Running head: Control of HAK1-mediated potassium transport

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The Ionic Environment Controls the Contribution of the Barley HvHAK1 Transporter to Potassium Acquisition

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ABSTRACT

The control of potassium (K⁺) acquisition is a critical requirement for plant growth. Although HAK1 transporters provide a pathway for K⁺ acquisition, the effect exerted by the ionic environment on their contribution to K⁺ capture remains essentially unknown. Here, the influence of the ionic environment on the accumulation of transcripts coding for the *Hordeum vulgare* HvHAK1 transporter as well as on HvHAK1-mediated K⁺ capture has been examined. *In situ* mRNA hybridization studies show that HvHAK1 expression occurs in most root cells, being augmented at the outermost cell layers. Accumulation of HvHAK1 transcripts is enhanced by K⁺-deprivation and transiently by exposure to high salt concentrations. In addition, studies on the accumulation of transcripts coding for HvHAK1 and its close homologue HvHAK1b revealed the presence of two K⁺ responsive pathways, one repressed and the other insensitive to ammonium. Experiments with *Arabidopsis thaliana* HvHAK1-expressing transgenic plants showed that K⁺ deprivation enhances the capture of K⁺ mediated by HvHAK1. A detailed study with HvHAK1-expressing *Saccharomyces cerevisiae* cells also unveils an increase of K⁺ uptake after K⁺ starvation. This increase does not occur in cells grown at high Na⁺ concentrations, but takes place for cells grown in the presence of NH₄⁺. 3,3´-Dihexyloxacarbocyanine iodide accumulation measurements indicate that the increased capture of K⁺ in HvHAK1-expressing yeast cells cannot be only explained by changes in the membrane potential. It is shown that the yeast PPZ1 phosphatase as well as the HAL4/HAL5 kinases negatively regulate the HvHAK1-mediated K⁺ transport.
INTRODUCTION

Potassium is the most abundant essential cation in almost all living cells. Besides having several major functions in normal physiology, $K^+$ plays an important role protecting plants during acclimation to saline-rich and ammonium-rich environments (Flowers and Läuchli, 1983; Cao et al., 1993). Acquisition of $K^+$ from the soil solution is primarily dependent on the activity of transport proteins located in the plasma membrane of soil bacteria, fungi and root epidermal cells of plants. Early on, it has been observed that the kinetics and energetics of $K^+$ transport in most fungi and plants share some common features that are not found in animal cells (Kochian and Lucas, 1988; Rodríguez-Navarro, 2000). This observation led to the concept that in most cases $K^+$ uptake is mediated by similar transport proteins in roots and fungal cells. Providing support for this claim, homologues of the fungal HAK and TRK transporters have been identified in plants (Véry and Sentenac, 2003). The possibility that regulatory elements involved in alkali cation homeostasis are functional in both plants and fungi has also been proposed (Gisbert et al., 2000; Quintero et al., 2002). Unlike their fungal counterparts, the plant homologues of TRK transporters, named HKT, appear to be mainly involved in $Na^+$ transport rather than in $K^+$ acquisition (García-deblás et al., 2003; Rus et al., 2004; Horie et al., 2007). On the other hand, members of the HAK1 subgroup of HAK-KUP-KT proteins have been found to play a major role in $K^+$ uptake in plants (Santa-María et al., 1997; Rubio et al., 2000; Gierth et al., 2005), being an additional route for $K^+$ uptake in roots provided by the Shaker-like inward rectifying $K^+$ channel AKT1 (Sentenac et al., 1992; Hirsch et al., 1998). Studies in yeasts indicate that $K^+$ transport mediated by HAK1 transporters is sensitive to the presence of $NH_4^+$, while studies with $akt1$ mutant plants demonstrate that the AKT1 channel is involved in the capture of $K^+$ for plants grown at high $NH_4^+$ concentrations (Hirsch et al., 1998; Santa-María et al., 2000). Other studies support the view that the $NH_4^+$-sensitive pathway involves the activity of more than
a single K⁺ transporter (Spalding et al., 1999; Gierth et al. 2005; Vallejo et al. 2005).

It has been shown that the acquisition of K⁺ by plants is a tightly regulated process. Perception of K⁺ deprivation rapidly occurs after K⁺ removal from the growth media leading to an enhancement of the K⁺ uptake capacity from diluted K⁺ solutions (Glass, 1976; Kochian and Lucas, 1982), which probably requires the accumulation of reactive oxygen species in a discrete root zone (Shin and Schachtman, 2004). Besides, the pharmacological properties and the thermodynamic constrains of K⁺ uptake from diluted K⁺ solutions in plants depend on the abundance of potassium, sodium and ammonium in the media encountered by roots during growth (Spalding et al., 1999; Santa-María et al., 2000; Kronzucker et al., 2003; Nieves-Cordones et al., 2007). When plants are grown in the presence of high NH₄⁺ or Na⁺ concentrations, the enhancement of the K⁺ uptake capacity resulting from K⁺-deprivation is affected, and a change in the contribution of the NH₄⁺-sensitive and NH₄⁺ insensitive components becomes evident. These results suggest a regulatory effect of the ionic environment on the contribution of AKT1 and HAK1 transporters. A clear influence of K⁺-starvation on the control of AKT1 activity has been recently reported (Li et al., 2006; Xu et al., 2006). However, the influence of the ionic environment found by plants during growth on the contribution of a HAK1 transporter remains essentially unknown. Here we introduce evidence for the presence of long-term regulatory mechanisms affecting the abundance of transcripts and the contribution to K⁺ capture of the canonical member of the HAK1 subgroup, the *Hordeum vulgare* HvHAK1 transporter.

RESULTS

**HvHAK1** is preferentially expressed in cells of the root outer-layers

As root cells expressing K⁺ transporters carry out the primary uptake of K⁺ from the soil, the site of *HvHAK1* expression in barley roots was determined. Because of
the existence of several HAK1 genes in Triticeae genomes and the high similitude among them (Santa-María et al., 1997), we used a specific probe mainly containing the 3´UTR for studying the HvHAK1 expression in transversal sections of roots. In situ hybridization studies in seminal barley roots showed that accumulation of HvHAK1 transcripts occurs in all cells layers, being the expression augmented in the outermost layers (Fig. 1A and Supplemental Fig. S1). No signal was detected with the sense probe, showing the suitability of the technique used (Fig. 1B and Supplemental Fig. S1).

The accumulation of transcripts coding for HAK1 transporters involves cross talk between NH$_4^+$-sensitive and NH$_4^+$-insensitive mechanisms

The specific accumulation of HvHAK1 mRNA was estimated by real time PCR coupled with reverse transcription. Long-term K$^+$-deprivation (no K$^+$ added to the complete aerated culture solution) led to increased accumulation of HvHAK1 transcripts (Fig. 2, A), being a similar pattern observed for plants grown in a MES-Ca$^{2+}$ solution in the absence of aeration (Supplemental Fig. S2). Experiments with plants grown at combined levels of NH$_4^+$ and K$^+$ showed that accumulation of HvHAK1 transcripts following K$^+$-deprivation occurs to the same extent in the presence and the absence of a high NH$_4^+$ concentration during plant culture (Fig. 2, B).

The accumulation of transcripts coding for HvHAK1b, a very close relative of HvHAK1, was also enhanced by long-term K$^+$ deprivation (Fig. 2, A). However, the accumulation of HvHAK1b transcripts following K$^+$-deprivation was significantly lower for plants grown at a high NH$_4^+$ concentration compared to those grown in the absence of NH$_4^+$ (Fig. 2, C). These results indicate that following K$^+$-deprivation the accumulation of transcripts coding for HAK1 transporters involves two different routes, being one of them sensitive and the other insensitive to NH$_4^+$.

HvHAK1 transcripts accumulation is up-regulated by NaCl salinization
Given that exposure to high Na⁺ concentrations interferes with K⁺ nutrition, the effect of NaCl on the accumulation of *HvHAK1* was analyzed. A 6 h exposure to 100 mM NaCl, in the presence of 1 mM K⁺, led to a significant increase in the amount of *HvHAK1* transcripts compared to that measured for control plants, effect that was reverted 48 h after salinization (Fig. 3, A). These results indicate a strong and transient NaCl-triggered *HvHAK1* up-regulation at transcript level, consistent with a possible role of HvHAK1 during the fast response of plants to salinity. Having in mind this possibility, we studied the influence of Na⁺ on the uptake of Rb⁺, a good analogue of K⁺ for HAK1 transporters, following salinization. The quotient between the uptake of Rb⁺ measured in the presence of 100 mM NaCl and that measured in the absence of this salt, increased significantly 6 h after salinization (Fig. 3, B). These results argue for a parallel control of K⁺ uptake properties and *HvHAK1* mRNA accumulation under saline conditions.

Because of the possibility that the rapid up regulation of *HvHAK1* transcripts accumulation could be the result of an osmotic shock, the effect of Sorbitol was explored. A 6 h exposure to 200 mM Sorbitol led to an accumulation of *HvHAK1* transcripts not significantly different from that determined for control plants and lower than that determined for 100 mM NaCl treated plants (Supplemental Fig. S3). While the contribution of an osmotic component can not be ruled out since it could account for a 44 % of that observed in NaCl stressed plants, these data indicate that non-osmotic components could be involved in the transient response. In turn, the 6 h effect of 100 mM NaCl on the accumulation of *HvHAK1* transcripts was not accompanied by a change in the total concentration of K⁺ in roots. Instead, the concentration of Na⁺ in roots clearly increased over that period (Supplemental Fig. S3).

*HvHAK1*-transgenic plants display an enhanced Rb⁺ transport when subjected to K⁺ deprivation
The results shown above indicate that K⁺-deprivation exerts a strong effect on the accumulation of transcripts coding for two HAK1 transporters (Fig. 2). It seems likely that these transporters, and others no examined here homologous to those already studied in Arabidopsis (Hirsch et al., 1998; Ahn et al., 2004; Gierth et al., 2005), contribute to the capture of K⁺ by roots. Therefore, in order to study the effect of K⁺-deprivation on the specific contribution of HvHAK1 to Rb⁺ capture we performed a stable transformation of Arabidopsis thaliana plants with a construct containing HvHAK1 under the control of the 35S promoter. All the HvHAK1-expressing transgenic lines assayed displayed a similar pattern (Fig. 4, A). A more detailed study performed with a selected transgenic line (J1) showed that the uptake of Rb⁺ was just slightly higher for HvHAK1-expressing than for HvHAK1-non expressing plants grown in the presence of 1mM KCl. When plants were deprived of K⁺ for 48h the subsequent transport of Rb⁺ was markedly higher in HvHAK1 expressing plants than in those that do not express HvHAK1 (Fig. 4, B). These results indicate that K⁺ availability controls the contribution of HvHAK1 to K⁺ capture in transgenic Arabidopsis plants.

**Rb⁺ uptake in yeasts cells expressing HvHAK1 is modulated by K⁺ supply**

In order to gain further insight on how the contribution of HvHAK1 is modulated by the composition of the environment, we performed a detailed study of Rb⁺ transport in Saccharomyces cerevisiae mutants compromised for K⁺ uptake. Expression of HvHAK1 in a yeast mutant lacking the TRK1 and the TRK2 K⁺ transporters, show that after K⁺ deprivation, the uptake of Rb⁺ mediated by HvHAK1 increases progressively until reaching a plateau (Fig. 5, A). After attaining this plateau the uptake of Rb⁺ was 3-4 fold higher in K⁺ deprived cells than in cells never subjected to K⁺ starvation. A reciprocal experiment showed that K⁺ re-supply led to a progressive decline of Rb⁺ uptake (Fig. 5, B), indicating that modulation of HvHAK1-mediated Rb⁺-transport is reversible. In measuring Rb⁺ uptake under these experimental conditions, special care should be taken to avoid the masking
effect caused by the release of K⁺ from cells to the medium during the Rb⁺ loading procedure, which may be particularly pronounced at the very beginning of K⁺ deprivation. We found that 15 and 210 min after K⁺ deprivation, the concentration of K⁺ in the loading solution was 8 µM and 4 µM, respectively, indicating the presence of only a minor interference of K⁺ on Rb⁺ uptake measurements made from a 100 µM Rb⁺ solution after 15 min of K⁺ deprivation. Consequently, further comparisons between K⁺ non-starved and K⁺ starved cells were done by comparing cells deprived of K⁺ for 15 and 210 min, respectively.

It has been previously shown that some HAK-KUP-KT transporters could mediate biphasic transport of K⁺ (Kim et al., 1998; Fu and Luan, 1998). No evidence for the modulation of HvHAK1 transport involving a switch between monophasic and biphasic modes of transport was found (Supplemental Fig. S4). Consistently, a single and saturable component, operative in the micromolar range of Rb⁺ concentrations was observed (Fig. 5, C). Values of Vmax were 0.66 ± 0.12 and 1.92 ± 0.32 nmol mg⁻¹ min⁻¹ for K⁺ non-starved and K⁺-starved cells, respectively, indicating a three-fold enhancement of the K⁺ transport capacity. On the other hand, Rb⁺ Km significantly increased from 5.6 ± 1.0 to 9.6 ± 1.3 µM in HvHAK1 expressing cells during the course of K⁺-starvation. Given that the K⁺ concentration in the loading solution is somewhat higher for K⁺-non-starved than for K⁺-starved cells (due to K⁺ loss), the actual difference in Rb⁺ Km may be even higher. An important question to address is whether this increased Km could be just a by-product of a response to K⁺ starvation with no acclimation value. Therefore, we explored whether changes in the Rb⁺ Km of HvHAK1-mediated transport are accompanied by a change in the sensitivity of Rb⁺ transport to Na⁺. The inhibitory effect of 100 mM NaCl on the transport of Rb⁺ was more pronounced for cells deprived of K⁺ for 210 min than for non K⁺-starved cells (Supplemental Fig. S4), a result compatible with a decreased K⁺/Na⁺ selectivity of the HvHAK1 transporter under conditions of K⁺ starvation. Unfortunately, attempts to detect differences in Na⁺ transport between K⁺-starved and K⁺-non-starved HvHAK1 expressing cells in different yeast mutants failed because of the presence of yeast...
low-affinity transporters that dominate the transport of Na⁺ over that attributed to the plant transporter.

An important question to be considered is whether the changes in Vmax above reported are linked, at least in part, to changes in the accumulation of HvHAK1 transcripts during the course of K⁺-deprivation. RT-PCR studies showed that the amount of HvHAK1 transcripts in HvHAK1-expressing yeasts was not significantly different between K⁺-starved and K⁺-non-starved cells (Fig. 5, D).

The contribution of HvHAK1 to K⁺ capture in yeast cells is modulated by the concentration of K⁺ and Na⁺, but not by NH₄⁺, in the growth media

We also tried to gain insight into the S. cerevisiae mechanisms leading to enhanced HvHAK1 K⁺ transport contribution in K⁺ starved cells. For this purpose, we exposed HvHAK1 expressing cells to a wide range of external alkali cation concentrations for 15 or 210 min and measured the subsequent uptake of Rb⁺ in a solution with no addition of K⁺, Na⁺ and NH₄⁺. We observed a similar internal K⁺ concentration and a similar uptake of Rb⁺ for cells exposed for 15 min to different external K⁺ concentrations (data not shown). For 210 min exposed cells, the internal K⁺ concentration was similar at different external K⁺ concentrations (data not shown), but the rate of Rb⁺ uptake depended on the new external K⁺ concentration in the growth media (Fig. 6, A). These results are consistent with a role of K⁺ trafficking across the membrane in controlling HvHAK1 transport. In order to perform an assessment on the specificity of K⁺ supply on changes in Rb⁺ uptake, we next investigated whether or not inclusion of Na⁺ and NH₄⁺ in the growth media interfere with the course of K⁺ deprivation. The addition of Na⁺ during the first 15 min of K⁺ starvation does not exert any effect on the subsequent Rb⁺ transport (data not shown). However, the addition of NaCl during a 210 min K⁺-deprivation period results in a decrease of the subsequent Rb⁺ uptake (Fig. 6, B), indicating that the presence of high Na⁺ concentrations during growth interferes with the stimulatory effect of K⁺ starvation on HvHAK1 contribution to Rb⁺
transport. However, not all monovalent cations possess this capacity. Complementary experiments highlighted that culture at high NH$_4^+$ concentrations does not interfere with the enhancement of HvHAK1-mediated Rb$^+$ transport observed in yeast cells after 210 min of K$^+$ deprivation (Fig. 6, C).

The observed dependence of Rb$^+$ uptake on the external concentration of K$^+$ used for yeast culture is consistent with the possibility that changes in HvHAK1 transport are associated with changes in membrane potential. In order to provide an estimate of changes in membrane potential following K$^+$ deprivation, we measured the accumulation of the fluorescent dye 3,3’-Dihexyloxacarbocyanine iodide (DiOC$_6$), by flow cytometry, which has been formerly validated as an indicator of membrane potential for yeast cells grown under the same conditions (Madrid et al., 1998). HvHAK1-expressing cells deprived of K$^+$ for 210 min accumulated more DiOC$_6$ than non-K$^+$ starved cells, indicating that hyperpolarization takes place during K$^+$ starvation (Fig. 6, D). Addition of the uncoupler CCCP abolished this high accumulation of DiOC$_6$, providing a control on the reliability of membrane potential measurements here performed. The presence of high NH$_4$Cl concentrations during long-term K$^+$-deprivation, in turn, affects neither the subsequent membrane potential difference nor the transport of Rb$^+$ (Fig. 6, D and 6, C, respectively). If the contribution of HvHAK1 would be strictly associated with an increased hyperpolarization, it should be expected that any condition leading to a more negative membrane potential must result in a higher Rb$^+$ uptake. However, we found that when cells are long-term deprived of K$^+$ in the presence of 100 mM NaCl, they become hyperpolarized and exhibit a low Rb$^+$ transport (Fig. 6, D and 6, B, respectively). Therefore, other factors should contribute to set the transport of K$^+$ mediated by HvHAK1.

Results obtained for HvHAK1-expressing yeast cells grown in the presence of Na$^+$ prompted us to analyze the role of Na$^+$ exclusion on HvHAK1 activity. For this purpose, we transformed HvHAK1 into yeast cells carrying a disruption of the genes coding for the Na$^+$-ATPases ENA1-4 and the Na$^+$/H$^+$ antiporter NHA1 in addition to the disruption of genes coding for the TRK1 and TRK2 K$^+$ transporters.
Pre-treatment with moderate NaCl concentrations during the course of K⁺ deprivation leads to a higher relative reduction of the subsequent Rb⁺-uptake by cells lacking the systems involved in Na⁺-exclusion than by cells where active Na⁺ exclusion takes place (Fig. 7).

Modulation of HvHAK1 contribution to K⁺ capture in yeast cells involves PPZ1 and HAL4-HAL5 proteins

Results shown above indicate a complex control on the contribution of HvHAK1 to K⁺ transport. In yeast cells, regulation of K⁺ influx mediated by TRK transporters involves, among other components, PPZ1/2 phosphatases as well as HAL4/5 kinases. HvHAK1-expressing yeast cells lacking HAL4 and HAL5 in addition to the lack of TRK1 and TRK2 showed a higher Rb⁺ transport than that displayed by cells only lacking TRK1 and TRK2 when deprived of K⁺ for 210 min (Fig. 8, A). Since hal4∆hal5∆ disruption in a TRK1-TRK2 background causes membrane hyperpolarization (Mulet et al., 1999), we next tried to determine whether results obtained in Rb⁺-uptake experiments could be explained by differences in membrane potential between trk1∆trk2∆hal4∆hal5∆ and trk1∆trk2∆HAL4HAL5 cells expressing HvHAK1. Ruling out this possibility, we found a similar DiOC₆ accumulation in both kinds of cells (Fig. 8, C). As such, we concluded that HAL4/HAL5 proteins down-regulate the contribution of HvHAK1 to K⁺ transport.

To study the role of PPZ1 we transformed trk1∆trk2∆ and trk1∆trk2∆ppz1∆ cells with a p424 plasmid containing the HvHAK1 cDNA. Expression of HvHAK1 into p424 did not restore the growth of yeast cells lacking TRK1 and TRK2. However, in a background also lacking PPZ1, expression of HvHAK1 into p424 restores the capacity of yeast cells to grow at low external K⁺ concentrations, indicating a control of HvHAK1 contribution to K⁺ capture (Supplemental Fig. S5). The role of PPZ1 was directly tested by measuring Rb⁺ uptake after 15 or 210 min of K⁺ starvation in trk1∆trk2∆ or trk1∆trk2∆ppz1∆ HvHAK1-expressing cells. A strong response of Rb⁺ uptake to K⁺ starvation was clearly found in cells lacking PPZ1
(Fig. 8, B) suggesting a role of the encoded protein in down-regulating HvHAK1 contribution to K⁺ capture. Studies on the accumulation of DiOC₆ revealed that after a 210 min period of K⁺ starvation, HvHAK1-expressing ppz1Δtrk1Δtrk2Δ cells are hyperpolarized compared to HvHAK1-expressing trk1Δtrk2Δ cells (Fig. 8, D).

**DISCUSSION**

The HAK-KT-KUP-type of transporters constitutes a large family of proteins thought to play a pivotal role in the maintenance of K⁺ homeostasis in plants. Here we introduced evidence demonstrating that the ionic environment regulates the contribution of the HvHAK1 K⁺-transporter to K⁺ movement. This regulation may be critical to ensure K⁺ capture in potassium deficient environments and during the early response to salinity.

It has been previously shown that HAK1 transporters provide a route for K⁺ uptake in HAK1-expressing yeasts that is similar to that previously found in plants suffering from K⁺ deficiency (Epstein et al., 1963; Santa-María et al., 1997; Rubio et al., 2000). The contribution of HAK1 transporters to high-affinity K⁺ transport has been later confirmed in an Arabidopsis mutant lacking the HvHAK1 homologue, AtHAK5, which displays a reduced capture of Rb⁺ under conditions of K⁺ starvation (Gierth et al., 2005). The evidence that HvHAK1 accumulation is augmented in the outermost layers of the root (Fig. 1) supports a role of the encoded transporter at the boundary between the external medium and the roots. This tissue location (Fig. 1), the enhanced accumulation of HvHAK1 transcripts upon K⁺-deprivation in barley roots (Fig. 2), as well as the enhanced capture of K⁺ displayed by HvHAK1-transgenic plants following K⁺ withdrawal (Fig. 4), are consistent with a role of this transporter in the inducible component of K⁺ uptake from diluted K⁺ solutions.

Studies with transgenic Arabidopsis plants expressing HvHAK1 indicate a strong effect of K⁺-deprivation on the contribution of HvHAK1. Studies with yeast cells expressing HvHAK1 allowed us to dissect the way by which K⁺-deprivation sets the contribution of a HAK-KUP-KT transporter in a model organism where the
endogenous transport of \( K^+ \) from diluted \( K^+ \) solutions is nil. In this organism, changes in the contribution of HvHAK1 to \( Rb^+ \) capture following \( K^+ \)-starvation are not linked to changes in the accumulation of \( HvHAK1 \) transcripts (Fig 5, D), indicating that \( K^+ \) deprivation should act on the amount and/or on the activity of the HvHAK1 transporter. A primary phenomenon that takes place in plants and yeasts suffering \( K^+ \) deficiency is plasma membrane hyperpolarization (Maathuis & Sanders, 1994; Walker et al., 1996; Hirsch et al., 1998; Madrid et al., 1998). In this context, an important question to address is whether the enhancement of \( Rb^+ \) uptake here observed (Fig. 5, A; Fig 6, A) just results from a long-term change in the membrane potential. Measurements of DiOC6 accumulation indicate that \( K^+ \) starvation in \( HvHAK1 \)-expressing yeast cells also leads to plasma membrane hyperpolarization (Fig. 6, D and Fig. 8). These data are consistent with a possible role of membrane potential on changes in the transport of \( Rb^+ \) mediated by HvHAK1 by affecting either the driving force and/or a signaling cascade. Evidence here offered (Fig. 6, D and 8, C) indicates that changes in the contribution of HvHAK1 to \( K^+ \) capture cannot be entirely accounted for by changes driven by membrane potential. Therefore, other mechanisms modulating HvHAK1 contribution to \( K^+ \) capture in yeasts should be taken into consideration.

In most fungi, \( K^+ \) transport results from the activity of TRK and HAK transporters (Benito et al., 2004). The mechanisms controlling \( K^+ \) homeostasis through TRK1 have been deeply explored in \( S. cerevisiae \) revealing that TRK1 is activated by the HAL4 and -5 kinases, and inhibited by the PPZ1 and -2 phosphatases (Mulet et al., 1999; Yenush et al., 2002). Interestingly, genetic evidence obtained along this work supports the hypothesis that HAL4-5 kinases act as down-regulators of HvHAK1 contribution (Fig. 8, A), which is opposite to their effect on TRK1. On the other hand, current evidence indicates that PPZ1 is involved in determining the upper limits of potassium accumulation in yeast cells, mainly in a TRK1-dependent manner (Yenush et al., 2005). Our results indicate that PPZ1 could exert this regulatory role, critical for \( K^+ \) homeostasis, by setting the contribution of a HAK1 transporter to \( K^+ \) capture (Fig. 8, B). The fact that both
HAL4/5 kinases and the PPZ1 phosphatase act as down-regulators of the contribution of HvHAK1 to K⁺ transport, could be explained by their action at different levels of regulation. Although the precise mode by which PPZ1 and HAL4/5 modulates HvHAK1 contribution is still uncertain, our results illustrate that both phosphorylation and dephosphorylation processes act in concert to determine the uptake of K⁺ mediated by a HAK1 transporter in yeasts. In this context, the search for plant functional counterparts of PPZ1 phosphatase and HAL4-5 kinases could help to determine whether or not the mechanisms setting the contribution of HvHAK1 in yeast cells are operative in plants. Besides, the finding that a HAK1 transporter is modulated by PPZ1 and HAL4-5 in S. cerevisiae, where no HAK genes have been found, posses important questions regarding the conservation of the regulatory network controlling K⁺ homeostasis in fungi.

Transcriptome studies revealed the existence of a cross-talk of signals associated with the perception of nitrogen and potassium status in plants (Wang et al., 2002; Armengaud et al., 2004). Besides, the presence of high NH₄⁺ concentrations has been used as a tool to dissect the components involved in K⁺ transport (Hirsch et al., 1998; Spalding et al., 1999; Nieves-Cordones et al., 2007). In barley, the presence of high NH₄⁺ concentrations in the growth media generates a switch on the properties of Rb⁺ uptake from diluted K⁺ solutions (Santa-María et al., 2000). This switch involves two parallel processes, being the first one the dominance of channel-like features, partially explained by a thermodynamic gradient favorable to channel participation (Kronzucker et al., 2003). The second process involved in that switch is the reduced size of the inducible component of Rb⁺ transport sensitive to NH₄⁺. Our results indicate that the reduction of this inducible component does not involve a long-term down-regulation of HvHAK1 transcripts accumulation mediated by high ammonium levels (Fig. 2, B). Therefore, it seems possible that the reduced relative contribution of the NH₄⁺-sensitive component of Rb⁺ transport after long-term growth at high NH₄⁺ supplies could be—at least in part- the result of a down-regulation on the accumulation of transcripts coding for other transporters. Gene expression studies (Fig. 2, C) suggest that
HvHAK1b could be a target for such a regulation. Therefore, the accumulation of transcripts coding for HAK1 transporters presumably involved in the NH₄⁺-sensitive pathway of K⁺ transport, occurs through two signaling routes: one NH₄⁺-sensitive acting on HvHAK1b, and the other insensitive to NH₄⁺ acting on HvHAK1.

The effect of sodium salts on K⁺ homeostasis has been a main subject in salinity studies (Flowers and Läuchli, 1983). In Arabidopsis current evidence indicates that, for plants grown in the presence of ammonium, the inhibitory effect of Na⁺ on K⁺ uptake results from an effect of Na⁺ on the activity of the AKT1 K⁺ channel (Qi and Spalding, 2004). The evidence contributed in our work indicates that culturing at high NaCl concentrations precludes the subsequent enhancement of K⁺ transport mediated by a HAK1 transporter in K⁺-deprived yeasts (Fig. 6, B and Fig. 7). While this effect tends to diminish the contribution of HvHAK1 to K⁺ capture, the transient enhanced accumulation of HvHAK1 transcripts observed in barley roots after a 6 h exposure to a high external NaCl concentration (Fig. 3) could lead to the opposite outcome and is likely to play a role in determining the low inhibitory effect of Na⁺ on Rb⁺ transport observed in barley roots 6 h after salinization (Fig. 3). Whereas our data (Supplemental Figure S3) do not allow ruling out the contribution of an osmotic component they indicate a role of non-osmotic components on the peak of accumulation of HvHAK1 transcripts. Evidence for a cross talk between osmotic and non osmotic signals during the early transcriptome response to salt stress has been offered for barley plants (Ueda et al., 2004). The enhancement and decline in the amount of HvHAK1 transcripts here observed (Figure 3) is similar to that reported for a member of the group II of HAK transporters in Mesembryanthemum crystallinum (Su et al., 2002) and essentially mimics that formerly observed for the genes implicated in the early coordinated gene response to salt stress described in the barley close relative species Lophopyrum elongatum (Gulick and Dvořák, 1992; Galvez et al., 1993). Since a similar response is elicited by abscisic acid (Galvez et al., 1993) and a transient change in the balance between methyl jasmonate and abscisic acid has been observed during the first hours of exposure to high NaCl concentrations.
(Moons et al., 1997), being a prominent role of jasmonic acid on the K⁺-dependent transcriptome also advanced (Armengaud et al., 2004), the possibility that hormone signaling plays a role in setting the response of HvHAK1 to salt stress should not be discarded.

In conclusion, the present study indicates the presence of mechanisms driven by the ionic environment that determine the contribution of a HAK1 transporter to K⁺ capture. Furthermore, studies with yeast cells reveal a role of phosphorylation and dephosphorylation processes in setting this contribution.

MATERIALS AND METHODS

Plant materials and culture

Seeds of barley (Hordeum vulgare cv Golden promise) were germinated in the dark on moistened filter paper for 48 h. Seedlings were then transferred to a 0.8 L plastic pot filled with a complete nutrient solution of the following composition: 1.0 mM Ca(NO₃)₂, 0.5 mM MgSO₄, 0.5 mM H₃PO₄, 50 µM FeEDTA, 50 µM CaCl₂, 25 µM H₃BO₃, 2 µM ZnSO₄, 2 µM MnSO₄, 0.5 µM CuSO₄, 0.5 µM molybdic acid, 2.5 mM 2-(N-morpholino)-ethanesulfonic acid (MES), with or without the addition of 1 mM K⁺, except where indicated. The pH was brought to 6.0 by the addition of Ca(OH)₂ and the solution was aerated. When added, K⁺, Na⁺ and NH₄⁺ were provided as chloride salts. Temperature in the growth chamber was set to 22°C (day/night), and the relative humidity was kept at 85%. The photon flux density at the plant level was set at 70 µmol m⁻² s⁻¹ over a photoperiod of 14 h. Experiments were carried out with one week old plants, except for the experiment reported in Fig. 2 B and C where plants were 13 d old. Root and shoot samples were extracted with 0.5 N HCl to release free cations, and K⁺ (or Rb⁺ in Rb⁺ uptake experiments) was determined with a Perkin Elmer AA 100 spectrophotometer, emission mode (Perkin Elmer Instruments, Norwalk, CT, USA). Arabidopsis thaliana (Columbia ecotype) seeds were sowed on plates containing the media above described plus
0.8% agar. After 10 d seedlings were transferred to 0.125 L plastic pots, were only roots were in contact with the nutrient solution. Plants grew for another two weeks, until the experiments were performed.

**Rb⁺ uptake measurements in plants**

Roots of intact plants were transferred for 5 min to a solution with the same composition as used for growth, but without K⁺. This allows elution of this chemical species from the root apoplast, and thus helps to minimize the effect of ionic perturbations on the subsequent measurement. Loading was performed in 50 mL plastic pots containing the complete nutrient solution with no K⁺ added. This solution, heavily aerated, contained 100 µM Rb⁺. Loading was extended for 60 min for barley and for 120 min for Arabidopsis. Subsequently, roots were washed two times for a total of 6 min with the same solution used for loading but without Rb⁺. Results are expressed on a fresh weight basis.

**Quantitation of plant mRNAs**

For the quantitation of plant mRNAs, RNA extracted from whole barley roots was used. Extraction of total RNA was performed through the use of RNeasy Plant Mini Kit (Qiagen Science, Maryland, USA). After extraction, total RNA was treated with RQ1 DNase (Promega, Madison, WI, USA) and the presence of contamination from genomic origin was specifically tested for by PCR. For each DNA-free RNA sample several independent reverse transcription (RT) reactions were performed. RT was carried out with Superscript II (Gibco-BRL, Gaithersburg, MD, USA) on 20 ng of total RNA using an Oligonucleotide-dT₁₈ as 3’primer. Real-time PCR was performed by duplicate for each RT reaction using an ABI Prism 5700 Sequence Detection System (Perkin Elmer Applied Biosystems, Foster City, CA, USA) and SYBRGreen PCR Master Mix (Perkin Elmer Applied Biosystems Warrington, UK). Primers used for PCR for each gene, as well as amplicon sizes, have been
formerly described (Vallejo et al., 2005). Amplification was carried out with an initial step at 50°C for 2 min, followed by 1 cycle at 95°C for 10 min and then by 40 amplification cycles. After each PCR, the dissociation curve of the PCR product was analyzed. The cDNA content of \( \beta \)-tubulin for each running was also estimated. In addition, two negative controls were included to exclude the possibility of genomic DNA contamination: one of them consisted in a reaction without cDNA, while the other contained an aliquot of the DNA-free RNA sample. Subsequently, the accumulation of transcripts was estimated by the use of the delta-delta Ct method.

**In situ hybridization**

Barley plants one week old, grown without aeration in a 10 mM MES solution brought to pH 6.0 ± 0.1 with Ca(OH)\(_2\), were used for *in situ* hybridization studies. Roots were cut into 10 mm long fragments, from 10 to 60 mm from the root apex. For the fixation protocol roots were treated with 3%(v/v) paraformaldehyde and 0.25%(v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, at room temperature, dehydrated in graded ethanol and xylene series and embedded in paraplast. Sections, 2 \( \mu \)m thick, were attached to poly-L-lysine coated slides. Sections were deparaffinized with xylene and rehydrated through a graded ethanol series. They were subsequently pretreated with 1 \( \mu \)g ml\(^{-1}\) proteinase K in 200 mM Tris-HCl, pH 7.5, 2 mM CaCl\(_2\) at 37°C for 30 min and with 0.25%(v/v) acetic anhydride in 0.1 M triethanolamine, pH 8.0, at room temperature for 10 min, dehydrated in a graded ethanol series and air dried. Sections were hybridized with either sense or antisense RNA \(^{35}\)S-labeled probes.

A pGEM-T easy vector (Promega, Madison, WI, USA) containing a 325 bp cDNA of the *HvHAK1* 3′UTR, was linearized with PstI or NcoI to be used as DNA template for in vitro synthesis of sense and antisense RNA probes, respectively. Sense and antisense RNA probes were synthesized by the incorporation of \( \alpha \)-\(^{35}\)S-UTP using an RNA labeling kit following the manufacturer’s instructions.
Southern blot analyses with barley genomic DNA previously showed a single hybridization band with this 325 bp probe. Hybridization and detection of the radioactive signal were performed as previously described (Langdale et al., 1988). Photographs were taken using an automatic camera coupled to a light microscope (Axiophot, Zeiss, Columbus, OH, USA).

Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains used along this work were WΔ3 (W303.1A *trk1Δ::LEU2 trk2Δ::HIS3*), which is deficient in the endogenous K⁺ uptake systems TRK1 and TRK2, MA5 (W303.1A *trk1Δ::LEU2 trk2Δ::HIS3 nha1Δ::LEU2 ena1-4Δ::HIS3*) which derived from WΔ3, being also deficient in the Na⁺ efflux systems ENA1-4 and NHA1 (Benito et al., 2004) and JM110 (W303.1A *trk1Δ::LEU2 trk2Δ::HIS3 hal4Δ::TRP1 hal5Δ::KanMX*) also derived from WΔ3 which carries a disruption on the genes coding for the protein kinases HAL4 and HAL5 (Mulet et al., 1999). These strains were the recipient of the *pYPGE15* plasmid or its derivative containing the *HvHAK1* cDNA, *pGF718*. Strains ESV212 (DBY746 *trk1Δ::LEU2 trk2Δ::HIS3*) and its derivative MAR70 (DBY746 *trk1Δ::LEU2 trk2Δ::HIS3 ppz1Δ::URA3*), which carries a disruption of the gene coding for the PPZ1 phosphatase in addition to the disruption of TRK1 and TRK2 (Ruiz et al., 2004), were also used. In this case the *HvHAK1* cDNA was cloned into the *p424* plasmid. Yeast cells were grown in arginine (AP) medium supplemented with 30 mM KCl (Santa-María et al., 1997). Cells were washed twice and transferred to AP K⁺-non added medium. The basal concentration of K⁺ in that medium was 2.5 µM. At different times from the beginning of K⁺ deprivation Rb⁺-uptake measurements were performed. Cells were suspended in 2% glucose and 10 mM MES buffer brought to pH 6.0 with Ca(OH)₂. Unless specifically indicated (Supplemental Fig. S4, B) Rb⁺-uptake measurements were performed in the absence of K⁺, Na⁺ or NH₄⁺ in the loading solution. At intervals cells were taken, filtered through a 0.8 µm
pore nitrocellulose membrane (Millipore, Bedford, MA) and washed with 20 mM MgCl₂. Filters were incubated overnight in 0.5 N HCl and Rb⁺ determined by atomic emission spectrophotometry. Results are expressed on a cell dry weight basis. Vmax and Km values were estimated by non-linear regression.

Accumulation of DiOC₆ was estimated for yeast cells grown during 210 min in the presence or absence of different alkali cations. These cells did not receive any other treatment and were suspended in MES-Ca²⁺ and exposed to 1 nM DiOC₆ for 30 min at 28°C in the dark. To test cell viability, propidium iodide was used. Flow cytometry analysis were performed in a FACSCalibur (Becton Dickinson, San José, CA, USA).

Plant transformation

The HvHAK1 718 cDNA was cloned into the plant binary vector pB112 containing the nptII kanamycin resistance marker. Agrobacterium tumefaciens pgv3101 was the recipient of the HvHAK1-containing plasmid, which was later used to transform Arabidopsis thaliana plants, Col-0 Ecotype, by the floral dip procedure (Clough and Bent, 1998). Plants displaying resistance to the marker were selected for further analysis. The presence of the transgene was evaluated by PCR on genomic DNA. To identify homozygous plants, studies on the segregation of kanamycin resistance were performed. Expression of HvHAK1 for each homozygous line was determined by RT-PCR using DNA-free RNA.

Except were indicated, results obtained along this work were analyzed by two factors ANOVA, being post-hoc comparisons made by the Duncan´s test. The analysis was performed by using the Statistica 6.0 Program (StatSoft*).

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.
Supplemental Material

The following materials are available in the online version of this article.

**Supplemental Figure S1.** *In situ* hybridization studies show accumulation of HvHAK1 transcripts in most root cells.

**Supplemental Figure S2.** Effect of K⁺-deprivation, in a no aerated MES-Ca²⁺ solution, on the accumulation of HvHAK1 transcripts.

**Supplemental Figure S3.** Comparative effect of Sorbitol and NaCl on the accumulation of HvHAK1 transcripts. Effect of NaCl exposure on the concentration of K⁺ and Na⁺ in roots.

**Supplemental Figure S4.** Rb⁺ uptake properties by yeast cells expressing HvHAK1 following K⁺ withdrawal.

**Supplemental Figure S5.** HVHAK1-expressing yeast cells lacking the PPZ1 phosphatase display an enhanced growth capacity at low K⁺ concentrations.

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Figure 1. *In situ* hybridization of *HvHAK1* mRNA in barley roots.

Cross section of a seminal *Hordeum vulgare* root located 10-20 mm from the tip is shown. The expression of *HvHAK1* is indicated by dark granules. A, antisense probe. B, sense probe. Bars correspond to 100 µm. C, cortex; E, endodermis; VC, vascular cylinder.
Figure 2. Expression of potassium-transporter coding genes is enhanced by K⁺-starvation through a pathway ammonium-sensitive and a pathway ammonium-insensitive.

A, Accumulation of HvHAK1 and HvHAK1b transcripts in 1 week old plants grown with or without K⁺ since germination (n=3). Delta-delta Ct corresponds to the difference in transcript accumulation between the presence of K⁺ (control condition) and its absence (no K⁺ added). The asterisk denotes a significant effect of K⁺-deprivation. In B and C, effect of the presence or the absence of K⁺ and NH₄⁺ (100 µM and 5mM, respectively) during a 3 d period on the accumulation of HvHAK1, and HvHAK1b transcripts (n=6). Error bars represent ± SE. Different letters indicate significantly different values (P<0.05 for A and B, P<0.001 for C, respectively).
Figure 3. Expression of HvHAK1 is transiently enhanced by salinization and parallels changes in the reduction of Na⁺ inhibitory effects on Rb⁺ uptake in barley roots.

Plants were exposed to NaCl 100 mM in the presence of 1 mM KCl. A, accumulation of HvHAK1 transcripts measured at 0, 6 and 48 h since the beginning of salt stress (n=5). In B, Rb⁺ uptake from a 100 µM Rb⁺ solution, after those periods, was measured both in the presence and in the absence of 100 mM NaCl, and the quotient between the uptake measured in those conditions is shown (n=6). Error bars represent ± SE. Different letters indicate significantly different values (P<0.005 for A, P<0.05 for B, respectively).
Figure 4. Arabidopsis thaliana plants expressing HvHAK1 display an enhanced Rb⁺ uptake following K⁺ withdrawal. A, four homozygous HvHAK1-expressing Arabidopsis thaliana lines (named as J1, B1, A28 and A41) display an enhanced uptake of Rb⁺ from a 100 µM Rb⁺ solution relative to WT plants, when deprived of K⁺ for 48 h (on average, n=7). B, detailed analysis for the HvHAK1-expressing J1 line. Results are the mean of 13 experiments (each one consisting of 7 independent replicates, on average). Error bars represent ± SE. Different letters indicate significantly different values (P<0.05 for A, P<0.01 for B, respectively).
Figure 5. Rb⁺ uptake is enhanced by K⁺ starvation in HvHAK1-expressing yeast cells.
A, Rb⁺-uptake from a 100 µM Rb⁺ solution by HvHAK1-expressing yeast cells grown overnight at 30 mM KCl deprived of K⁺ (no K⁺ added) for different time periods (n=6). B, reciprocal experiment (n=5) with 210 min K⁺-deprived cells transferred to a 30 mM KCl medium. C, Rb⁺ uptake (n=6) from a wide range of micromolar Rb⁺ concentrations for K⁺-starved (white triangles) and K⁺-non starved cells (black triangles). D, effect of K⁺-deprivation on the accumulation of HvHAK1 transcripts in yeast cells (n=7). Error bars represent ± SE.
Figure 6. High sodium, but not high ammonium, concentrations interfere with the increased contribution of HvHAK1 following potassium starvation in yeast cells.

HvHAK1-expressing yeast cells were grown overnight at 30 mM KCl, and then exposed for 210 min to different external concentrations of alkali cations. A, Rb⁺-uptake from a 100 µM Rb⁺ solution after cells were exposed to different external K⁺ concentrations (n=4). B, and C, Rb⁺ uptake for cells exposed for 210 min to the absence of K⁺ in the presence of different Na⁺ (n=3) or NH₄⁺ (n=5) concentrations, respectively. D, accumulation of the fluorescent dye DiOC₆ measured in yeast cells grown for 210 min in the absence of K⁺ with or without 30 mM NH₄⁺, 100 mM Na⁺ or 20 µM of the CCCP uncoupler, as well as in the presence of 30 mM KCl (n=5). Error bars represent ± SE. Different letters indicate significantly different values (P<0.05 for A, B and C, P<0.005 for D).
Figure 7. Culture at moderate NaCl concentrations affects Rb⁺-transport mediated by HvHAK1 in yeast cells lacking Na⁺ exclusion systems. Rb⁺ uptake from a 100 µM Rb⁺ solution (n=4) was measured after 210 min of K⁺ starvation in the presence of different NaCl concentrations for HvHAK1-expressing trk1Δtrk2Δ cells (black) and trk1Δtrk2Δena1-4Δnha1Δ cells, which lack the Na⁺ excluding systems ENA1-4 and NHA1 (dots). Data for K⁺ non-starved cells (left columns) has been included for comparative purposes. Error bars represent ± SE. Different letters indicate significantly different values (P<0.01).
Figure 8. The PPZ1 phosphatase and the HAL4-5 kinases control the inducible transport of Rb⁺ mediated by HvHAK1 in yeast cells. 

A, Rb⁺ uptake from a 100 µM Rb⁺ solution measured in K⁺ starved (210 min) and K⁺-non starved trk1Δtrk2Δ and trk1Δtrk2Δhal4Δhal5Δ HvHAK1-expressing yeast cells (n=5). B, under the same experimental conditions Rb⁺-uptake was also measured in trk1Δtrk2Δ or trk1Δtrk2Δppz1Δ HvHAK1-expressing yeast cells derived from the DBY746 strain (n=5). C and D, accumulation of the DiOC₆ fluorescent dye for cells grown in the presence or the absence of K⁺ is shown (n=3). Error bars represent ± SE. Different letters indicate significantly different values (P<0.01 for A and D, P<0.001 for B and C, respectively).