High-affinity sodium uptake in land plants

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(Received September 11, 2009; Accepted November 17, 2009)

High-affinity Na+ uptake in plants and its mediation by HKT transporters have been studied in very few species. This study expands the knowledge of high-affinity Na+ uptake in land plants for both uptake characteristics and involvement of HKT transporters. In non-flowering plants, we analyzed the Na+ content of wild mosses, carried out experiments on K+ and Na+ uptake in the micromolar range of concentrations with the moss Physcomitrella patens and the liverwort Riccia fluitans, studied a Δhkt1 mutant of P. patens and identified the HKT genes of the lycopodiophyta (clubmoss) Selaginella moellendorffii. In flowering plants we studied Na+ uptake in the micromolar range of concentrations in 16 crop plant species, identified the HKT transporters that could mediate high-affinity Na+ uptake in several species of the Triticeae tribe, and described some characteristics of high-affinity Na+ uptake in other species. Our results suggest that high-affinity Na+ uptake occurs in most land plants. In very few of them, rice and species in the Triticeae and Aveneae tribes of the Poaceae family, it is probably mediated by HKT transporters. In other plants, high-affinity Na+ uptake is mediated by one or several transporters whose responses to the presence of K+ or Ba2+ are fundamentally different from those of HKT transporters.

Keywords: HKT transporters • Land plants • Physcomitrella patens • Sodium transport.

Abbreviations: AP, arginine phosphate; KFM, potassium free medium; RT–PCR, reverse transcription–PCR.

The nucleotide sequences reported in this paper have been submitted to the GenBank database under accession numbers FN401372 (PpHKT1), FN401373 (Tm1), FN401382 (Tm2), FN401374 (Tt1), FN401377 (Tt2), FN401376 (Tt3), FN401378 (At1), FN401379 (Ta2), FN401375 (Ta3), FN401381 (As1), FN401380 (Sc1).

Introduction

Potassium is the most abundant cation in the cytoplasm of probably all living cells and it is widely accepted that the substitution of Na+ for a significant part of the cytoplasmic K+ produces toxicity. This general rule applies to plants, in which epidermal and cortical root cells have to take up sufficient K+ for the whole plant, with the greatest possible avoidance of Na+ uptake. The absolute requirement for K+ of plants and the variable composition of plant environments, from which plants cannot escape, explain the rather large number of K+ transport systems in plants (Véry and Sentenac 2003, Ashley et al. 2006, Amrutha et al. 2007, Gierth and Mäser 2007, Britto and Kronzucker 2008, Zhao et al. 2008).

In apparent opposition to the paradigm of near exclusivity of K+ as the normal alkali cation of plant cells, it has been found that some plant species can take up Na+ from external concentrations as low as those at which K+ can be taken up (Rodríguez-Navarro and Rubio 2006). This also occurs in fungi (Benito et al. 2004) and a likely rational explanation for this physiological trait is that Na+ is a suitable substitute for K+ when the low availability of K+ limits growth (Camacho et al. 1981, Flowers and Läuchli 1983, Marschner 1995, Subbarao et al. 2003, Horie et al. 2007). If this explanation is correct high-affinity Na+ uptake should be an ability shared by all, or at least by most, land plants, from bryophytes to flowering plants, because low availability of K+ was probably frequent in the early evolution of land plants (Beerbower 1985). However, neither this explanation nor the consequent conjecture can be assessed because the capacity of most land plants to perform high-affinity Na+ uptake is unknown. Although there are many reports on Na+ uptake by plant roots (reviewed in Amtmann and Sanders 1999) probably in only four species: barley (Hordeum vulgare), wheat (Triticum durum), rice (Oryza sativa) and sunflower (Helianthus annuus) (Rains and Epstein 1967, Garciadeblás et al. 2003) has high-affinity Na+ uptake been clearly demonstrated. In contrast to this low level of information regarding Na+ uptake at the low concentrations prevailing in normal soils, the substitution of K+ by Na+ and even the possibility of applying Na+ fertilizers to crop plants have been classical subjects in plant mineral nutrition (reviewed by Flowers and Läuchli 1983, Marschner 1995, Subbarao et al. 2003). These different levels of research into different aspects of the same problem generate different perspectives of the field of high-affinity Na+ uptake that are revealed by recent reviews, which may specifically address the matter (Rodríguez-Navarro and Rubio 2006) or pay almost no

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attention to it (Apse and Blumwald 2007). All of this indicates that more in-depth research is required in order to establish a comprehensive overview of plant Na⁺ uptake in the micromolar concentration range.

The high-affinity Na⁺ transporters that have been cloned so far belong to the TRK–HKT family, and the best-studied transporter is OsHKT1. For this transporter the comparison of Na⁺ influx in rice roots and in yeast cells expressing the OsHKT1 cDNA (García-de-Blás et al. 2003) as well as reverse genetics (Horie et al. 2007) have proved that OsHKT1 is the micromolar Na⁺ uptake system of rice. For two other transporters, TaHKT1 and HvHKT1, the similar kinetic characteristics of Na⁺ influx in yeast cells expressing them and in the corresponding plant roots support the notion that they mediate high-affinity Na⁺ uptake in wheat and barley (Haro et al. 2005). Although these results leave open the possibility that the rice model is applicable to other plants (García-de-Blás et al. 2003), that extension is problematic because, among HKT transporters, OsHKT1 is more an exception than a rule. Fungal TRK and plant HKT proteins have four MPM motifs (Rodríguez-Navarro 2000, Véry and Sentenac 2003). In flowering plants HKT transporters are grouped in two subfamilies and all of them have a characteristic Ser or Gly residue in the first pore-forming P-loop of the protein that may be related to their function (Mäser et al. 2002, García-de-Blás et al. 2003, Platten et al. 2006). Considering these characteristics of HKT transporters, for four reasons most plants do not fit into the model defined by OsHKT1 and rice: i) OsHKT1 belongs to subfamily 2 but HKT transporters from dicot species are all in subfamily 1, ii) no HKT transporter in subfamily 1 has ever been reported to mediate high-affinity Na⁺ uptake; instead they mediate the low-affinity Na⁺ uptake that removes Na⁺ from the xylem sap and apoplastic plant tissues (Mäser et al. 2002, Berthomieu et al. 2003, Ren et al. 2005, Sunarpi et al. 2005, Davenport et al. 2007); iii) OsHKT1 is the exception in subfamily 2 because only in OsHKT1 is the Ser or Gly characteristic residue in the Pₐ Ser (García-de-Blás et al. 2003, Platten et al. 2006), and iv) OsHKT1 may be functionally different from both TaHKT1 and HvHKT1 because these transporters, but not OsHKT1, mediate high-affinity K⁺ influx and Na⁺–K⁺ cotransport in yeast cells, in addition to high-affinity Na⁺ uptake (Haro et al. 2005, Bañuelos et al. 2008). In conclusion, as described above for the process of micromolar Na⁺ uptake, more in-depth research is required on the transporters that mediate it.

Therefore, this study sought to investigate whether the capacity of taking up Na⁺ from micromolar concentrations is a general characteristic of land plants and whether HKT transporters universally mediate this uptake. In particular we studied high-affinity Na⁺ uptake in bryophytes because ancestral plants with the characteristics of extant bryophytes colonized terrestrial environments in the Paleozoic era (Kenrick and Crane 1997) and, therefore, bryophytes may conserve the systems that early plants used to take up Na⁺.

In this report the new nomenclature of HKT transporters (Platten et al. 2006) is not used because it cannot be directly applied to the HKT transporters of the moss and the clubmoss described herein. The problem of nomenclature that has arisen is not addressed in this study.

Results

Micromolar Na⁺ uptake in bryophytes

We analyzed samples of mosses growing on rocks, granite and limestone, in conditions in which only the rock and rainwater could provide mineral nutrients. These samples contained a very low amount of K⁺, and Na⁺ constituted up to 15% of the K⁺ content. In mosses growing in soils we found a much higher K⁺ content than those on rocks and Na⁺ was practically absent.

To simulate the low K⁺ supply in a moss colony growing on a rock, we grew Physcomitrella patens in KFM solid medium with 0.1 mM K⁺ and 0.2 mM Na⁺. In colonies with a diameter of 10 mm, which were mainly gametophores and had probably exhausted most of the K⁺ in the surroundings of the colonies, the Na⁺ content was half the K⁺ content. A similar experiment in liquid cultures with the liverwort Riccia fluitans produced a similar result except for a slightly lower Na⁺/K⁺ ratio. To describe in more detail these observations, we studied the time course of Na⁺ depletion as a function of the K⁺ concentration in the growth medium. For that purpose, P. patens and R. fluitans were grown in liquid cultures with 200–500 µM K⁺ and 80–150 µM Na⁺ and the changes in the K⁺ and Na⁺ concentrations of the medium that occurred as a consequence of the growth of the plants were recorded. These results revealed that both species took Na⁺ from low micromolar concentrations and that this uptake started after K⁺ had been exhausted (Fig. 1).

Cloning of the PpHKT1 gene

To date HKT transporters are the only transporters known to mediate high-affinity uptake of Na⁺ in plants. Therefore, we studied the transporters of this type in P. patens. BLAST searches using several sequences of HKT transporters as queries identified a single HKT gene in the genome of P. patens (García-de-Blás et al. 2007). The gene was cloned as well as the corresponding cDNA. Comparison of the DNA and cDNA sequences showed that the PpHKT1 gene had two introns of 137 and 297 bp that were in the positions described for the rice HKT genes (García-de-Blás et al. 2003). The gene could encode a protein of 616 amino acids and with topology similar to that of fungal and plant TRK–HKT transporters (Rodríguez-Navarro 2000, Véry and Sentenac 2003).

To establish the phylogenetic relationships of PpHKT1 with other HKT transporters we carried out BLAST searches in the recently sequenced genomes of the lycophyta (clubmoss) Selaginella moellendorffii and the flowering plants Sorghum bicolor, Zea mays, Vitis vinifera, Populus trichocarpa, Brachypodium distachyon and Glycine max. With the identified HKT genes and many previously identified we constructed a comprehensive phylogenetic tree of HKT transporters (Fig. 2). The tree paid special attention to the two previously described subfamilies of HKT transporters (Platten et al. 2006) and the
two clusters into which subfamily 2 can be divided. The predicted proteins encoded by all the newly identified genes showed the typical MPM motifs of TRK–HKT transporters. In *S. moellendorffii* we identified six genes, two of which were very recent duplications. Remarkably, the six *S. moellendorffii* HKT transporters and the one from *P. patens* had a Gly residue in the P₆ loop, similarly to fungal TRK transporters and plant HKT transporters in subfamily 2, with the only known exception of OsHKT1.

The phylogenetic tree of TRK and HKT transporters revealed that HKT transporters of mosses and clubmosses did not belong to either of the two previously proposed subfamilies (Platten et al. 2006). Although phylogenetic analysis with the sequence of the HKT complete proteins (Fig. 2) suggests the possibility of grouping the moss and clubmoss transporters in a subfamily 3 of HKT transporters, phylogenetic analyses of independent motifs led to variable patterns of clustering. A similar finding has been reported for TRK proteins from ascomycete fungi, in which the slower evolution rate of the MPM4 motif with reference to the other MPM motifs may be related to a separate function of Cl⁻ channeling (Miranda et al. 2009). The ambiguous phylogenetic position of moss and clubmoss HKT transporters and the uncertainty about their function raises interesting questions about these transporters, but they are beyond the scope of this study. Therefore, until further studies are carried out we preferred to keep the classical one number nomenclature of HKT transporters for them instead of adding a subfamily with a doubtful phylogenetic and functional support.

**PpHKT1 does not mediate high-affinity Na⁺ uptake**

Expression of the *PpHKT1* cDNA in a yeast mutant defective in *K⁺* uptake, which does not take up *Na⁺* from low *Na⁺* concentrations, proved that *PpHKT1* mediated *K⁺* and *Na⁺* influx. *Na⁺* uptake tests in yeast cells, which did not contain *Na⁺* at the beginning of the experiment, demonstrated that *PpHKT1* mediated *Na⁺* influx that exhibited a hyperbolic *Na⁺* concentration dependence (*Kₘ* = 1.6 mM *Na⁺*; Fig. 3A). According to these kinetics *PpHKT1* could not be considered the high-affinity *Na⁺* transporter of *P. patens*, which was consistent with further results described below (see Fig. 5). Similar experiments for *K⁺* influx could not be carried out because *K⁺* uptake cannot be detected in cells that contain much *K⁺*. This creates an experimental problem when, as in this case, the influx at micromolar concentrations is too slow to produce detectable *K⁺* depletion in the external medium. Moreover, the use of *Rb⁺* as a *K⁺* analogue in TRK–HKT transporters may be misleading because the affinities of *K⁺* and *Rb⁺* for the transporter may be quite different (Rodríguez-Navarro 2000), or the two cations may be transported with different mechanisms, *K⁺* uniport versus *Rb⁺−Na⁺* symport (Haro et al. 2005). Therefore, we carried out yeast growth tests. These experiments did not provide kinetic information but demonstrated that the *PpHKT1* cDNA suppressed the defective growth of the yeast mutant at low *K⁺* concentrations (Fig. 3B), as a consequence of supplementary *K⁺* uptake. In summary, *PpHKT1* might be described as a *Na⁺* transporter that mediated slow but growth-supporting *K⁺* uptake in yeast cells.

Because there is a single *HKT* gene in the released sequence of the *P. patens* genome we continued the study testing the effects on *Na⁺* uptake of disrupting this gene. The targeted disruption (Fig. 4) was carried out by standard procedures (Frank et al. 2005), and PCR amplification and sequencing of the *PpHKT1* locus in *PpΔhkt1* plants confirmed that the disruption occurred as expected (data not shown). We also confirmed that a second copy of the disruption cassette was not inserted elsewhere in the genome of the mutant by Southern blot analyses with genomic DNA samples (Fig. 4C).

Parallel growth experiments of wild-type and *pphkt1-1* plants at different *K⁺* and *Na⁺* concentrations did not reveal any effect of the mutation either on the growth rate, or on the *K⁺* or
Na⁺ content. Furthermore, in experiments such as those shown in Fig. 1, the mutation apparently did not affect Na⁺ uptake (not shown). However, in long-term experiments of this type a significant reduction in the uptake rate, e.g. 30%, might be undetectable. Therefore, we carried out short-term K⁺ and Na⁺ uptake experiments in K⁺-starved plants, in which uptake rates can be determined with precision. Again, we did not find differences between the two lines of plants in their uptake rates of the two cations (Fig. 5). These results indicated that PP-HKT1 was not the system involved in high-affinity Na⁺ uptake in *P. patens*. In search of additional information about the function of PP-HKT1 we tested the transcript expression levels of PP-HKT1 in several cultural conditions: K⁺ starvation, medium pH from 3.0 to 9.0, and in the presence of Na⁺; in all these experiments the expression of PP-HKT1 transcript was low and not significantly affected by the cultural conditions (data not shown).

If high-affinity Na⁺ uptake in *P. patens* was mediated by a non-HKT transporter some distinctive features of this uptake could be expected. A constant characteristic of high-affinity Na⁺ uptake in plant species in which the mediation of an HKT transporter is certain or probable: rice, wheat and barley, is its sensitivity to Ba²⁺ (approximately 30% in Fig. 6A). The effect of 4 mM Ba²⁺ (this concentration produces instantaneous inhibition of OsHKT1, see Garcadebláes et al. 2003) varied from no inhibition to a very slight inhibition (<10%), depending on the experiment. Therefore, we increased the concentration to 8 mM Ba²⁺ finding that in most experiments the inhibition was not increased (Fig. 6B) and in a very few there was a slight increase (data not shown). Because the presence of Ca²⁺ decreases the
Ba\(^{2+}\) sensitivity of HKT transporters (Garciadeblás et al. 2003) we carried out experiments in the complete absence of Ca\(^{2+}\), which confirmed that high-affinity Na\(^{+}\) uptake in P. patens is insensitive to Ba\(^{2+}\) and that the weak effect of Ba\(^{2+}\) might be unrelated to the high-affinity Na\(^{+}\) transporter.

In summary, the results obtained with P. patens suggested that in some land plants high-affinity Na\(^{+}\) uptake was mediated by non-HKT transporters, which were insensitive to K\(^{+}\) or Ba\(^{2+}\).

**High-affinity Na\(^{+}\) uptake in flowering plants**

High-affinity Na\(^{+}\) uptake has been demonstrated in barley, wheat, rice and sunflower (Garciadeblás et al. 2003). To investigate whether it takes place in other species we prepared K\(^{+}\)-starved seedlings of rye (Secale cereale), sorghum (S. bicolor), maize (Z. mays), onion (Allium cepa), lentil (Lens culinaris), alfalfa (Medicago sativa), squash (Cucurbita moschata), melon (Cucumis melo), lettuce (Lactuca sativa), chard (Beta vulgaris), carrot (Daucus carota), celery (Apium graveolens) and eggplant (Solanum melongena), and tested their capacity to deplete Na\(^{+}\) from 30 to \(\leq 5\) µM. All species passed this uptake test producing similar results with excised roots and with whole plants. Then, we carried out detailed time courses of Na\(^{+}\) depletion in some species, which were used for kinetic analyses (see Material and Methods section). Table 1 summarizes the \(K_{1/2}\) constant of Na\(^{+}\) influx in several species.

When we constructed the phylogenetic tree of HKT transporters presented above (Fig. 2) we found that no transporter from S. bicolor or Z. mays belonged to cluster 1 in subfamily 2. This absence raised the question of whether any HKT transporter in S. bicolor or Z. mays could mediate high-affinity Na\(^{+}\) uptake, because all studied high-affinity Na\(^{+}\) transporters, TaHKT1, HvHKT1 and OsHKT1, belong to cluster 1 in subfamily 2.

![Fig. 3 Transport of Na\(^{+}\) and K\(^{+}\) by yeast cells expressing the PpHKT1 cDNA.](image)

(A) Concentration dependence of Na\(^{+}\) influx in K\(^{+}\)-starved cells of the WΔ6 strain carrying the ∆trk1 ∆trk2 mutations and transformed with PpHKT1 cDNA; in the test conditions Na\(^{+}\) influx of cells transformed with the empty plasmid, pYGE15, amounted to <5% of the recorded values. Inset, double reciprocal plot of the data. (B) Growth of the WΔ6 strain transformed with the PpHKT1 cDNA in AP medium containing the recorded K\(^{+}\) concentrations.

![Fig. 4 Targeted disruption of the PpHKT1 gene by double homologous recombination.](image)

(A) The disruption fragment that was transformed into P. patens is shown below the PpHKT1 gene; aph4 indicates the encoded hygromycin B phosphotransferase marker protein; the introns are shown as clearer grey fragments, the small gray arrows show the positions of primers used for screening the transformants. (B) Scheme of the wild-type and disrupted loci; the figures indicate the size of the restriction fragments. (C) Southern blot detection of the electrophoresed restriction fragments produced by complete digestion of the genomic DNA from pphkt1-1 plants with the restriction enzymes indicated in each line.
Although there are no established criteria for assessing whether a particular HKT transporter performs this function, it has been reported that $K^+$ starvation highly enhances the transcript levels of $HvHKT1$, $TaHKT1$, and $OsHKT1$ in roots (Wang et al. 1998, García-de Blas et al. 2003). In contrast, this treatment does not enhance or produce a weak enhancement of rice HKT transporters different from OsHKT1 (García-de Blas et al. 2003). Therefore, we tested the HKT transcript levels in sorghum roots before and after $K^+$ starvation, i.e. when high-affinity $Na^+$ uptake does not exist or after it has been developed, respectively, in search of a relationship between transcript abundance and the development of high-affinity $Na^+$ uptake. The results showed that none of the HKT genes of sorghum displayed notable transcript enhancement ($>3$-fold) after $K^+$ deprivation (data not shown).

Taken together, the phylogenetic and expression findings suggested that high-affinity $Na^+$ uptake in sorghum might not be mediated by an HKT transporter.

**Only OsHKT1 has the characteristic $P_A$ loop Ser residue in subfamily 2 of HKT transporters**

OsHKT1 mediates root $Na^+$ uptake in rice roots (Horie et al. 2007), and TaHKT1 and HvHKT1 have been proposed to carry out the same function in wheat and barley (Haró et al. 2005). As mentioned above, HKT transporters have a characteristic Ser or Gly residue in the $P_A$ loop (Mäser et al. 2002b, García-de Blas et al. 2003, Platten et al. 2006) and in all HKT transporters of subfamily 1 that have been identified up to now this residue is Ser whereas in all transporters of subfamily 2 it is Gly, with the exception of OsHKT1. Thus, the two HKT transporters of cluster 1 in subfamily 2 that have been isolated in wheat and barley both have Gly residues (Schachtman and Schroeder 1994, Haró et al. 2005). However, the existence of a second HKT1 transporter in cluster 1 of subfamily 2 in these species with a Ser residue is possible because this is the case for rice Pokkali, in which OsHKT1 has a Ser residue and OsHKT2 a Gly residue (Horie et al. 2001), and because two HKT1 genes exist in the A, B and D wheat genomes (Huang et al. 2008). Although Ser/Gly and Gly/Ser mutations of these characteristic residues change the transport capacity of HKT transporters (Mäser et al. 2002b, Horie et al. 2008), it is unlikely that this residue determines the selectivity of the transporters by itself (Platten et al. 2006).
However, considerable confusion would arise if grasses had two HKT transporters with very similar sequences but different transport capacities in cluster 1 of subfamily 2, one with a Gly residue and another with a Ser residue. If this were the case, previous studies of transcript expression and root Na⁺ uptake might actually correspond to the transporters with the Ser residue. In contrast, the transporters cloned and expressed in yeast cells were those with a Gly residue, because only these might actually correspond to the transporters with the Ser residue and another with a Ser residue. If this were the case, two types of high-affinity Na⁺ uptake in flowering plants could be mediated by non-HKT transporters. Considering the types of HKT transporter, land plant species can be divided into four groups (Fig. 2): (i) plants with

<table>
<thead>
<tr>
<th>Species</th>
<th>K₁/₂ (µM)</th>
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<tbody>
<tr>
<td>Rye (S. cereale)</td>
<td>50</td>
</tr>
<tr>
<td>Sorghum (S. bicolor)</td>
<td>45</td>
</tr>
<tr>
<td>Maize (Z. mays)</td>
<td>60</td>
</tr>
<tr>
<td>Onion (A. cepa)</td>
<td>26</td>
</tr>
<tr>
<td>Lentil (L. culinaris)</td>
<td>32</td>
</tr>
<tr>
<td>Alfalfa (M. sativa)</td>
<td>25</td>
</tr>
<tr>
<td>Melon (C. melo)</td>
<td>20</td>
</tr>
<tr>
<td>Carrot (D. carota)</td>
<td>28</td>
</tr>
<tr>
<td>P. patens</td>
<td>120⁺</td>
</tr>
<tr>
<td>Barley (H. vulgare)</td>
<td>40⁻</td>
</tr>
<tr>
<td>Rice (O. sativa)</td>
<td>60⁻</td>
</tr>
<tr>
<td>Wheat (T. durum)</td>
<td>15⁻</td>
</tr>
<tr>
<td>Sunflower (H. annus)</td>
<td>25⁻</td>
</tr>
</tbody>
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*K₁/₂ was calculated from K⁺ depletion curves as described in the Material and Methods section.

**Table 1** High-affinity Na⁺ uptake in plant species

Fig. 7 Sequence analysis of 520 bp fragments of HKT1 genes in species of the Triticaceae and Aveneae tribes. These fragments encode the first pore-forming P-loop (Pₐ) of the transporters. OsHKT3 and OsHKT9 have been included as an outgroup. (A) Alignment of the Pₐ and M₂A translated sequences. (B) Phylogenetic tree constructed with the sequences of the DNA fragments; the inset with the Accession numbers: T. monoccum (AA) (Tm1, FN401373; Tm2, FN401382; T. turgidum (AABB)) (Tt1, FN401374; Tt2, FN401377; Tt3, FN401376); A. tauschii (DD) (At1, FN401378); T. aestivum (AABBD) (Ta1, TaHKT1; Ta2, FN401379; Ta3, FN401375); A. sativa, FN401381; S. cereale, FN401380, Oryza rufipogon, DQ374773; Oryza glumaeaputala, DQ374754; Oryza longistaminata, DQ374753; Oryza meridianalis, DQ374751; Oryza barthii, DQ374748; Oryza nivara, DQ374757. The sequences of Ta2 and At1, and those of Tm1 and Tt1, respectively, are identical.
HKT transporters in subfamily 1 and in the two clusters of subfamily 2 (genera *Triticum*, *Hordeum*, *Secale*, *Avena*, *Brachypodium* and *Oryza*); (ii) plants with HKT transporters in subfamily 1 and exclusively in cluster 2 of subfamily 2 (genera *Zea* and *Sorghum*); (iii) plants with HKT transporters only in subfamily 1 (probably all dicot species); (iv) plants with HKT transporters in neither of the two HKT subfamilies (mosses and clubmosses). To date, only HKT transporters in cluster 1 of subfamily 2 have been proposed to mediate high-affinity Na\(^+\) uptake. Therefore, with the current exceptions of species from the genera *Triticum*, *Hordeum*, *Secale*, *Avena*, *Brachypodium* and *Oryza*, high-affinity Na\(^+\) uptake in land plants might not be mediated by HKT transporters, as found in *P. patens*. To explore this possibility, we carried out identical tests to determine the sensitivity to K\(^+\) and Ba\(^{2+}\) of high-affinity Na\(^+\) uptake in one species, rice, sorghum and sunflower, of each of the first three plant types described above (Fig. 8). In rice the sensitivity to K\(^+\) or Ba\(^{2+}\) was high, as previously described (García-de-la-Banda et al. 2003); in sorghum it was very low; and in sunflower plants K\(^+\) or Ba\(^{2+}\) did not inhibit. We also tested rye, and several cultivars of barley, and of durum and bread wheat. All of them displayed high-affinity Na\(^+\) uptake similar to that of rice, but with slight variants regarding Ba\(^{2+}\) sensitivity that have not been further investigated.

**Discussion**

The first general question addressed in this study was about the number of plant species that can take up Na\(^+\) from very low concentrations, i.e. as low as those at which K\(^+\) can be taken up. We studied this uptake in two bryophytes, *P. patens* and *R. fluitans*, and in seven monocot and nine dicot species belonging to several plant families. All of these species showed high-affinity Na\(^+\) uptake. In some of them, the rate of Na\(^+\) uptake was as rapid as that of K\(^+\) uptake at cation concentrations of 100 \(\mu\)M (Fig. 8). In others it was slower but of the same order of magnitude (Fig. 5). This sample of plant species is too small to make conclusions for all land plants. However, we found significant amounts of Na\(^+\) in mosses growing on rocks that were not rich in Na\(^+\), and the flowering plant species that we tested were selected only because they belong to different plant families and have seeds of a size appropriate for our testing, among those seeds available from a local seller. Therefore, we used a random sample and our findings lend support to the notion that Na\(^+\) uptake from very low concentrations is probably a normal feature of land plants. This trait is no biological surprise, because low K\(^+\) availability was probably normal in the early evolution of plants (Beerbower 1985), and because the use of Na\(^+\) to partially substitute for K\(^+\) is a conceivable solution.

For the Na\(^+\) uptake described herein we continue to use the name of high-affinity Na\(^+\) uptake (Rodríguez-Navarro and Rubio 2006), which is the term given for the K\(^+\) transport that saturates at low K\(^+\) concentrations (Epstein et al. 1963). Although this fact is evident in the depletion curves shown in Figs. 1, 6, 8 (see Materials and Methods section) the name may be questioned because of two specific characteristics of this transport. The first is that precisely because of the existence of high-affinity Na\(^+\) transporters and the unavoidable presence of Na\(^+\) in growth media it is impossible to prepare K\(^+\)-starved plant roots without Na\(^+\) and difficult to prepare them with a low Na\(^+\) content. Therefore, if special conditions are not met, the Na\(^+\) content in the roots of K\(^+\)-starved plants is high and it down-regulates the kinetics of Na\(^+\) influx. The Na\(^+\) content of roots probably explains why in a study on OsHKT1 (Horie et al. 2007) the originally reported high-affinity Na\(^+\) uptake of rice roots (García-de-la-Banda et al. 2003) was not found and was called into question. In relation to this problem our current results emphasize the convenience of considering the Na\(^+\) content in studies with K\(^+\)-starved plants. This content, which may be significant with reference to the K\(^+\) content, has traditionally been neglected (Horie et al. 2007, Armengaud et al. 2009). The second characteristic is that fungal TRK and plant HKT transporters have two binding sites (Haro and Rodríguez-Navarro 2002, Rodríguez-Navarro and Rubio 2006) and may exhibit a sigmoid concentration dependence (Haro and Rodríguez-Navarro 2002; see also the sigmoidal response of OsHKT2;1 in Fig. 7B, Horie et al. 2007) that might complicate a discussion of kinetics.

The second question addressed in this study was the identity of the transporters that mediate high-affinity Na\(^+\) uptake in plants. Because OsHKT1 mediates high-affinity Na\(^+\) uptake in rice (Horie et al. 2007) and rice was proposed as a model for this transport (García-de-la-Banda et al. 2003), the question also involves the consideration of whether the rice model applies to other plant species. Attending to sequence analysis, OsHKT1 is exceptional because no other HKT transporter in subfamily 2 has its...
characteristic Ser residue in the P_{A} loop (Figs. 2, 7). If this residue determined the function, high-affinity Na\(^{+}\) uptake in rice would not have a counterpart in any other plant species. However, this may not be the case because in terms of K\(^{+}\) and Ba\(^{2+}\) sensitivity, high-affinity Na\(^{+}\) uptake in wheat, barley and rye, all with HKT transporters in cluster 1 of subfamily 2, cannot be distinguished from that of rice. In contrast, sorghum, which has no HKT transporter in cluster 1 of subfamily 2 (Fig. 2), is different from wheat, barley and rye in terms of K\(^{+}\) and Ba\(^{2+}\) sensitivity (Fig. 8). These findings suggest that the rice model applies only to itself and probably to other species with transporters in cluster 1 of subfamily 2.

High-affinity Na\(^{+}\) uptake in P. patens is not mediated by PpHKT1 because Pp\(\Delta\)hkt1 plants maintained normal K\(^{+}\) and Na\(^{+}\) influx (Fig. 5). Moreover, according to the current release of the genome sequence, HKT1 is the only HKT transporter in P. patens. Both observations together indicate that high-affinity Na\(^{+}\) uptake in this species is not mediated by an HKT transporter. This conclusion is also suggested by the K\(^{+}\) or Ba\(^{2+}\) insensitivity of Na\(^{+}\) uptake (Fig. 6).

In flowering plant species without transporters in cluster 1 of subfamily 2, high-affinity Na\(^{+}\) uptake is unlikely to be mediated by an HKT transporter. This hypothesis is supported by the experiments on HKT gene expression in sorghum. High transcript levels of the HvHKT1, TaHKT1 and OsHKT1 genes in K\(^{-}\)-starved plants (Wang et al. 1998, Horie et al. 2001, Garcia-de Blas et al. 2003, Horie et al. 2007) suggest that transcripts of high-affinity Na\(^{+}\) transporters might be highly expressed only in K\(^{-}\)-starved plants, which is in coherence with the fact that in all the species that we have studied high-affinity Na\(^{+}\) uptake does not exist in normal plants and appears after K\(^{-}\) starvation. Therefore, the lack of a significant induction of any HKT gene in sorghum under K\(^{-}\) starvation suggests that high-affinity Na\(^{+}\) uptake in these species is not mediated by the transporters encoded by the HKT genes. A further support of this hypothesis is the K\(^{+}\) and Ba\(^{2+}\) insensitivity of high-affinity Na\(^{+}\) uptake in sorghum and sunflower, which show similarity with P. patens but are different from rice, barley, wheat and rye.

The existence of at least two types of transporter mediating very similar processes of high-affinity Na\(^{+}\) uptake in land plants lends further support to the notion already discussed of the importance of high-affinity Na\(^{+}\) uptake in the physiology of plants. Because in flowering plants we only tested domesticated crop plants, it can be concluded that domestication has not eliminated the process and, therefore, that high-affinity Na\(^{+}\) uptake benefits the plant at certain moments in crop production. This possibility has already been considered (Flowers and Lüchicht 1983, Marschner 1995, Subbarao et al. 2003), but it still requires further investigation. For example, maize, sorghum and rye are considered to be plant species in which Na\(^{+}\) is not beneficial, but we found that all three of them carry out high-affinity Na\(^{+}\) uptake.

We found six HKT genes in S. moellendorffii. Because S. moellendorffii is a vascular plant some of the encoded transporters may have the function of Na\(^{+}\) recirculation by removing Na\(^{+}\) from the xylem sap and loading Na\(^{+}\) into the phloem sap as some subfamily 1 HKT transporters do in flowering plants (Mäser et al. 2002a, Berthomieu et al. 2003, Ren et al. 2005, Sunarpi et al. 2005, Davenport et al. 2007). Subfamily 1 HKT transporters have a characteristic Ser residue in the P_{A} loop in which all the S. moellendorffii transporters have a Gly residue. Therefore, if some S. moellendorffii and subfamily 1 HKT transporters had the same function the Ser or Gly residue in the P_{A} loop may be more closely related to the evolution of the transporters than to their functions. In any case it would be interesting to find out whether the Gly transporters are more or less effective than the Ser transporters for performing the task of removing Na\(^{+}\) from the xylem sap. The possibility that extant representatives of early vascular plants may be a source of genes for improving salt tolerance in crop plants is worth exploring.

### Materials and Methods

**Plants and growth conditions**

The moss *P. patens* (Ashton et al. 1979b) was maintained axenically in BCDAT medium (Nishiyama et al. 2000) supplemented with 7 g liter\(^{-1}\) agar when required, in a phytochamber as described elsewhere (Garcia-de Blas et al. 2007). A K\(^{+}\)-free mineral medium (KFM), which contained 1.5 mM Ca(NO\(_{3}\))\(_{2}\), 0.5 mM PO\(_{4}\)H\(_{3}\), 1 mM MgSO\(_{4}\), 45 µM FeSO\(_{4}\) and oligoelements in the amounts previously described (Ashton et al. 1979a), was adjusted to pH 5.8 with Ca(OH)\(_{2}\) and supplemented with either 2 mM K\(^{+}\) for control plants and 0.4 mM K\(^{+}\) for preparing K\(^{-}\)-starved plants. The Na\(^{+}\) content of this medium was 2–5 µM and, when required in solid form, it was supplemented with agarose instead of agar because of the lower Na\(^{+}\) content of agarose. Plants were normally grown in glass bottles with air bubbling in a phytochamber with continuous white light at 25°C. The bottles were inoculated with plant suspensions containing a high proportion of gametophores. Consequently, the cultures for uptake experiments were almost exclusively gametophores. Control plants contained 2020 ± 180 K\(^{+}\) and 9 ± 2 Na\(^{+}\) nmol mg\(^{-1}\) (dry weight). K\(^{-}\)-starved plants were prepared by growing the plants in the low K\(^{+}\) medium for 2 weeks. Then they were transferred to KFM and incubated in this medium for 10–15 d. These plants contained 520 ± 120 K\(^{+}\) and 15 ± 4 Na\(^{+}\) nmol mg\(^{-1}\). The liverwort *R. fluitans* was maintained and grown as previously described (Ballesteros et al. 1998). When these plants were maintained for a long time in this standard medium they contained a considerable amount of Na\(^{+}\). For the experiment in Fig. 1, a young culture in the standard medium was transferred to 2 mM K\(^{+}\) KFM for 15 d and then to KFM containing the recorded K\(^{+}\) and Na\(^{+}\) concentrations. To prepare K\(^{-}\)-starved seedlings, seeds of the studied species were surface sterilized and germinated in wetted filter paper. Then the germinated seeds were transferred to 5-liter plastic containers with a 2 mM CaCl\(_{2}\) solution as previously described (Garcia-de Blas et al. 2003). Selected seeds produced by seed companies were bought from a local seller.
Bacterial and yeast strains, media and growth conditions

The Escherichia coli strain DH5α was routinely used for plasmidic DNA propagation. The yeast strain W3-56 (Mata ade2 ura3 trp1 trk1Δ::LEU2 trk2A::HIS3) (Haro and Rodríguez-Navarro 2003) deficient in the endogenous K⁺ uptake systems TRK1 and TRK2 was used for functional complementation and transport assays. Yeast transformants were routinely grown in SD medium (Sherman 1991) supplemented with 50 mM K⁺. For yeast growth experiments at low K⁺ concentrations, serial dilution drops of yeast cells were inoculated on arginine phosphate (AP) medium (Rodríguez-Navarro and Ramos 1984) supplemented with the indicated K⁺ concentrations. The K⁺-starved cells were obtained by transferring actively growing cells in 50 mM K⁺ AP medium into K⁺-free AP medium, and incubating them for 4 h.

Recombinant DNA techniques

Manipulation of nucleic acids was performed by standard protocols or, when appropriate, according to the manufacturer’s instructions. PCRs were performed in a Perkin-Elmer thermocycler, using the Expand-High-Fidelity PCR System (Roche Molecular Biochemicals, Summerville, NJ, USA). Total plant RNA and DNA were prepared using the RNeasy Plant Kit and DNeasy Plant Kit (Qiagen, Valencia, CA, USA). PCR amplifications of cDNAs were carried out on double-stranded cDNA using the Synthesis System Kit (GE Healthcare, Barcelona, Spain). The PCR fragments were first cloned into the PCR2.1-Topo vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). DNA sequencing was performed in an automated ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). DNA sequencing was performed in an automated ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The full-length PpHKT1 cDNA was amplified from total RNA of K⁺-starved P. patens plants by standard RT–PCR methods using specific primers designed from the genome sequence (http://genome.jgi-psf.org/Phypha1_1/Phypha1_1_home.html). However, the annotated sequence had an improbable initiation of translation and the 5’ end of the PpHKT1 mRNA was obtained by using the 5’/3’RACE Kit (Roche Diagnostic). The gene was cloned from genomic DNA using specific primers designed from the previously determined 5’ and 3’ untranslated regions of the PpHKT1 mRNA. For yeast expression, the BamHl–KpnI cDNA fragment was cloned into vector pYPGE15 (Brunelli and Pall 1993) and transformed into the trk1 trk2 Saccharomyces cerevisiae strain. PCR amplifications of 520 bp fragments of HKT1 genes in species of Triticaceae and Aveneae tribes were carried out using primers designed from the known sequences in barley, wheat and rice. Two forward and two reverse primers were used in the four possible combinations. Forward primers HKT1-A1 (5’-GT(T/C)T(A/G)TTTCGTCGCATACCATGC-3’) and HKT1-A2 (5’-GGTGTGCTTGCACAGCT-3’) and reverse primers, HKT1-B1R (5’-CCATATGCTCTGTCG(G/C)GGG-3’) and HKT1-B2R (5’-CCATGCTCTTGTGGCAG-3’).

Generation of the Pphkt1-1 knockout line

The PpHKT1 deletion fragment was constructed in plasmid pTN186 (http://moss.nibb.ac.jp/) with two fragments of 1137 and 1221 bp of the PpHKT1 gene flanking the hygromycin-B resistance cassette (the apha4 ORF under the control of 35S promoter and termination sequences) as shown in Fig. 4. The 5’ fragment extended from nucleotide –1985 to –849 and the 3’ fragment from nucleotide 2001 to 3221. The pphkt1-1 knockout line was generated by transforming P. patens protoplasts with 25 µg of a linear fragment obtained by digesting the pTN186(pphkt1) plasmid with Stul and Rcal restriction enzymes. Stable antibiotic-resistant clones were selected after two rounds of incubation in BCDAT medium supplemented with 30 µg ml⁻¹ hygromycin-B. The first screening of putative disrupted clones was carried out by PCRs on genomic DNA purified from transformant plants, one spanning the complete targeted region, and two amplifying a part of the resistance cassette and part of the 5’ and 3’ gene regions outside the deletion construct. In clones in which these amplifications produced fragments of the expected sizes, the fragments were sequenced to verify that integration had occurred as designed. Southern blot analyses of knockout P. patens lines were carried out according to standard procedures. Genomic DNA (15 µg) was digested with EcoRI and HindIII enzymes and hybridized with a probe that includes part of the hygromycin resistance cassette and part of the 5’ region of the PpHKT1 gene, as shown in Fig. 4. The DNA probe was synthesized and digoxigenin labeled by PCR. Hybridization and detection were carried out according to the supplier’s manual (Roche Applied Science).

Real-time PCR assays

For P. patens the RNA was extracted from the K⁺-starved plants described above and from plants growing in 2 mM K⁺ KFM as described (control plants), and plants growing in 2 mM K⁺ KFM adjusted to different pH values or supplemented with 50 mM NaCl. In sorghum the RNA was extracted from seedlings growing in 2 mM CaCl₂, K⁺-starved seedlings, or in 2 mM CaCl₂ and 2 mM KCl, control seedlings. Real-time PCRs were performed using an ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems) and SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems). PCR amplifications were carried out according to standard procedures as described in García-Deblais et al. (2003). The results were expressed as the transcript level ratios between the studied HKT and actin genes in the same preparation. All samples were analyzed in two PCR runs in which all samples were triplicates. The amplified fragments are shown below (if not stated, accession numbers are given in the legend to Fig. 2 and nucleotide numbers are referred to the corresponding sequences). In P. patens: PpACT5 (nucleotides 1281–1422, sequence 109052 in JGI genome) and PpHKT1 (nucleotides 1504–1646). In sorghum: SbHKT6 (1326–1517), SbHKT7 (1467–1647), SbHKT8 (1201–1391), SbHKT9 (1398–1529), SbAct1 (823–1020, sequence Sb05g003880 in JGI S. bicolor genome).
Cation uptake experiments in plants and yeast cells

Cation uptake experiments with whole seedlings or excised roots and with *P. patens* plants were performed by following the depletion of the tested cation by atomic emission spectrophotometry, as described previously (Bañuelos et al. 2002, García-deblás et al. 2003) in 60 ml of buffer for roots and KFM for *P. patens*. In García-deblás et al. (2003) the effect of Ba2+ was tested in the absence of Ca2+. However, the absence of Ca2+ produced defective Na+ uptake in some of the tested species. Therefore, all of the root uptake tests in this study were carried out in aerated 2 mM MES–Ca2+ buffer pH 6.0. Experiments with *P. patens* in the absence of Ca2+ were carried out in modified KFM, in which arginine was substituted for Ca2+. In experiments of Na+ uptake in the presence of K+, K+ had to be added continuously to compensate for K+ uptake. For this purpose, the K+ concentration was determined in each sample of external medium and the result used to calculate the K+ addition that was necessary to restore the K+ concentration. With this procedure the K+ concentration varied <10% with reference to the nominal value. For Na+ uptake experiments in yeast cells, K+-starved cells were suspended in testing buffer: 2% glucose, 10 mM MES–Ca2+ pH 6.0, and, at intervals after the addition of Na+, samples were taken, filtered through 0.8 µm pore nitrocellulose membranes filters and washed with 20 mM MgCl2 in the same filter. Filters were incubated overnight in 0.1 M HCl, and Na+ was determined by atomic emission spectrophotometry of acid-extracted cells. All experiments were repeated several times. Statistical analyses of Na+ influx in *P. patens* have not been carried out but result deviations are small if plants are carefully standardized in terms of K+ and Na+ contents and the length of the K+ starvation periods. For example, using different preparations of well-standardized plants the result deviations of experiments reported in Fig. 6 were <20% of their value. Seedling experiments were also repeated several times and several preliminary experiments had to be carried out to determine the most appropriate size and K+ content of seedlings. The effects of K+ or Ba2+ on Na+ uptake (Figs. 6, 8) were tested in parallel experiments with the same batch of plants or seedlings, using approximately the same amounts of plants or roots. Results from different plant preparations produced almost identical results. The time courses of cation depletion can be used for kinetic analyzing considering that Na+ influx at any given Na+ concentration is the slope of the tangent to the depletion curve at that point. The methods for computing Vmax and Km as well as possible errors of the method have been described elsewhere (Bañuelos et al. 2002). TRK–HKT transporters have two binding sites and may exhibit complex kinetics (Rodríguez-Navarro and Rubio 2006) that we did not compute from depletion curves. Therefore in Table 1 we record the K1/2, the Na+ concentration for half-maximal rate, which can be easily calculated in the saturation kinetics that we found in all cases.

Protein alignments and phylogenetic tree constructions

Protein or nucleotide alignments and phylogenetic trees were obtained using the CLUSTAL X program (Thompson 1997).

Funding

This work was supported by the Spanish Ministerio de Ciencia e Innovación and by the ERDF European program through grant no. AGL2007-61075. Additional financial support was provided by DGUI-UPM Research Group Program.

Acknowledgments

We thank Mitsuyasu Hasebe and Tomoaki Nishiyama for the gift of plasmid pTN86, José A. Fernández for providing the culture of *R. fluitans* and Jesús Muñoz for the taxonomic identification of wild mosses. The help of Ana C. Ureta and Ana Villa in many experiments is greatly acknowledged.

References


High-affinity sodium uptake in land plants


