent increase in the inward rectification of currents (Figure 4A and B). However, analysis of mean zero-current potentials indicated that the cytosolic Na⁺ concentration was lower in oocytes expressing SAS2-1 than in those expressing AtHK1 (~25 and ~100 mM, respectively, assuming a Nernstian behavior of the transporters in the presence of high Na⁺ concentrations). It is very likely that the former oocytes, due to their lower Na⁺ transport capacity, had taken up less Na⁺ than the latter ones during the 2 days of incubation in the ~100 mM Na⁺ external solution (Burth solution) following cRNA injection. Thus, the increase in rectification observed in SAS2-1-expressing oocytes was most likely due to their reduced cytosolic Na⁺ concentration rather than to a reduced ability of SAS2-1 transporters to mediate outward fluxes of Na⁺.

**AtHK1 is expressed in the phloem tissues**

Northern analysis detected AtHK1 mRNA in both roots and shoots. Relative to total RNA, the level of AtHK1 mRNA was much higher in roots than in shoots (data not shown). It did not change significantly in response to NaCl stress at any of the NaCl concentrations tested (0, 25, 50 or 100 mM NaCl; data not shown). These results were similar to those obtained by Uozumi et al. (2000).

The spatial expression of the AtHK1 gene was investigated by analyzing 20 independent transgenic *Arabidopsis* lines containing the β-glucoronidase (GUS) reporter gene under the control of the AtHK1 promoter (Figure 5). All the transgenic lines displayed GUS expression in the vascular tissues of every organ, in agreement with the recent report of Mäser et al. (2002b). In cross-sections of roots, leaves and flower peduncles, GUS expression was only detectable in the phloem tissues (Figure 5). Salt stress (50 mM NaCl) did not alter either the pattern of GUS expression or the intensity of the histochemical GUS staining in plants grown hydroponically or

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**Fig. 4.** Functional characterization of SAS2-1 in *Xenopus* oocytes. Experiments were performed in parallel on SAS2-1 and on wild-type AtHK1. Recordings were carried out 2 days after cRNA injection. Oocytes (all from the same batch) were successively voltage clamped in bath solutions containing either 0.3 mM Na⁺ plus varying K⁺ concentrations (0.3, 3 and 10 mM), or 0.3 mM K⁺ plus varying Na⁺ concentrations (1 or 10 mM). Applied membrane potentials ranged from −20 to −140 mV. (A–C) Mean steady-state currents (± SE) recorded in oocytes injected with 50 ng of either wild-type AtHK1 (A, n = 6) or sas2-1 (B, n = 11) cRNA, or with H₂O (C; n = 5). Note the difference in scale of current between (A) and (B and C). (D) Comparison of currents recorded at −140 mV in oocytes expressing either AtHK1 (wt) or SAS2-1, or in control oocytes injected with H₂O (cont) in the presence of 0.3 mM K⁺ plus 10 mM Na⁺. Means ± SE [data from (A), (B) and (C)].
in vitro (data not shown), in agreement with the northern blot data. Since a role for AtHKT1 in root Na⁺ uptake had been previously hypothesized (Rus et al., 2001; see Discussion), exclusive AtHKT1 expression in root stelar tissues was further confirmed by in situ hybridization (Figure 5H–J).

The sas2-1 mutation results in altered Na⁺ transport in the phloem sap

The above results suggested that phloem Na⁺ transport could be altered in sas2 plants. This hypothesis was checked by directly analyzing the phloem sap of sas2-1 and wild-type plants. In order to reduce the difference in leaf Na⁺ content between the two types of plants, Na⁺ was introduced at a lower concentration in the culture medium of the sas2-1 plants than in that of the wild-type plants. Plant leaves were cut and the phloem sieve was collected in EDTA solution (King and Zeevaart, 1974). For experimental reasons, the volume of the collected phloem sieve is highly variable and cannot be measured directly. Thus, both Na⁺ and glutamine were assayed in the EDTA extracts, and the amount of Na⁺ was expressed relative to that of glutamine. Glutamine was used as an internal standard since this amino acid has been shown to be abundant in Arabidopsis phloem sap and to remain constant during the entire 24 h cycle (Corbesier et al., 2001). The amounts of glutamine in the EDTA extracts varied from 13 to 56 nmol (mean value: 29 ± 16 nmol, n = 6) for wild-type plants, and from 13 to 83 nmol (mean value: 44 ± 32 nmol, n = 6) for sas2-1 plants. Sodium to glutamine concentration ratios in the phloem extracts were plotted versus leaf Na⁺ contents in Figure 6. The mean ratio was seven times lower in sas2-1 (4.5 ± 2.6, n = 6) than in wild-type (29 ± 3.9, n = 6) plants, despite the sas2-1 leaf Na⁺ content [830 ± 200 µmol/g dry weight (DW)] being 25% higher than wild type (660 ± 140 µmol/g DW). Similar results were obtained in other culture conditions. The whole set of data indicated that the sas2-1 mutation resulted in a dramatic reduction in Na⁺ concentration in the phloem sieve.
The *sas2-1* mutation does not affect Na⁺ translocation from root to shoot

Direct recirculation of Na⁺ ions from xylem to phloem tissues in the upper parts of the roots and in the stem is thought to play a role in the control of Na⁺ translocation towards the shoots. The hypothesis that Na⁺ overaccumulation in *sas2* aerial organs might also result from an alteration in the ascending Na⁺ stream was therefore checked. Translocation of Na⁺ to the aerial organs depends mainly on the mass flow due to transpiration. The rate of transpiration (expressed on a leaf area basis) was measured in hydroponically grown wild-type and *sas2-1* plants under three different conditions: during the night at 63% relative humidity or during the day at either 63 or 45% relative humidity. This revealed no significant difference between the two genotypes (data not shown). Thus, Na⁺ overaccumulation in *sas2* leaves could not be ascribed to an increase in Na⁺ mass flow due to increased transpiration rates. Na⁺ and K⁺ were then assayed in xylem sap obtained from pressurized shoots (Figure 7). The Na⁺ concentrations in the xylem sap were on average higher in *sas2-1* plants (3.9 ± 2.1 mmol/l; n = 14) than in wild-type plants (2.0 ± 1.4 mmol/l; n = 13). However, the difference was not significant (P > 0.01). Altogether, these results suggest that the ascending Na⁺ flow is not altered in *sas2-1* plants.

The *sas2* mutations cause increased Na⁺ accumulation in aerial organs but decreased Na⁺ accumulation in roots

We first checked whether the *sas2* mutation resulted in Na⁺ overaccumulation in every aerial organ, whatever its sink or source status. Bud, flower, green silique, cauline leaf, rosette leaf and stem collected from greenhouse-cultivated *sas2-1* and wild-type plants were assayed for Na⁺ (Table 1A). Higher Na⁺ contents were found in the *sas2-1* organs, except in stems which accumulated Na⁺ at similar levels in both genotypes. The difference in Na⁺ accumulation between the two genotypes was dependent on the organ, ranging from 200% (bud) to 700% (cauline leaf).

The effect of the *sas2* mutation on the root Na⁺ content was then examined, using plants grown *in vitro* and hydroponically. *sas2-1* plants had higher shoot Na⁺ content (as expected) but lower root Na⁺ contents than wild-type plants (Table 1B and C). A similar decrease in Na⁺ root content was also observed in the *sas2-2* mutant (0.48 ± 0.06 μmol/g DW in 6-week-old *sas2-2* plants grown hydroponically during 4 days in the presence of 100 mM NaCl, and 1.03 ± 0.2 μmol/g DW in the wild-type plants).

The *sas2-1* mutation does not result in reduced Na⁺ uptake by roots

*AtHK1* had been suggested to be involved in Na⁺ uptake by roots in *Arabidopsis* (Rus et al., 2001; Discussion). We thus examined whether Na⁺ uptake was altered in *sas2-1* roots. Surprisingly, Na⁺ influx was 20% higher in *sas2-1* than in wild-type roots (2.33 ± 0.19 and 1.86 ± 0.09 μmol/min·g fresh weight, respectively; n = 6). This indicates that *AtHK1* is not involved in Na⁺ uptake by roots in *Arabidopsis*.

The *sas2* mutation affects plant growth and survival upon salt stress

Control of Na⁺ accumulation in shoots is of major importance in the plant adaptation to salt stress. The hypothesis that the *sas2-1* mutation could be detrimental to plant development in the presence of NaCl was examined using plants grown hydroponically. NaCl was added to the standard culture medium of 26-day-old *sas2-1* and wild-type plants at different concentrations: 0, 25, 50, 100 or 200 mmol/l. In the absence of NaCl, the two genotypes displayed quite similar growth rates (Figure 8). None of the plants could cope with the two highest NaCl concentrations, 100 and 200 mM, although the mutant plants died a few days prior to the wild-type ones (data not shown). Exposure to 25 and 50 mM NaCl made clearer the difference in salt sensitivity between the two genotypes (Figure 8A and D). Growth of *sas2-1* plants was markedly reduced compared with that of wild-type plants. It was inhibited by 25 mM NaCl, while growth of wild-type plants was stimulated by this treatment. Most *sas2-1* plants...
Fig. 8. The sas2-1 mutation results in NaCl hypersensitivity. Four-week-old plants grown in hydroponics were either subjected to NaCl treatment (standard solution supplemented with 25 or 50 mM NaCl) or further cultivated on standard solution. (A–C) Shoot DW (A), shoot Na⁺ content (B) and shoot K⁺ content (C) during the 18 days following the application of NaCl treatment. Open and closed symbols correspond to wild-type and sas2-1 plants, respectively. Squares, circles and triangles correspond to plants grown on media supplemented with 0, 25 and 50 mM NaCl, respectively. Means ± SE, n = 10. (D) sas2-1 (top) and wild-type (bottom) rosettes after 17 days of growth on the standard culture medium supplemented with 0 (left) or 50 (right) mM NaCl. Scale bars = 1 cm.

died after an average of 20 days of exposure to 50 mM NaCl, whereas the wild-type plants could complete their cycle on this medium, with a growth similar to that observed in NaCl-free medium. Cation assays confirmed that these differences in growth and development were associated with higher Na⁺ content (and lower K⁺ content) in shoots of sas2-1 plants (Figure 8B and C). Our results are in agreement with the recent report that mutant AtHKT1 plants show Na⁺ hypersensitivity in long-term growth assays performed in vitro (Mäser et al., 2002b).

Discussion

sas2 is a new locus involved in the control of Na⁺ accumulation in shoots and corresponds to the Na⁺ transporter gene AtHKT1

We report the isolation and characterization of two new mutants, sas2-1 and sas2-2, displaying Na⁺ overaccumulation in shoots. They harbor allelic mutations that correspond to a different locus from the sasl locus identified previously (Nublat et al., 2001). sas2 and sasl plants display many physiological characteristics in common. The process of overaccumulation in shoots specifically concerns Na⁺ since these mutants do not overaccumulate K⁺, Ca²⁺ or Mg²⁺. It is worth noting that the specificity is even higher in sasl2, which does not overaccumulate Li⁺. Also, like sasl plants (Nublat et al., 2001), sas2 plants display increased sensitivity to salt stress compared with wild-type plants. In sasl, Na⁺ overaccumulation in shoots was shown to result from impaired control of Na⁺ translocation from roots to shoots (Nublat et al., 2001). The sas2 mutants identify another process playing a crucial role in the control of the shoot Na⁺ content, as discussed below.

Positional cloning and sequencing of the two mutated alleles indicated that the sas2 locus corresponds to the Na⁺ transporter AtHKT1 gene. AtHKT1 was first identified (Uozumi et al., 2000) as a homolog of the wheat TaHKT1 transporter (Schachtman and Schroeder, 1994). Transporters constituting the plant HKT family display sequence and topological similarities to the yeast Trk1,2 and the Escherichia coli TrkH transporters, which are
Table 1. Na⁺ accumulation in different organs of intact plants

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of repeats</th>
<th>Na⁺ content</th>
<th>sas2-1 vs WT ratio</th>
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</thead>
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<tr>
<td>(A) Bud</td>
<td>6</td>
<td>25 ± 2</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>Flower</td>
<td>6</td>
<td>35 ± 3</td>
<td>150 ± 16</td>
</tr>
<tr>
<td>Green siliqua</td>
<td>6</td>
<td>41 ± 6</td>
<td>200 ± 29</td>
</tr>
<tr>
<td>Cauline leaf</td>
<td>6</td>
<td>41 ± 6</td>
<td>340 ± 34</td>
</tr>
<tr>
<td>Stem</td>
<td>6</td>
<td>42 ± 2</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>Rosette leaf</td>
<td>6</td>
<td>75 ± 4</td>
<td>320 ± 50</td>
</tr>
<tr>
<td>Shoot in vitro</td>
<td>6</td>
<td>1160 ± 120</td>
<td>1860 ± 70</td>
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<td>Root in vitro</td>
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<tr>
<td>Shoot in hydroponic culture</td>
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<td>990 ± 230</td>
<td>2060 ± 75</td>
</tr>
<tr>
<td>Root in hydroponic culture</td>
<td>10</td>
<td>380 ± 17</td>
<td>150 ± 4</td>
</tr>
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</table>

*The aerial plant tissues (A) were collected from 6-week-old mature plants grown on compost in the greenhouse. Plants cultivated *in vitro* (B) or in hydroponic culture (C) were grown on standard medium for 11 or 26 days, respectively, and then for an additional 6 days on standard medium supplemented with 35 or 50 mM NaCl, respectively. 
*The samples were collected as pools of five plants for greenhouse-cultivated plants, as pools of 20 plants for plants cultivated *in vitro*, or as individual plants for plants grown in hydroponic culture.

Na⁺ content expressed in μmol/g DW ± SD.

involved in K⁺ acquisition (Durell and Guy, 1999 and references therein). In Arabidopsis, the HKT family is represented by a single member, AtHKT1. Based on their functional properties when expressed in Xenopus oocytes, plant transporters of the HKT family can be sorted into two subfamilies. One subfamily would contain transporters similar to the wheat transporter TaHKT1, which is able to mediate both K⁺ and Na⁺ transport when expressed in Xenopus oocytes. At low external Na⁺ concentrations, TaHKT1 is endowed with an Na⁺:K⁺ symport activity allowing active K⁺ uptake. In the presence of high external Na⁺ concentrations, it mediates only Na⁺ transport (Rubio et al., 1995). The other subfamily would group one of the six rice HKT transporters (Po-OsHKT1; Horie et al., 2001) and the Arabidopsis AtHKT1. AtHKT1 and Po-OsHKT1 can mediate Na⁺ transport in Xenopus oocytes but are not significantly permeable to K⁺, whatever the external Na⁺ concentration. These systems are therefore probably devoted to specific Na⁺ transport. The distinctive functional properties of the plant members of the HKT family, probably coupled with different expression patterns—TaHKT1 is expressed in the root epidermis and cortex and in leaf vasculature (Schachtman and Schroeder, 1994), whereas AtHKT1 is only expressed in the phloem tissues (this work)—might underlie different functions of these genes in planta.

A role for AtHKT1 in Na⁺ recirculation by the phloem sap

AtHKT1 was initially proposed to control Na⁺ entry in Arabidopsis roots, based on the phenotype of a double athkt1Δ sos3 mutant obtained from the screening for mutations suppressing the salt over sensitive phenotype of the sos3 mutant (Rus et al., 2001). The Arabidopsis SOS3 gene encodes a calcineurin-like protein involved in control of Na⁺ transport in the root stele (Zhu, 2002). The double athkt1Δ sos3 mutant was found to display lower Na⁺ contents (whole plant extracts) than both the wild-type control and the sos3 mutant. On this experimental basis, AtHKT1 was proposed to be involved in Na⁺ uptake in roots; neither Na⁺ influx in roots nor Na⁺ release to the culture medium was checked in the double athkt1Δ sos3 mutant (Rus et al., 2001). The hypothesis that AtHKT1 mediates Na⁺ entry in roots is, however, not supported by our data showing that the sas2-1 mutation does not result in reduced root Na⁺ uptake and that AtHKT1 is not expressed in root peripheral cells.

AtHKT1 expression is restricted to phloem tissues. The sas2-1 mutation, which strongly decreases AtHKT1 Na⁺ transport activity in Xenopus oocytes, results in a marked decrease in Na⁺ concentration in the phloem sap exuding from leaves, in higher Na⁺ shoot content and in lower Na⁺ root content. Recently, disruption of the AtHKT1 gene (T-DNA insertion) has also been reported to result in higher Na⁺ shoot content and lower Na⁺ root content (Mäser et al., 2002b). Thus, the simplest hypothesis that can be drawn from our data is that AtHKT1 is involved in Na⁺ recirculation from shoots to roots by the phloem sap. Electrophysiological analyses indicate that AtHKT1 can act in a wide range of membrane potentials, is poorly rectifying and able to mediate both influx and efflux of Na⁺, depending on the electrochemical gradient of Na⁺. We propose that this functional plasticity allows AtHKT1 to fulfill different functions in leaves and roots: it would mediate Na⁺ loading into the phloem sap in leaves and unloading in roots. A similar hypothesis has been proposed, also in the absence of any direct thermodynamic support because measurement of transmembrane electrochemical gradients in the phloem vasculature is not straightforward (Deeken et al., 2002), for two transport systems expressed in the phloem of both leaves and roots in Arabidopsis: the H⁺:sucrose symporter AtSUC2 (Truernit and Sauer, 1995) and the K⁺ channel AKT2 (Lacombe et al., 2000). The root steady-state Na⁺ content depends on the rates of net Na⁺ uptake from the culture medium (i.e. balance between Na⁺ influx and efflux into/from the root), Na⁺ translocation to the shoots and Na⁺ release from the phloem. Within the framework of the above hypotheses, we propose that the decrease in root Na⁺ content observed in sas2 mutants is due to reduced Na⁺ release from the phloem in the root stele. This model is also supported by the report from Laurie et al. (2002) showing that reduction
of TaHKT1 expression in wheat resulted in a marked decrease in the root stele Na⁺ content while poorly affecting the root epidermal and cortical contents.

The above model may provide a clue to understand the interaction between AtHKT1 and SOS3 shown by Rus et al. (2001). It is tempting to speculate that tight regulation of Na⁺ transport and accumulation in, and Na⁺ exchanges between, stelar tissues is required in the root stele. This regulation could involve, for instance, the SOS3/SOS2 pathway via the control that SOS3 and SOS2 exercise over SOS1 (Zhu, 2002), a plasma membrane Na⁺/H⁺ antiporter expressed in root xylem parenchyma cells which is suggested to mediate either Na⁺ release into or Na⁺ retrieval from the xylem stream, depending on salt stress intensity (Shi et al., 2002). Functional interactions in the regulation of Na⁺ accumulation in the root stele occurring between SOS1, under the control of SOS3, and AtHKT1, might explain why mutations in the AtHKT1 gene suppress the sos3 mutant phenotype, as reported in Rus et al. (2001).

**AtHKT1 is part of the mechanism of salt tolerance**

The role of Na⁺ recirculation from leaves to roots in plant tolerance to salinity has been debated. The fact that salt-sensitive species were found to display higher Na⁺ concentrations in the phloem sap and a greater ability to recirculate Na⁺ from the leaves to the roots than moderately or strongly salt-tolerant species was puzzling (Munn et al., 1988; Jeschke and Pate, 1991; Wolf et al., 1991; Lohaus et al., 2000). It was even sometimes suggested that, in fact, Na⁺ recirculation by the phloem sap contributed to salt sensitivity by favoring Na⁺ translocation from mature leaves to young (sensitive) organs (Jeschke et al., 1987). In contrast to these interpretations, our results provide direct evidence, in the model plant Arabidopsis, that Na⁺ recirculation by the phloem vasculature plays a major role in plant tolerance to salt by contributing to Na⁺ exclusion from aerial organs.

Genetic and molecular information now supports a model in which two mechanisms act synergistically in Arabidopsis leaves to prevent deleterious Na⁺ build-up in the leaf aposlast and symplast: Na⁺ sequestration into vacuoles, involving the tonoplast Na⁺/H⁺ antiporter AtNHX1 (Apse et al., 1999), and Na⁺ recirculation to roots, involving AtHKT1. These two mechanisms can protect the leaf aposlast and symplast as long as their combined efficiency is greater than the rate of Na⁺ delivery to the leaves. The balance of their relative contribution in this function should however determine the level of Na⁺ accumulation in the whole leaf. A high efficiency of sequestering Na⁺ to the vacuole, compared with that of recirculating Na⁺ to the roots, would result in high Na⁺ levels in the leaf. This has been observed in the most salt-tolerant species which display high leaf Na⁺ content (Wolf et al., 1991). Conversely, a low efficiency of sequestering Na⁺ to the vacuole, compared with that of recirculating Na⁺ to the roots, would result in low Na⁺ levels in the leaf, as observed in the most salt-sensitive species (Munn et al., 1988). It is worth noting that, in Arabidopsis, this model of functional interaction between AtHKT1 and the tonoplastic Na⁺/H⁺ antiporter AtNHX1 can explain the otherwise puzzling result that transgenic plants overexpressing AtNHX1 accumulated ~30% more Na⁺ in leaves than control plants (Apse et al., 1999).

In conclusion, this report provides the first demonstration that Na⁺ recirculation from shoots to roots via the phloem vasculature plays a crucial role in protecting aerial tissues from Na⁺ invasion. The phloem Na⁺ transporter AtHKT1 is identified as playing a key role in this function. Besides their basic interest for plant transport biology, these results might help in improving salt tolerance in cultivated species since Na⁺ exclusion is the main mechanism that these species use to face salt stress.

**Materials and methods**

**Plant materials**

The sos2-1 mutant was identified from the screening of 6625 M₂ seedlings resulting from ~800 M₁ parents of a commercial population derived from A.thaliana ecotype Landsberg erecta after mutagenesis with EMS (Lehle Seeds, Round Rock, TX; reference M2E-4.2). The sos2-2 (or 444B) mutant was identified from the screening of M₂ seedlings derived from A.thaliana ecotype Wassilewskija after EMS mutagenesis (a kind gift from Dr Bellini, INRA Versailles, France); 10–20 M₃ progenies were screened for each of 350 M₂ parents. Wild-type A.thaliana of ecotypes Landsberg erecta, Wassilewskija and Columbia were obtained from the Nottingham Arabidopsis Stock Center (Nottingham, UK) under the references NW20, N915 and N907, respectively.

**Growth conditions and screening procedures**

Plants were grown in vitro, in hydroponics and in the greenhouse as described by Nublat et al. (2001).

The sos2-1 mutant was identified using the following screening procedure. Seedlings at 10–12 days old, grown in vitro on standard medium, were transferred to plates containing standard medium plus 35 mM NaCl. Four to 6 days after transfer, one of the two oldest leaves was removed from each plant to be tested, and its Na⁺ concentration was determined. The sos2-2 mutant was identified as follows. Two-week-old plants grown on compost in the greenhouse were watered once with tap water supplemented with 100 mM NaCl. One week later, the third or fourth leaf was collected and its Na⁺ concentration determined. For both screening procedures, plants that accumulated Na⁺ at a concentration exceeding the average Na⁺ concentration of the population plus three times its standard deviation were characterized further.

**Cartography of the sos2-1 mutation**

The sos2-1 mutant was used as the male parent in the genetic crosses. Mapping analysis was performed using CAPS (GapC, GL1, ASA1, NCC1, GPA1, DFR, KELP and g8300) and microsatellite (ngab, nga8 and nga280) markers described in the TAIR database (www.arabidopsis.org), together with new CAPS (F178AM, CER464979, T5L195E and F242G44E) and SSLP (CER4511801) markers created for this work (Figure 2; Table II).

**Genetic constructs and transgenic plants**

A 10.7-kb-long Sgel–SpeI genomic DNA fragment comprising the whole AtHKT1 gene was cloned from the BAC T9A4 into the binary vector pBIB-HYG (Becker, 1990) by conventional means. The resulting construct was named pBIBHAHKT1.

A 2.3-kb-long fragment corresponding to the AtHKT1 promoter region was amplified from genomic DNA (ecotype Columbia) by PCR with the Eurobio-Taq polymerase (Eurobio, France), using a primer introducing a unique NcoI site around the ATG start codon of the AtHKT1 coding sequence. The PCR product was digested with the NcoI restriction enzyme and introduced into pBl320.X (R.Derosse, RHOBIO, Evry, France). pBl320.X bears a unique NcoI site at the start codon of a promoterless GUS coding sequence located upstream of the nopaline synthase (NOS) terminator. The recombinant clone thus harbored a transcriptional fusion between the AtHKT1 mRNA 5'-untranslated sequence and the GUS coding sequence. The AtHKT1 promoter sequence of the selected clone was identical to the corresponding published sequence except for one T→C substitution at position –1166 relative to the ATG start codon. The complete expression cassette comprising the AtHKT1 promoter, the GUS coding sequence and the NOS terminator was subsequently transferred into pBIB-HYG. The resulting construct was called pBIBHAHKT1::GUS, pBIBHAHKT1 and pBIBHAHKT1::GUS were transferred from E.coli into the Agl0 (Lazo et al., 1991) and the GV3101 (Koncz and
Table II. CAPS and SSLP markers created in this work

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers</th>
<th>Restriction enzyme</th>
<th>Fragment sizes&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
</table>
| (A) SSLP markers  
CER4511801<sup>b</sup> | GTTTAGGCCGCTTAGTTCCAC  
TGTCGAAGATTCGTCAGGCCACC | –                  | Landsberg erecta  
670 |
| (B) CAPS markers  
F17A8M  
CER446979<sup>b</sup>  
TSL19SE  
F24G24SE | ATATACACTACAGACACTAGT  
GAGGAGACAGAGCAAGACAGGAT  
CAAGATCTCAACGGTGTTAGGG  
ATGTTCTCCTGGAATCAACCGAG  
GATGATAGCCACCTGCAAAACC | XbaI  
TaqI  
RsaI  
XbaI | Columbia  
410+205  
815  
155+100+100  
820+440+305 |

<sup>a</sup>Fragment sizes are expressed in base pairs.

<sup>b</sup>Markers CER446979 and CER4511801 were constructed from data delivered in TAIR by Cereon Genomics.

Schell, 1986) Agrobacterium strains, respectively, by triparental mating using the pRK2013 E.coli strain as a helper. Five-week-old greenhouse-cultivated sas2 and wild-type Columbia plants were transformed with the pBIBHahHKTI-containing AgI0 and the pBIBHahHKTI:GUS-containing GY3101 strains. respectively, using the floral dip transformation procedure (Clough and Bent, 1998). Transgenic plants were screened in vitro on a Murashige and Skoog medium (Sigma M5519) with 30 mg/l hygromycin B (Sigma H7772).

β-glucoronidase histochemical assay
GUS histochemical staining was performed as described by Lagarde et al. (1996), except that the embedding resin was the hydroxymethyl methacrylate Technovit 7100 (Heraus-Kulzer GmbH, Wehrheim, Germany).

In situ hybridization
Root samples were fixed in Histochoice MB fixative (Amresco, Solon, OH) for 3 h, dehydrated in ethanol, cleared in Safesolv (Labonord, Templemars, France) and embedded in ParaplastPlus. Two antisense and one sense 200-b-long AthKTI riboprobes were labeled with biotin (Lig’nScribe, MAXIScript and BrightStar Psoralen-Biotin kits; Ambion, Austin, TX). A probe concentration of 2.5 μg/ml was used in an overnight hybridization of 8 μm thick tissue sections. Detection was performed with NBT-BCIP for 2 days (Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA).

<sup>22</sup>Na influx assay
Plants were grown as described by Demidchik and Tester (2002) for 19 days. Fluxes were then measured as described by Davenport and Tester (2000), with the following modifications resulting from adaptation of the protocol to Arabidopsis. The 3 h sorbitol pre-treatment prior to fluxes was omitted. No sorbitol was used at any further step of the protocol. After 2 min of incubation in labeled solution (50 mM NaCl, 0.5 mM CaCl₂), the roots (excised from 10 plants for each measurement) were rinsed twice (for 2 and then 3 min) with ice-cold 200 mM NaCl and 10 mM CaCl₂.

Heterologous expression in Xenopus oocytes
pNU111 (Uozumi et al., 2000) is a modified pYES2 vector (Invitrogen) harboring the full-length cDNA of wild-type AthKTI, which was both (i) inserted between the T7 promoter and a poly(A) sequence and (ii) placed under the control of the yeast Gal1 promoter. An ~3-3kb-long BstEII-XhoRI fragment containing the sas2-1 mutation was subcloned into pNU111, replacing the corresponding wild-type fragment. The resulting construct was called pNU1as2-1. Capped complementary RNAs (cRNA) of AthKTI and sas2-1 were prepared from pNU111 and pNU1as2-1 with the mMESSAGE mMACHINE kit (Ambion, Austin, TX). Electrophysiological analyses were performed by Véry et al. (1995). The same batch of oocytes was used for all injections. The oocytes were injected with 50 ng of RNA. The control oocytes received an equivalent volume of water. The two-electrode voltage clamp measurements were performed 2 days after injection, using bath solutions containing 6 mM MgCl₂, 1.8 mM CaCl₂, various concentrations of K⁺ and Na⁺ (as glutamate salts), 10 mM Mes-1,3-bis[tris(hydroxymethyl)-methylamino]propane pH 5.5 and 240 mM t-mannitol.

Phloem sieve analysis
The protocol was derived from King and Zeevaart (1974). Wild-type and sas2-1 plants were grown hydroponically for 8 weeks in standard solution. Then, NaCl was introduced into the solution, at a final concentration of 5 mM for sas2-1 plants and of 50 mM for wild-type plants (the difference in Na⁺ concentration being aimed at reducing the difference in Na⁺ shoot content between the two genotypes). The culture medium was renewed 3 days later and phloem sap was collected the following day. A mature rosette leaf was excised, its petiole was recut under 20 mM K₂EDTA pH 7.5 and the cut extremity was kept in the same solution for at least 1 min. The leaf, with its petiole dipping in 1 ml of 15 mM K₂EDTA pH 7.5, was then left for 4 h in an illuminated growth room under a water-saturated atmosphere to reduce xylem transpiration. The leaf was then dried at 80°C and weighed. The amounts of Na⁺ and glutamine released in the EDTA solutions were determined by flame photometry and HPLC, respectively. The Na⁺ to glutamine concentration ratio was used to standardize the Na⁺ data.

Xylem sap analysis
Xylem sap was obtained from pressurized shoot of mature plants grown hydroponically in a culture medium supplemented with 10 mM NaCl and displaying floral humps. The roots and the top of the hump were cut off. The rosette was placed in a pressure chamber with the rest of the hump emerging out. Pneumatic pressure (~11 bars) was applied. The first two drops emerging were discarded to prevent contamination of the xylem sap with contents from damaged cells or phloem sap. Xylem sap was then collected as leaf exudate during the following 5 min, and stored at ~20°C pending Na⁺ and K⁺ concentration measurements.

Determination of ion contents
Ion concentrations were determined by flame photometry as described by Nublat et al. (2001).

Acknowledgements
We thank S.Joulie, N.Garcia, S.Burey, H.Baudot, F.Moreau, G.Bonamy, K.Chenu, F.Leccooq and S.Gelin for technical help, and J.C.Davidian for helpful discussions. Part of the oocyte recordings were supported by CREST of Japan Science and Technology to N.U.

References


Received September 29, 2002; revised March 5, 2003; accepted March 6, 2003