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 overaccumulation 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⁺ underaccumulation 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

Introduction

Soil salinity is one of the most significant abiotic stresses for crop plants. Adaptation to NaCl is an extensively investigated, complex process involving both metabolic reactions and transport phenomena (Yeo, 1998). A specific feature of salt stress is Na⁺ invasion of plant tissues. Upon prolonged exposure of a plant to NaCl, Na⁺ is translocated

from the roots to the transpiring leaves where it can increase to toxic levels. In the leaf, Na⁺ toxicity results from Na⁺ build-up in cell walls and cytoplasm; this process can rapidly lead to cell death since these two compartments are of limited volume (Munns, 1993, 2002). Therefore, the ability to postpone the time at which Na⁺ reaches toxic levels in these compartments is crucial for NaCl tolerance. Numerous investigations have led to the conclusion that Na⁺ sequestration in vacuoles is an efficient way to protect leaf cells from Na⁺ injury. Recently, direct evidence has been obtained in various species, including *Arabidopsis*, that improvement of the Na⁺ sequestering capacity of the vacuole results in increased salt tolerance (Apse *et al.*, 1999; Zhang and Blumwald, 2001). However, the most salt-sensitive plants display poor ability to sequester Na⁺ in leaf vacuoles; they have to rely on another mechanism to cope with the Na⁺ delivered to leaf cells. The hypothesis that Na⁺ recirculation from shoots to roots by the phloem sap could be important for salt tolerance, limiting Na⁺ accumulation in leaves, has barely been considered (Munns, 2002). Na⁺ assays in the phloem sap sometimes revealed high concentrations, up to 80 mM in some species (Jeschke *et al.*, 1992 and references therein), but the physiological significance of such data was contradictorily evaluated (Munns *et al.*, 1988; Munns, 2002). Here we describe the isolation in *Arabidopsis* of two new allelic mutants displaying sodium overaccumulation in shoots, *sas2-1* and *sas2-2*, and the identification of the corresponding gene, *AtHKT1*, which encodes an Na⁺ transporter. Functional analyses indicate that the encoded protein is involved in Na⁺ recirculation from shoots to roots and that this process plays a crucial role in plant tolerance to salinity.

Results

Identification of the *sas2* locus

The screening procedure previously described for the isolation of the *sas1* mutant (Nublat *et al.*, 2001), applied to M₂ seed derived from *Arabidopsis thaliana* ecotype Landsberg *erecta* after mutagenesis with ethyl methane-sulfonate (EMS), led to the identification of a second mutant line, *sas2*, which also displayed the Sas⁻ phenotype, i.e. sodium overaccumulation in shoot. Another mutant line overaccumulating Na⁺ in shoot, 444B, was isolated later from the screening of a M₂ 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

Fig. 1. *sas2* plants overaccumulate Na⁺ in shoots. Twelve-day-old plants grown on compost in the greenhouse were irrigated twice within an additional 6-day period, with tap water supplemented or not with 50 mM NaCl. The shoot Na⁺ content of *sas2-1* (left) and *sas2-2* (right) plants (initially named *sas2* and 444B, respectively; see main text) were compared with those of the corresponding wild types in two independent experiments. Means $\pm$ SD, $n \geq 30$.

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 Sas⁻ 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 types of the corresponding ecotypes. For both *sas2* and 444B, the Sas⁻ 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 ($\chi^2 = 0.24$, $P > 0.05$ and $\chi^2 = 0.83$, $P > 0.05$, 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 Sas⁻ phenotype.

The *sas2* locus was mapped by genetically analyzing 43 plants of the Sas⁻ 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 Sas⁻ 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 (T₂) 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 Sas⁻ phenotype, contained the transgene. Thus, the presence of the wild-type *AtHKT1* transgene in the *sas2-1* plants reverted the Sas⁻ 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; Diatloff *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 AtHKT1 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 AtHKT1 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 , Na_2SO_4 or NaNO_3) 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 AtHKT1 and *sas2-1* cDNAs in *Xenopus* oocytes

The effect of the *sas2-1* mutation on functional properties of the AtHKT1 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 AtHKT1 in oocytes (Figure 4A and C)

Fig. 3. Comparison of Na^+ , K^+ , Li^+ , Ca^{2+} and Mg^{2+} accumulation in shoots between *sas2-1* and wild-type plants. Twelve-day-old *in vitro* grown plants were cultivated for 6 additional days on standard medium supplemented with NaCl (Na^+), KCl (K^+), CaCl_2 (Ca^{2+}) or MgCl_2 (Mg^{2+}) at a final concentration of 35 mmol/l, or with LiCl (Li^+) at a final concentration of 4 mmol/l. For each treatment, the shoot content of the cation that had been added to the standard culture medium was determined from a pool of 20 plants. Ion content in *sas2-1* shoots was expressed as a percentage of the wild-type value, which was standardized to 100. Wild-type ion contents were 1.6 ± 0.1 mmol/g DW for Na^+ , 3.5 ± 0.3 mmol/g DW for K^+ , 19.8 ± 1.2 $\mu\text{mol/g}$ DW for Li^+ , 0.7 ± 0.1 mmol/g DW for Ca^{2+} and 0.8 ± 0.08 mmol/g DW for Mg^{2+} . Means $\pm$ SD; $n = 6$.

Fig. 2. Positional cloning of the *sas2* locus. (A) Genetic and physical mapping of the *sas2* locus. Top: genetic map of the region of chromosome IV encompassing the *sas2* locus. Below: physical map (to scale) including the five BACs, F17A8, T5L19, F28M11, T9A4 and F24G24 (data from the AGI consortium sequencing effort, www.arabidopsis.org). On both maps, numbers in bold italics are the numbers of recombination events (identified by the analysis of 107 recombinant plants) between the corresponding adjacent genetic markers, or between the *sas2* locus and the neighboring genetic markers. The physical distance separating the two genetic markers most closely flanking *sas2* (F17A8M and F24G24SE) and the position of the *AtHKT1* gene are indicated on the physical map. (B and C) Protein sequence alignments of plant members of the HKT1 family in the regions containing the *sas2-1* (B) and *sas2-2* (C) mutations. Accession numbers of the *A.thaliana*, *eucalyptus-1* and *-2*, *rice-1* and *-2*, wheat and *Mesembryanthemum crystallinum* sequences are AAF68393, AAF97728, AAD53890, BAB61790, BAB61791, AAA52749 and AAK52962, respectively. The *sas2-1* and *sas2-2* mutations (S282 to L282 and G325 to E325, respectively) are marked with circles. A light gray background highlights the amino acid positions common to at least all the members of the family but one. Numbers above the sequence alignments relate to the position of the corresponding amino acids in the *Arabidopsis* AtHKT1 sequence. (D) The *sas2-1* and *sas2-2* mutations are marked by stars on the structural model (Kato *et al.*, 2001) of the AtHKT1 protein.

Fig. 4. Functional characterization of SAS2-1 in *Xenopus* oocytes. Experiments were performed in parallel on SAS2-1 and on wild-type AtHKT1. 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 ($\pm$ SE) recorded in oocytes injected with 50 ng of either wild-type *AtHKT1* (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 AtHKT1 (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 $\pm$ SE [data from (A), (B) and (C)].

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 AtHKT1-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 AtHKT1 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 *AtHKT1* 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 AtHKT1 (~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 (Barth 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⁺.

AtHKT1 is expressed in the phloem tissues

Northern analysis detected *AtHKT1* mRNA in both roots and shoots. Relative to total RNA, the level of *AtHKT1* 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 *AtHKT1* gene was investigated by analyzing 20 independent transgenic *Arabidopsis* lines containing the β -glucuronidase (GUS) reporter gene under the control of the *AtHKT1* 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

Fig. 5. *AtHKT1* is expressed in the phloem tissues. (A–G) β -glucuronidase staining in excised organs from transgenic *Arabidopsis* plants expressing the GUS coding sequence under the control of the *AtHKT1* promoter. Three-week-old plant (A); leaf (B), flower (D), root (F) and flower peduncle (G) cross-sections; flower (C); roots (E). The cross-sections were counterstained with Schiff reagent. (H–J) *In situ* hybridization performed with sense (H) or antisense (I and J) *AtHKT* riboprobes on root longitudinal (H and I) or cross (J) sections. Plants were grown *in vitro* (A, B, E, F, H, I and J) or in hydroponics (C, D and G). c, cortex; e, endodermis; ep, epidermis; f, filament; ms, mesophyll; p, petal; ph, phloem; s, sepal; st, stele; sty, style; x, xylem. Scale bars = 25 μ m (B, F, H, I and J), 50 μ m (G) and 200 μ m (D).

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 Zeveaart, 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 $\mu\text{mol/g}$ dry weight (DW)] being 25% higher than wild type (660 ± 140 $\mu\text{mol/g}$ 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.

Fig. 6. The *sas2-1* mutation results in decreased Na⁺ concentration in the phloem sap. Eight-week-old hydroponically grown plants were cultivated for 4 additional days on standard medium supplemented with 5 mM NaCl (*sas2-1* plants) or 50 mM NaCl (wild-type plants) before phloem sap was collected in EDTA solutions. The Na⁺ to glutamine molar ratios in the EDTA extracts were plotted versus the leaf Na⁺ content. Each symbol corresponds to a single plant.

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 IA). 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).

Fig. 7. *sas2-1* and wild-type plants display similar Na⁺ content in xylem sap. Eight-week-old hydroponically grown plants were cultivated for 4 additional days on standard medium supplemented with 10 mM NaCl. Na⁺ and K⁺ concentrations were determined in xylem sap collected as leaf exudate using a pressure chamber. Each symbol corresponds to a single plant.

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 IB and C). A similar decrease in Na⁺ root content was also observed in the *sas2-2* mutant (0.48 ± 0.06 $\mu\text{mol/g DW}$ in 6-week-old *sas2-2* plants grown hydroponically during 4 days in the presence of 100 mM NaCl, and 1.03 ± 0.2 $\mu\text{mol/g DW}$ in the wild-type plants).

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

AtHKT1 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 (2.23 ± 0.19 and 1.86 ± 0.09 $\mu\text{mol/min/g fresh weight}$, respectively; $n = 6$). This indicates that AtHKT1 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 $\pm$ 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 *sas1* locus identified previously (Nublat *et al.*, 2001). *sas2* and *sas1* 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 *sas2*, which does not overaccumulate Li⁺. Also, like *sas1* plants (Nublat *et al.*, 2001), *sas2* plants display increased sensitivity to salt stress compared with wild-type plants. In *sas1*, 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

	Tissue ^a	No. of repeats ^b	Na ⁺ content ^c		<i>sas2-1</i> versus WT ratio
			Wild type	<i>sas2-1</i>	
(A)	Bud	6	25 ± 2	80 ± 5	3.2
	Flower	6	35 ± 3	150 ± 16	4.3
	Green silique	6	41 ± 6	200 ± 29	4.9
	Cauline leaf	6	41 ± 6	340 ± 34	8.3
	Stem	6	42 ± 2	40 ± 4	1
	Rosette leaf	6	75 ± 4	320 ± 50	4.3
(B)	Shoot <i>in vitro</i>	6	1160 ± 120	1860 ± 70	1.6
	Root <i>in vitro</i>	6	250 ± 20	153 ± 12	0.6
(C)	Shoot in hydroponic culture	10	990 ± 230	2060 ± 75	2.1
	Root in hydroponic culture	10	380 ± 17	150 ± 4	0.4

^aThe 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.

^bThe 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.

^cNa⁺ 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 overly 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 (Munns *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 apoplast 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 apoplast 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 (Munns *et al.*, 1988). It is worth noting that, in *Arabidopsis*, this model of functional interaction between *AtHKT1* and the tonoplast 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 *sas2-1* 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 *sas2-2* (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 *M₂* progenies were screened for each of 350 *M₁* 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 *sas2-1* 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 *sas2-2* 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 *sas2-1* mutation

The *sas2-1* mutant was used as the male parent in the genetic crosses. Mapping analysis was performed using CAPS (GapC, GL1, ASA1, NCC1, GPA1, DFR, KLP and g8300) and microsatellite (nga6, nga8 and nga280) markers described in the TAIR database (www.arabidopsis.org), together with new CAPS (F17A8M, CER446979, T5L19SE and F24G24SE) and SLP (CER451180/1) markers created for this work (Figure 2; Table II).

Genetic constructs and transgenic plants

A 10.7-kb-long *SpeI*–*SpeI* genomic DNA fragment comprising the whole *AtHKT1* gene was subcloned from the BAC T9A4 into the binary vector pBIB-HYG (Becker, 1990) by conventional means. The resulting construct was named pBIBHAtHKT1.

A 2.3-kb-long fragment corresponding to the *AtHKT1* promoter region was amplified from genomic DNA (ecotype Columbia) by PCR with the EurobioTAQ 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 pBi320.X (R.Derose, RHOBIO, Evry, France). pBi320.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 subcloned into pBIB-HYG. The resulting construct was called pBIBHAtHKT1::GUS.

pBIBHAtHKT1 and pBIBHAtHKT1::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

Name		Primers	Restriction enzyme	Fragment sizes ^a	
				Landsberg <i>erecta</i>	Columbia
(A)	SSLP markers CER451180/1 ^b	GTTTTAGGCCGGTTTAGTTCAC TGTCGAAGATTCGGTCCAAGAACC	—	570	670
(B)	CAPS markers F17A8M	ATATACCACTAGACACTAGACT GTGTTTTGGCAAGAATTAGTTGTTGGA	<i>Xba</i> I	615	410+205
	CER446979 ^b	GGAGAAGCAGACAGCAAGCAAGAGT CACCTTTTAGCAGAACGAGCCAGT	<i>Taq</i> I	455+360	815
	T5L19SE	CAAGATTCAAACGGTTGAGGTGGG CAAGACTATCATGCTGACATGTCG	<i>Rsa</i> I	775+255+210 +175+100	775+210+175 +155+100+100
	F24G24SE	ATGTTTCCCTGGAATCAACCGAG GATGATATAGCCACCTGCAAAACC	<i>Xba</i> I	1260+305	820+440+305

^aFragment sizes are expressed in base pairs.

^bMarkers CER446979 and CER451180/1 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 pBIBHAtHKT1-containing Agl0 and the pBIBHAtHKT1::GUS-containing GV3101 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*-glucuronidase histochemical assay**

GUS histochemical staining was performed as described by Lagarde *et al.* (1996), except that the embedding resin was the hydroxyethyl methacrylate Technovit 7100 (Heraeus-Kulzer GmbH, Wehrin, 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 *AtHKT1* 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).

²²Na 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 *AtHKT1*, which was both (i) inserted between the T7 promoter and a poly(A) sequence and (ii) placed under the control of the yeast *Gall* promoter. An ~0.3-kb-long *Bcl*I–*Eco*RI fragment comprising the *sas2-1* mutation was subcloned into pNU111, replacing the corresponding wild-type fragment. The resulting construct was called pNUsas2-1. Capped complementary RNAs (cRNA) of *AtHKT1* and *sas2-1* were prepared from pNU111 and pNUsas2-1 with the mMESSAGE mMACHINE kit (Ambion, Austin, TX). Electrophysiological analyses were performed as described by Véry *et al.* (1995). The same batch of oocytes was used for all injections. The oocytes were injected with 50 ng of cRNA. 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 D-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 detached, 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 hamp. The roots and the top of the hamp were cut off. The rosette was placed in a pressure chamber with the rest of the hamp 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).

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References

- Apse,M.P., Aharon,G.S., Snedden,W.A. and Blumwald,E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na⁺/H⁺ antiport in *Arabidopsis*. *Science*, **285**, 1256–1258.
- Becker,D. (1990) Binary vectors which allow the exchange of plant selectable markers and reporter genes. *Nucleic Acids Res.*, **18**, 203.

- Clough,S.J. and Bent,A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.*, **16**, 735–743.
- Corbesier,L., Havelange,A., Lejeune,P., Bernier,G. and Périlleux,C. (2001) N content of phloem and xylem exudates during the transition to flowering in *Sinapis alba* and *Arabidopsis thaliana*. *Plant Cell Environ.*, **24**, 367–375.
- Davenport,R.J. and Tester,M. (2000) A weakly voltage-dependent, nonselective cation channel mediates toxic sodium influx in wheat. *Plant Physiol.*, **122**, 823–834.
- Deeken,R., Geiger,D., Fromm,J., Koroleva,O., Ache,P., Langenfeld-Heyser,R., Sauer,N., May,S.T. and Hedrich,R. (2002) Loss of the AKT2/3 potassium channel affects sugar loading into the phloem of *Arabidopsis*. *Planta*, **216**, 334–344.
- Demidchik,V. and Tester,M. (2002) Sodium fluxes through non-selective cation channels in the plasma membrane of protoplasts from *Arabidopsis thaliana* roots. *Plant Physiol.*, **128**, 379–387.
- Diatloff,E., Kumar,R. and Schachtmann,D.P. (1998) Site directed mutagenesis reduces the Na⁺ affinity of HKT1, an Na⁺ energized high affinity K⁺ transporter. *FEBS Lett.*, **432**, 31–36.
- Durell,S.R. and Guy,H.R. (1999) Structural models of the KtrB, TrkH, and TrkI,2 symporters based on the structure of the KcsA K⁺ channel. *Biophys. J.*, **77**, 789–807.
- Horie,T., Yoshida,K., Nakayama,H., Yamada,K., Oiki,S. and Shimmyo,A. (2001) Two types of HKT transporters with different properties of Na⁺ and K⁺ transport in *Oryza sativa*. *Plant J.*, **27**, 129–138.
- Jeschke,W.D. and Pate,J.S. (1991) Cation and chloride partitioning through xylem and phloem within the whole plant of *Ricinus communis* L. under conditions of salt stress. *J. Exp. Bot.*, **42**, 1105–1116.
- Jeschke,W.D., Pate,J.S. and Atkins,C.A. (1987) Partitioning of K⁺, Na⁺, Mg²⁺, and Ca²⁺ through xylem and phloem component organs and nodulated white lupin under mild salinity. *J. Plant Physiol.*, **128**, 77–93.
- Jeschke,W.D., Wolf,O. and Hartung,W. (1992) Effect of NaCl on flows and partitioning of C, N, and mineral ions in whole plants of white lupin, *Lupinus albus* L. *J. Exp. Bot.*, **43**, 777–788.
- Kato,Y., Sakaguchi,M., Mori,Y., Saito,K., Nakamura,T., Bakker,E.P., Sato,Y., Goshima,S. and Uozumi,N. (2001) Evidence in support of a four transmembrane-pore-transmembrane topology model for the *Arabidopsis thaliana* Na⁺/K⁺ translocating AtHKT1 protein, a member of the superfamily of K⁺ transporter. *Proc. Natl Acad. Sci. USA*, **98**, 6488–6493.
- King,R.W. and Zeevaert,J.A.D. (1974) Enhancement of phloem exudation from cut petioles by chelating agents. *Plant Physiol.*, **53**, 96–103.
- Koncz,C. and Schell,J. (1986) The promoter of T₁-DNA gene 5 controls the tissue specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.*, **204**, 383–396.
- Lacombe,B., Pilot,G., Michard,E., Gaymard,F., Sentenac,H. and Thibaud,J.-B. (2000) A shaker-like K⁺ channel with weak rectification is expressed in both source and sink phloem tissues of *Arabidopsis*. *Plant Cell*, **12**, 837–851.
- Lagarde,D., Basset,M., Lepetit,M., Conejero,G., Gaymard,F., Astruc,S. and Grignon,C. (1996) Tissue-specific expression of *Arabidopsis AKT1* gene is consistent with a role in K⁺ nutrition. *Plant J.*, **9**, 195–203.
- Laurie,S., Feeney,K.A., Maathuis,F.J., Heard,P.J., Brown,S.J. and Leigh,R.A. (2002) A role for HKT1 in sodium uptake by wheat roots. *Plant J.*, **32**, 139–149.
- Lazo,G.R., Stein,P.A. and Ludwig,R.A. (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Biotechnology*, **9**, 963–967.
- Liu,W., Schachtmann,D.P. and Zhang,W. (2000) Partial deletion of a loop region in the high affinity K⁺ transporter HKT1 changes ionic permeability leading to increased salt tolerance. *J. Biol. Chem.*, **275**, 27924–27932.
- Lohaus,G., Hussmann,M., Pennewiss,K., Schneider,H., Zhu,J.-J. and Sattelmacher,B. (2000) Solute balance of a maize (*Zea mays* L.) source leaf as affected by salt treatment with special emphasis on phloem retranslocation and ion leaching. *J. Exp. Bot.*, **51**, 1721–1732.
- Mäser,P. *et al.* (2002a) Glycine residues in potassium channel-like selectivity filters determine potassium selectivity in four-loop-per-subunit HKT transporters from plants. *Proc. Natl Acad. Sci. USA*, **99**, 6428–6433.
- Mäser,P. *et al.* (2002b) Altered shoot/root Na⁺ distribution and bifurcating salt sensitivity in *Arabidopsis* by genetic disruption of the Na⁺ transporter AtHKT1. *FEBS Lett.*, **6**, 157–161.
- Munns,R. (1993) Physiological processes limiting plant growth in saline soil: some dogmas and hypotheses. *Plant Cell Environ.*, **16**, 15–24.
- Munns,R. (2002) Comparative physiology of salt and water stress. *Plant Cell Environ.*, **25**, 239–250.
- Munns,R., Tonnet,M.L., Shennan,C. and Gardner,P.A. (1988) Effect of high external NaCl concentration on ion transport within the shoot of *Lupinus albus*. II. Ions in phloem sap. *Plant Cell Environ.*, **11**, 291–300.
- Nublat,A., Desplans,J., Casse,F. and Berthomieu,P. (2001) *sas1*, an *Arabidopsis* mutant overaccumulating sodium in the shoot, shows deficiency in the control of the root radial transport of sodium. *Plant Cell*, **13**, 125–137.
- Rubio,F., Gassmann,W. and Schroeder,J.I. (1995) Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. *Science*, **270**, 1660–1663.
- Rubio,F., Schwarz,M., Gassmann,W. and Schroeder,J.I. (1999) Genetic selection of mutations in the high affinity K⁺ transporter HKT1 that define functions of a loop site for reduced Na⁺ permeability and increased Na⁺ tolerance. *J. Biol. Chem.*, **274**, 6839–6847.
- Rus,A. *et al.* (2001) AtHKT1 is a salt tolerance determinant that controls Na⁺ entry into plant roots. *Proc. Natl Acad. Sci. USA*, **98**, 14150–14155.
- Schachtman,D.P. and Schroeder,J.I. (1994) Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. *Nature*, **370**, 655–658.
- Shi,H., Quintero,F.J., Pardo,J.M. and Zhu,J.-K. (2002) The putative plasma membrane Na⁺/H⁺ antiporter SOS1 controls long-distance Na⁺ transport in plants. *Plant Cell*, **14**, 465–477.
- Truernit,E. and Sauer,N. (1995) The promoter of the *Arabidopsis thaliana* SUC2 sucrose-H⁺ symporter gene directs expression of glucuronidase to the phloem: evidence for phloem loading by SUC2. *Planta*, **196**, 564–570.
- Uozumi,N., Kim,E.J., Rubio,F., Yamaguchi,T., Muto,S., Tsuboi,A., Bakker,E.P., Nakamura,T. and Schroeder,J.I. (2000) The *Arabidopsis* HKT1 gene homolog mediates inward Na⁺ currents in *Xenopus laevis* oocytes and Na⁺ uptake in *Saccharomyces cerevisiae*. *Plant Physiol.*, **122**, 1249–1259.
- Véry,A.-A., Gaymard,F., Bosseux,C., Sentenac,H. and Thibaud,J.-B. (1995) Expression of a cloned plant K⁺ channel in *Xenopus* oocytes: analysis of macroscopic currents. *Plant J.*, **7**, 321–332.
- Wolf,O., Munns,R., Tonnet,M.L. and Jeschke,W.D. (1991) The role of the stem in the partitioning of Na⁺ and K⁺ in salt-treated barley. *J. Exp. Bot.*, **42**, 697–704.
- Yeo,A. (1998) Molecular biology of salt tolerance in the context of whole-plant physiology. *J. Exp. Bot.*, **49**, 915–929.
- Zhang,H.-X. and Blumwald,E. (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nat. Biotechnol.*, **19**, 765–768.
- Zhu,J.-K. (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.*, **53**, 247–273.

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