Insights into the mechanisms of transport and regulation of the arabidopsis high-affinity K⁺ transporter HAK5

Reyes Ródenas, Paula Ragel, Manuel Nieves-Cordones, Almudena Martínez-Martínez, Jesús Amo, Alberto Lara, Vicente Martínez, Francisco J. Quintero, Jose M. Pardo and Francisco Rubio

1 Departmento de Nutrición Vegetal, Centro de Edafología y Biología Aplicada del Segura, Consejo Superior de Investigaciones Científicas, Campus de Espinardo, 30100 Murcia, Spain
2 Instituto de Bioquímica Vegetal y Fotosíntesis, cic-Cartuja, Consejo Superior de Investigaciones Científicas-Universidad de Sevilla, 41092 Sevilla, Spain

*Author for communication: frubio@cebas.csic.es

Abstract

The high-affinity K⁺ transporter HAK5 from Arabidopsis (Arabidopsis thaliana) is essential for K⁺ acquisition and plant growth at low micromolar K⁺ concentrations. Despite its functional relevance in plant nutrition, information about functional domains of HAK5 is scarce. Its activity is enhanced by phosphorylation via the AtCIPK23/AtCBL1-9 complex. Based on the recently published three-dimensional structure of the bacterial ortholog KimA from Bacillus subtilis, we have modeled AtHAK5 and, by a mutational approach, identified residues G67, Y70, G71, D72, D201, and E312 as essential for transporter function. According to the structural model, residues D72, D201, and E312 may bind K⁺, whereas residues G67, Y70, and G71 may shape the selective filter for K⁺, which resembles that of K⁺ shaker-like channels. In addition, we show that phosphorylation of residue S35 by AtCIPK23 is required for reaching maximal transport activity. Serial deletions of the AtHAK5 C-terminus disclosed the presence of an autoinhibitory domain located between residues 571 and 633 together with an AtCIPK23-dependent activation domain downstream of position 633. Presumably, autoinhibition of AtHAK5 is counteracted by phosphorylation of S35 by AtCIPK23. Our results provide a molecular model for K⁺ transport and describe CIPK-CBL-mediated regulation of plant HAK transporters.
Introduction

Potassium (K⁺) is the most abundant inorganic cation in plants, composing up to 10% of the dry weight in well-fed plants (Leigh and Wyn Jones, 1984). This macronutrient fulfills many important functions in cellular physiology and plant development and contributes to abiotic and biotic stress tolerance (Shabala and Pottosin, 2014). K⁺ is taken up from the soil by roots and distributed to the different plant organs through the xylem and phloem. Within the cells, K⁺ is accumulated in different organelles and especially in the vacuoles, where it fulfills osmotic and electrical balance functions (White and Karley, 2010). K⁺ uptake and distribution involve the movement of K⁺ across cell membranes, which requires the integrated operation of specific K⁺ transport systems. Different families of K⁺ transport systems have been described in plants: the Shaker-like (AKT/KAT), tandem-pore, and two-pore channels, the K⁺ transporters of the HKT and the HAK/KUP/KT families, and the K⁺/H⁺ antiporters of the CPA family (Maser et al., 2001; Ragel et al., 2019).

The HAK/KUP/KT family belongs to the amino acid-polyamine-organocation (APC) transporter superfamily (Vastermark et al., 2014) and comprises members that operate at different membranes and plant organs, fulfilling a wide range of functions (Santa-María et al., 2018; Ragel et al., 2019). High-affinity K⁺ uptake at the root is a distinctive function of some members of this family. The barley (Hordeum vulgare) HvHAK1 was the first high-affinity K⁺ transporter of the HAK/KUP/KT family identified in plants (Santa-María et al., 1997). Studies with mutant lines impairing HvHAK1 homologs from other species, such as the Arabidopsis (Arabidopsis thaliana) AtHAK5 (Rubio et al., 2008; Rubio et al., 2010), the rice (Oryza sativa) OsHAK1 (Chen et al., 2015), or the tomato (Solanum lycopersicum) SlHAK5 (Nieves-Cordones et al., 2020), demonstrated the crucial role of this type of transporters for acquiring K⁺ from diluted solutions. These high-affinity, HAK5-like transporters are thought to operate as K⁺/H⁺ symporters (Scherzer et al., 2015; Böhm et al., 2018). However, some family members are involved in physiological processes other than root K⁺ uptake, such as K⁺ efflux at the root or in guard cells (Osakabe et al., 2013), long-distance K⁺ transport (Yang et al., 2014; Han et al., 2016; Nieves-Cordones et al., 2019), and pollen K⁺ uptake (Nieves-Cordones et al., 2020), and their mechanism of transport remains uncertain. Finally, some HAK/KUP/KT transporters have been implicated in Na⁺ transport (Benito et al., 2012; Zhang et al., 2019; Wang et al., 2020) and other important physiological processes such as shoot cell expansion (Elumalai et al., 2002) or auxin homeostasis (Osakabe et al., 2013; Rigas et al., 2013; Zhang et al., 2020). With respect to protein subcellular localization, representatives of the HAK/KUP/KT family are found at the plasma membrane (Qi et al., 2008; Yang et al., 2014; Chen et al., 2015; Shen et al., 2015), and in inner cell membranes such as the tonoplast (Banuelos et al., 2002) or the endoplasmic reticulum membrane (Haro et al., 2013; Rigas et al., 2013; Zhang et al., 2020).

In spite of the relevance of HAK/KUP/KT transporters for plant K⁺ homeostasis and mineral nutrition, their structural and functional domains remain poorly understood. This contrasts with the large amount of structure–function studies for K⁺ channels (Gajdanowicz et al., 2009; Nieves-Cordones et al., 2014; Jegla et al., 2018; Sánchez-Barrena et al., 2020). The HAK/KUP/KT family does not show remarkable similarities with other families of K⁺ transport systems and, within the family, its members show a low degree of sequence homology (Rodríguez-Navarro, 2000; Nieves-Cordones et al., 2016; Santa-María et al., 2018). Thus, identification of putative conserved functional domains is a difficult task. Three-dimensional modeling using high-resolution templates of prokaryotic APC representatives (Santa-María et al., 2018) and topology studies based on protein–reporter fusions (Sato et al., 2014) support the existence of a hydrophobic core with 12 transmembrane (TM) domains and three cytosolic domains in the N- and C-terminus and the intervening loop between TM2 and TM3. Sequence alignments of many HAK/KUP/KT representatives, including the Escherichia coli Kup, showed the existence of the conserved GVVYG motif in TM1 (Rodriguez-Navarro, 2000; Nieves-Cordones et al., 2016), which resembles the K⁺-selectivity filter GYGD motif of plant K⁺ channels (Jegla et al., 2018). Mutagenic approaches with the E. coli Kup protein have identified residues D23, E116, and E229, located in TM1, TM3, and TM6, respectively, as essential for K⁺ transport (Sato et al., 2014). Recently, the cryo-EM structure of the Kup family K⁺/H⁺ symporter KimA from Bacillus subtilis has been reported (Tascón et al., 2020). This study identified D36 and D117, equivalent to the EeKup D23 and E116 residues, as involved in K⁺ ion coordination, and E233, equivalent to the EeKup E229, as possibly related to H⁺-coupling.

Studies with the high-affinity K⁺ transporters AtHAK5 and HvHAK1 suggested that the N- and C-termini of these proteins play a role in the regulation of transporter activity. The N-terminus of AtHAK5 is phosphorylated by a protein complex composed by the Ca²⁺ sensor AtCBL1 (or AtCBL8, AtCBL9, or AtCBL10) and the kinase AtCIPK23, which activates the transporter under K⁺ deprivation (Ragel et al., 2015). This regulatory mechanism seems to exist in AtHAK5 homologs from other species such as tomato, pepper (Capsicum annuum), quinoa (Chenopodium quinoa), or Venus flytrap (Dionaea muscipula; Ragel et al., 2015; Scherzer et al., 2015; Böhm et al., 2018). Regulation of transporter activity by phosphorylation has been also suggested for other members of the family, for example AtKUP6 by the SNRK2E/OST1/SNRK2.6 kinase (Osakabe et al., 2013) and AtKUP7 (Han et al., 2016). Compared to their bacterial counterparts, eukaryotic HAK/KUP/KT transporters have long cytoplasmic C-termini, and this feature is also present in transporters of other families, as for example Na⁺,K⁺/H⁺ antiporters of the CPA1 family (Quintero et al., 2011;
Núñez-Ramírez et al., 2012), the H+-ATPases (Fuglsang et al., 2007), or the Shaker-like K+ channels (Jegla et al., 2018). In all cases, these C-terminal tails include important regulatory domains. Likewise, the C-termini of AtHAK5 and HvHAK1 contain residues that, when mutated, produce proteins with an increased rate of K+ transport (Rubio et al., 2000; Mangano et al., 2008).

Given the varied and remarkable physiological roles that HAK/KUP/KT transporters play, insights into their regulation via functional domains may open avenues for plant biotechnology. In particular, an increase of K+ use efficiency of crops is especially required for future low-input agriculture within a context of global warming and an increasing population (Nature, 2010; Luan et al., 2017). Thus, we have undertaken a study to unravel structural determinants of functional domains in AtHAK5, one of the best-understudied transporters.

Results

Mutations affecting AtHAK5 functionality

To identify AtHAK5 amino acid residues essential for K+ transport, the three-dimensional (3D) structure of AtHAK5 was modeled with the structure reported for KimA as a template. The N- and C-termini of AtHAK5 were excluded from the modeling because the structure of N-terminus of KimA was not resolved, and the much longer C-terminus of AtHAK5 showed low homology with that of KimA. The modeled AtHAK5 structure showed that amino acid residues D72, D201, and E312, located in transmembrane TM6 of AtHAK5 that are essential for K+ transport and data that support the existence of residues in TM1, TM3, and TM6 of AtHAK5 that are essential for K+ transport and that may constitute a K+-coordination domain similar to that of KimA, and which resembles the K+ selectivity-filter of plant K+-channels. In addition, we identified the S35 residue in the N-terminal region as a phosphorylation target for AtCIPK23, together with the existence of distinct regulatory regions in the C-terminus of AtHAK5, including an autoinhibitory domain and an AtCIPK23-dependent activation domain.

Regulation of AtHAK5 by the C-terminal domain

To test if the C-terminal domain of AtHAK5 was involved in the regulation of its activity, AtHAK5 versions with deletions of the cytosolic tail were expressed in yeast (Figure 3). Selection of the deletion points was based on a secondary structure prediction obtained with the PROMALS 3D software (Figure 3A). This secondary structure study predicted the existence of several α-helix and β-sheet domains in the AtHAK5 C-terminus. The deletion points were initially chosen outside and flanking these secondary structures in order to maintain them unaltered when present in the deleted version of the transporter. An internal deletion was subsequently generated as indicated below. Deleted versions, cloned into the yeast expression vector pDR195, were expressed alone or with the regulatory complex AtCIPK23/AtCBL1 in the yeast strain 9.3 that were next assayed for growth in media with different K+ levels. Under K+ -sufficient conditions (50 mM K+), regulatory domains. Likewise, the C-termini of AtHAK5 and HvHAK1 contain residues that, when mutated, produce proteins with an increased rate of K+ transport (Rubio et al., 2000; Mangano et al., 2008).

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to vigorous yeast growth in the absence of the regulatory complex, similar to that of yeast expressing WT AtHAK5 and AtCIPK23/AtCBL1, and better than yeast expressing AtHAK5 alone. This result suggests that AtHAK5-Δ555 and AtHAK5-Δ571 are constitutively active transporters, allowing maximal growth in the absence of AtCIPK23/AtCBL1. In agreement with this idea, no improvement in yeast growth was detected when the AtCIPK23/AtCBL1 complex was co-expressed with the AtHAK5-Δ555 and AtHAK5-Δ571 transporters.

Expression of AtHAK5-Δ633 alone recapitulated the poor yeast growth promoted by WT AtHAK5 in the absence of AtCIPK23/AtCBL1. However, co-expression of AtCIPK23/AtCBL1 with AtHAK5-Δ633 did not improve growth. The higher rates of yeast growth mediated by AtHAK5-Δ571 in the absence of AtCIPK23/AtCBL1 in comparison with WT AtHAK5 suggested the presence of an autoinhibitory domain downstream position 571, which could be counteracted by AtCIPK23/AtCBL1 co-expression. This negative regulatory element seems to be contained within the next 62 residues, since the AtHAK5-Δ633 mutant conferred a poorer growth than AtHAK5-Δ571. To check this possibility, an AtHAK5 version with an internal deletion between positions 571 and 633, AtHAK5-Δ571–633, was obtained (Figure 3A). Expression of this internally truncated AtHAK5 version did not promote yeast growth at low K⁺, similarly to what was observed with the AtHAK5-Δ539 construct (Figure 3B). The lack of activation of AtHAK5-Δ633 by the AtCIPK23/AtCBL1 complex suggested the presence of an AtCIPK23/AtCBL1-related activation domain downstream of position 633. This activation domain seemed to extend to the end of the C-terminal tail because growth enhancement

Figure 1. Structural model of AtHAK5. (A) Overview of the AtHAK5 transmembrane regions. (B) Top view from the extracellular side of the putative K⁺ binding site of AtHAK5. Side chains from residues belonging to TM1, TM3, and TM6 that are critical for K⁺ transport are shown in framed boxes. TM number is shown in white boxes. (C) Lateral view of the same region. (D) Alignment of the conserved sequences in the TM1, TM3, and TM6 of AtHAK5, EcKUP, and KimA, and the selectivity filter of K⁺ channels. Acidic residues expected to bind K⁺ are shown in red. Conserved residues with the selectivity filter of voltage-gated K⁺ channels are underlined.
by AtCIPK23/AtCBL1 in the AtHAK5-D688 and -D744 mutants did not reach the levels observed for WT AtHAK5.

Immunoblot analysis of hemagglutinin epitope (HA)-tagged versions of the mutant AtHAK5 proteins showed that, with the exception of the AtHAK5-D539 and the AtHAK5-D571–633 mutants, all the truncated AtHAK5 proteins were expressed in yeast and no correlation between the amount of AtHAK5 protein and the complementation capacity could be observed (Supplemental Figure 2, B). Deletion of AtHAK5 at position 539 or between positions 571 and 633 seemed to produce proteins that are quickly degraded in yeast, since no signal was detected by immunoblot analysis. Likely, these mutated versions of AtHAK5 are detected by yeast cells as an abnormal or dysfunctional protein and sent for degradation. Unfortunately, the absence of AtHAK5-D571–633 protein did not allow to precisely characterize the role of the stretch between positions 571 and 633 in regulating AtHAK5 function.

Identification of AtHAK5 residues phosphorylated by the AtCIPK23 protein kinase

We have previously shown that AtCIPK23 phosphorylated an N-terminal polypeptide of AtHAK5 comprising the first 94 amino acids (Ragel et al., 2015). To identify the phosphorylated residue(s), three N-terminal fragments AtHAK5(1–94), AtHAK5(1–48), and AtHAK5(49–94) were expressed in E. coli as GST fusions and used as substrates for in vitro kinase assays with the constitutively active kinase AtCIPK23(T190D/D331; Ragel et al., 2015). In parallel, we searched the Arabidopsis Protein Phosphorylation Site Database, PhosPhAt 4.0 (Zulawski et al., 2013), for
phosphorylation sites identified in the AtHAK5 protein. A peptide belonging to the N-terminal part of AtHAK5 expanding from residues 30 to 49 was found, in which residues Y31, S35, and T43 appeared phosphorylated. Consequently, another N-terminal fragment, AtHAK5(30–49), was produced as GST fusion and included as substrate in the kinase assays. The results of the phosphorylation experiments are shown in Figure 4. A strong phosphorylation signal was detected only in the samples where AtHAK5(1–48) and AtHAK5(30–49) peptides were present in the reaction, demonstrating that target residues of AtCIPK23 were located between M1 and R49. Next, we aligned the N-terminal sequences of Arabidopsis HAK/KUP/KT proteins in order to identify phosphorylatable residues that were highly conserved. Serine at position 35 was the only amino acid meeting the criteria (Supplemental Figure 3). To demonstrate that this residue was indeed the target of the AtCIPK23 kinase, a serine-to-alanine mutation was introduced in position S35. The entire N-terminal region of AtHAK5 (residues 1–94), with and without the S35A mutation, was expressed and tested in AtCIPK23-dependent phosphorylation assays. Both GST fusions gave rise to peptides of lower molecular weight than expected. This may reflect partial degradation, likely induced by the presence of hydrophobic residues belonging to the putative TM1 of AtHAK5 at the end of the fusion peptide. Comparison of the phosphorylation pattern of WT and mutant AtHAK5(1–94) demonstrated that S35A prevented phosphorylation by AtCIPK23 (Figure 4). No other residues of AtHAK5 appeared to be phosphorylated by AtCIPK23 in the AtHAK5(1–94) polypeptide.

Residue S35 is essential for regulation of AtHAK5
To assess the requirement of the phosphorylation at S35 by AtCIPK23 for the activity of AtHAK5, complementation of yeast cells expressing WT AtHAK5 or the S35A mutant together with the AtCIPK23/AtCBL1 complex was assayed. As depicted in Figure 5, all yeast cells grew well in the presence of 50 mM K⁺. At lower K⁺ concentrations, the mutant protein S35A conferred a moderate growth capacity in the absence of AtCIPK23/AtCBL1. Co-expression of AtCIPK23/AtCBL1 with WT AtHAK5 produced a remarkable increase of yeast cells growth in medium containing low K⁺. By contrast, no increase of cell growth was observed when the S35A mutant was co-expressed with AtCIPK23/AtCBL1. These results suggest the existence of an autoinhibitory domain and an activation domain located in the C-terminus of AtHAK5 between the 571 and 633 positions and downstream of position 633, respectively. To study the interplay between the S35 residue in the N-terminus, the residues in the TM1 related to functionality, and the regulatory domains in the C-terminus, AtHAK5 constructs that included the S35A or the D72A mutation in combination with the Δ571 and Δ633 truncations were obtained. Growth tests of yeast expressing these AtHAK5 versions were performed under high- and low-K⁺ conditions (Figure 6). Yeast cells expressing the D72A mutant did not grow on low-K⁺ medium in the absence or presence of AtCIPK23/AtCBL1. Those expressing the S35A mutant showed mild growth both in the absence and presence of AtCIPK23/AtCBL1. Yeast cells expressing the Δ571 truncation grew well regardless of the presence of AtCIPK23/AtCBL1. Expression of the AtHAK5 version including both the D72A mutation and Δ571 truncation did not lead to

**Figure 4.** Protein kinase AtCIPK23 phosphorylates AtHAK5 at serine 35. Purified proteins corresponding to the indicated fragments of AtHAK5 were incubated with AtCIPK23(T190D/D331) in the presence of [γ-32P]ATP, resolved in SDS/PAGE (lower panel), and exposed to X-ray film (upper panel).
yeast growth either with or without AtCIPK23/AtCBL1. By contrast, when the AtHAK5 mutant combining both the S35A mutant and the Δ571 deletion was expressed, yeast cells grew well independently of co-expression of the regulatory complex. Finally, the S35A mutation combined with the Δ633 truncation promoted mild growth that was not activated by the presence of AtCIPK23/AtCBL1. Taken together, these results are coherent with the presence of an autoinhibitory domain between residues 571 and 633, whose activity is counteracted by phosphorylation at S35.

Rb⁺ uptake mediated by the AtHAK5 mutants

The effect of the mutations on AtHAK5-mediated K⁺ transport was studied in uptake experiments in yeast using Rb⁺ as a tracer for K⁺. Experimental conditions were chosen according to previous reports (Ragel et al., 2015) for optimal disclosure of high-affinity Rb⁺ uptake mediated by AtHAK5 and its activation by AtCIPK23/AtCBL1. Thus, Rb⁺ uptake from 100 μM external Rb⁺ was determined in K⁺-starved yeast cells expressing the WT and AtHAK5 mutants in the presence or absence of AtCIPK23/AtCBL1. A representative experiment of the accumulation of Rb⁺ versus time for yeast transformed with the empty vectors or expressing AtHAK5 alone or together with AtCIPK23/AtCBL1 is shown in Figure 7A. The initial rates of Rb⁺ uptake were calculated from several repetitions. Yeast transformed with the empty vectors showed a very low background rate of Rb⁺ uptake, of 0.0243 ± 0.002 nmol mg⁻¹ DW min⁻¹. When AtHAK5 was expressed, Rb⁺ uptake increased to 0.085 ± 0.01 nmol mg⁻¹ DW min⁻¹, and co-expression with AtCIPK23/AtCBL1 further activated Rb⁺ uptake to 0.327 ± 0.037 nmol mg⁻¹ DW min⁻¹ (Figure 7B). These results are consistent with a basal uptake capacity of AtHAK5 alone or together with AtCIPK23/AtCBL1 and a maximal level in the presence of the regulatory complex, in agreement with previous reports (Ragel et al., 2015).

The Rb⁺ uptake shown by yeast expressing D72A, D201A, and E312A, representative of the mutations located in TM1, TM3, and TM6, which did not promote yeast growth at low K⁺ (Figure 2), was not significantly different from that shown by yeast transformed with the empty vectors. In addition, no effect of the expression of AtCIPK23/AtCBL1 was observed (Figure 7, B). These results support that these mutations abolished K⁺ transport in AtHAK5.

Deletions of the C-terminus of AtHAK5 resulted in transporters with different Rb⁺ uptake capacities and with altered regulation by AtCIPK23/AtCBL1 (Figure 8). As expected, the AtHAK5-Δ539 and AtHAK5-Δ571–633 mutants did not mediate Rb⁺ uptake above the level of the strain transformed with the empty vectors, as these two AtHAK5 proteins were not detected in yeast (Supplemental Figure 2). The AtHAK5-Δ555 and AtHAK5-Δ571 mutants mediated high rates of Rb⁺ uptake regardless of the absence or presence of AtCIPK23/AtCBL1, and which were of similar magnitude to the maximal rate displayed by WT AtHAK5 in the absence of AtCIPK23/AtCBL1. Importantly, Rb⁺ uptake was not significantly increased in these mutants by the presence of AtCIPK23/AtCBL1. The results of Rb⁺ uptake experiments supported the conclusions obtained with the yeast growth experiments and were consistent with the presence of an autoinhibitory domain downstream of position 633.

The S35A mutation did not significantly affect the rate of Rb⁺ uptake mediated by WT AtHAK5 in the absence of AtCIPK23/AtCBL1. However, this S35A transporter was not activated by the regulatory complex (Figure 9). When the C-
terminus of the S35A transporter was truncated at position 571 (S35A-D571 mutant), an important increase in the rate of Rb⁺ uptake was observed in the absence of AtCIPK23/AtCBL1. Importantly, this S35A-D571 transporter was not further activated by the presence of AtCIPK23/AtCBL1 (Figure 9).

By contrast, truncation at position 633 in the S35A mutant produced no effect. Contrarily to the S35A mutant, truncation at position 571 in the D72A mutant did not produce any effect. Thus both the D72A and the D72A-D571 (Figure 9) transporters showed similar low rates of Rb⁺ uptake. In summary, these results indicate that: (1) phosphorylation of S35 by AtCIPK23/AtCBL1 is needed for AtHAK5 to reach maximal transport activity, (2) truncation of the C-terminus at position 571 reverts the low transport activity of the S35A mutant regardless of the presence of AtCIPK23/AtCBL1, (3) the low transport activity of the S35A mutant in the presence of AtCIPK23/AtCBL1 cannot be restored if the C-terminus is extended to position 633, and (4) the non-functional mutant D72A cannot be reverted by the ΔS71 truncation, supporting the essential role of D72 for K⁺ transport.

Discussion

In spite of the essential role that HAK transporters play in plant physiology, there is no information regarding their structural or functional domains involved in K⁺ transport or activity regulation. Based on the cryo-EM structure of the bacterial relative KimA, we have identified AtHAK5 residues essential for K⁺ transport that are probably involved in K⁺ coordination. AtHAK5, like most of the eukaryotic APC transporters, possesses significantly longer N- and C-terminal regions compared to its bacterial orthologues. These hydrophilic extensions allow for more complex regulation and could play essential functions in the activity of APC transporters, such as turnover, trafficking to the target membrane, oligomerization, or regulation of transport per se (Mikros and Diallinas, 2019). Indeed, we have identified important regulatory elements in these two extensions of AtHAK5.

Our study shows that G67, Y70, G71, and D72 in TM1, D201 in TM3, and E312 in TM6 are essential for K⁺ transport through AtHAK5. (A) Accumulation of Rb⁺ versus time from a 100 μM external Rb⁺ solution in K⁺-starved yeast cells transformed with the pDR195 and p14GPD empty vectors (EV) or expressing AtHAK5 alone or in combination with AtCIPK23/AtCBL1. (B) Rb⁺ uptake rates from 100 μM external Rb⁺ shown by K⁺-starved yeast transformed with the vectors (EV) or expressing AtHAK5 or the D72A, D201A, or E312A mutant versions in the absence or in the presence of AtCIPK23/AtCBL1. Bars show average values of at least three repetitions and error bars depict SE. Bars with different letters are significantly different according to LSD test (P < 0.05).

Figure 7. Residues G67, Y70, G71, and D72 in TM1, D201 in TM3, and E312 in TM6 are essential for K⁺ transport through AtHAK5. (A) Accumulation of Rb⁺ versus time from a 100 μM external Rb⁺ solution in K⁺-starved yeast cells transformed with the pDR195 and p14GPD empty vectors (EV) or expressing AtHAK5 alone or in combination with AtCIPK23/AtCBL1. (B) Rb⁺ uptake rates from 100 μM external Rb⁺ shown by K⁺-starved yeast transformed with the vectors (EV) or expressing AtHAK5 or the D72A, D201A, or E312A mutant versions in the absence or in the presence of AtCIPK23/AtCBL1. Bars show average values of at least three repetitions and error bars depict SE. Bars with different letters are significantly different according to LSD test (P < 0.05).

Figure 8. The AtHAK5 C-terminal tail contains autoinhibitory and activation domains essential for regulating AtHAK5-mediated K⁺ transport. Rb⁺ uptake rates from 100 μM external Rb⁺ shown by K⁺-starved yeast transformed with the pDR195 and p14GPD empty vectors (EV) or expressing AtHAK5 or different C-terminal AtHAK5 truncations in the absence or the presence of AtCIPK23/AtCBL1. Bars show average values of at least three repetitions and error bars depict SE. Bars with different letters are significantly different according to LSD test (P < 0.05).
due of KimA is too far from the K⁺-binding sites and that
However, Tasco´n et al. (2020) proposed that the E233 resi-
(Figures 2 and 7). Finally, the E233A mutation of KimA pro-
the equivalent AtHAK5-D201A mutant was not functional
whereas the D117A mutation produced no effect on KimA,
for the E312A mutation of AtHAK5 (Figures 2 and 7).
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model presented here for AtHAK5 (Figure 1) placed the
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average values of at least three repetitions and error bars depict SE. Bars with different letters are significantly different according to LSD test (P < 0.05).
respectively, equivalent to D72, D201, and E312 of AtHAK5
(Figure 1D), bind three K⁺ ions (Tascón et al., 2020). Tascón
et al. (2020) proposed that D36 of KimA is essential for K⁺
transport, binds two K⁺ ions, and is also involved in trans-
port regulation. This D36 residue of KimA is equivalent to
D23 of Kup (Figure 1D), also essential for Kup function
(Sato et al., 2014). Residue D117 of KimA is involved in K⁺
binding and may also play a sensing or regulatory role,
whereas E233 may be involved in H⁺ coupling. Similar to
the KimA-D36A mutant, the AtHAK5-D72A mutant was a
non-functional transporter (Figures 2 and 7). However,
whereas the D117A mutation produced no effect on KimA,
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model presented here for AtHAK5 (Figure 1) placed the
E233 residue at a position compatible with K⁺ binding.
Therefore, it is possible that the E312 residue of AtHAK5 is
in fact involved in K⁺ binding and H⁺ binding occurs
through a different residue. This is not surprising because
H⁺-coupling may occur in different ways. In the case of an-
other APC transporter, ApcT, it was suggested that proton-
ation of the K158 residue (in TMS5) is responsible for H⁺ co-
transport with its substrate by toggling the conformation of
TM1 (Shaffer et al., 2009). However, such a lysine is not con-
served in the TMS5 of HAK/KUP/KT K⁺ transporters
(Nieves-Cordones et al., 2016). Interestingly, different H⁺-
coupling mechanisms may operate even within the same
transporter family, as is the case for some NPFs transporters.
Structure of NRT1.1/NPF6.3 pointed to a key role of H356 in
H⁺-dependent NO₃⁻ transport, albeit this residue is not
conserved in other pH-dependent transporters, like
AtNRT1.5 and AtNRT1.8 (Sun et al., 2014).
The D72 residue present in TM1 of AtHAK5 is located
within the 67-GVYGYD-72 motif that is conserved in trans-
porters of the HAK/KUP/KT family (Rodríguez-Navarro,
2000; Nieves-Cordones et al., 2016). However, with the ex-
ception of the Asp residue, this motif is not present in
KimA (Tascón et al., 2020). This domain resembles the
GVYGD motif in the selectivity filter of voltage-gated K⁺
channels from animal and plant cells (MacKinnon, 2003;
Jegla et al., 2018), which provides the structural basis for
their K⁺ > Rb⁺ > Cs⁺ > Na⁺ selectivity sequence (Doyle
et al., 1998; Dreyer and Uozumi, 2011). Our results for
AtHAK5 show that, in addition to D72, the G67, Y70, and
G71 residues are also essential for its function (Figure 2) and
it is worth noting that AtHAK5 shows an ion sequence se-
lectivity similar to that of K⁺ channels (Rubio et al., 2000).
Thus, TM1 of AtHAK5, containing the GVYGYD domain,
also present in other K⁺ transporters of the family, may ac-
commodate a selectivity filter similar to that of K⁺ voltage-
gated channels. Further support for the involvement of TM1
of HAK transporters in determining their high selectivity for
K⁺ has been obtained from HvHAK1, the barley AtHAK5
homolog. Mutations G61S and T62I of HvHAK1, located in
TM1, and corresponding to G74 and T75 of AtHAK5, sub-
stantially decreased the affinity for K⁺ (Senn et al., 2001). In
addition, it is worth highlighting that TM1 participates in
the substrate recognition in other plant transporter families
like NRAMPs (also belonging to the APC superfamily) or
NPFs (Parker and Newstead, 2014; Pottier et al., 2015).
As mentioned above, the N- and C-termini of AtHAK5
could not be modeled for several reasons. The N-terminus
of KimA is missing in the structure resolved, there is no sig-
ificant homology between the C-terminal regions of
AtHAK5 and KimA, and the N- and C-termini of AtHAK5
are much longer than those of KimA. However, these seg-
ments are likely to contain important regulatory domains. In
fact, the N-terminus of AtHAK5 was previously shown to be
phosphorylated by the kinase AtCIPK23 (Ragel et al., 2015).
This posttranslational modification enhances the K⁺ trans-
port capacity of AtHAK5. Here, we identified S35 as the tar-
get for AtCIPK23 (Figure 4). This serine residue is not fully
conserved in all members of the plant HAK/KUP/KT family,
not even within the Arabidopsis representatives
(Supplemental Figure 3). Importantly, this serine is present
in many characterized high-affinity K⁺ transporters, some of
which, such as CaHAK5, SiHAK5, EsHAK5 (Ragel et al.,
2015), DmHAK5 (Scherzer et al., 2015), and CqHAK
(Böhm et al., 2018), are also regulated by AtCIPK23/AtCBL1
(Ragel et al., 2015). Therefore, this serine may be a central element
of AtCIPK23-mediated regulation of high-affinity K⁺ HAK
transporters, and its presence or absence may dictate

Figure 9. The AtHAK5 S35 residue is required for AtCIPK23/AtCBL1-
mediated regulation of AtHAK5 transport activity through its C-termi-
nal tail. Rb⁺ uptake rates from 100 μM external Rb⁺ shown by
K⁺-starved yeast transformed with the pDR195 and p414GPD empty
vectors (EV) or expressing AtHAK5, the S35A or the D72A mutant ver-
sions, or combinations of the mutant versions with C-terminal truncations
in the absence or the presence of AtCIPK23/AtCBL1. Bars show
average values of at least three repetitions and error bars depict SE. Bars with different letters are significantly different according to LSD test (P < 0.05).
whether a HAK/KUP/KT transporter will be subjected to regulation by CIPK23-like proteins or not. In addition to AtCIPK23, other kinases of the AtCIPK family have been recently reported to activate AtHAK5 in yeast (Lara et al., 2020). In particular, AtCIPK1 and AtCIPK9 have been shown to activate AtHAK5-mediated K⁺ uptake in planta (Lara et al., 2020). At present, it is unknown whether AtCIPK1 and AtCIPK9-mediated AtHAK5 activation also involves phosphorylation of S35 or if they modify another residue(s).

It would be interesting to identify the residues targeted by these other two kinases and how their interplay modulates high-affinity K⁺ uptake at the root. Phosphorylation, also described in other plant HAK/KUP/KT transporters, seems a common mechanism for regulation of their activity (Osakabe et al., 2013; Han et al., 2016).

The results obtained with the deletion approach undertaken here are consistent with the presence in the AtHAK5 C-terminus of an autoinhibitory domain between positions 571 and 633, and an AtCIPK23-dependent activation domain downstream position 633 (Figures 3 and 8). The presence of an autoinhibitory domain in APC transporters has been described in the C-terminus of the glutamate-GABA antiporter GadC from *E. coli*. Unlike other prokaryotic APC transporters, GadC possesses an extended C-terminal fragment, containing a domain named C-plug that blocks the path to the substrate binding site. Displacement of this C-plug is required for the transport activity (Ma et al., 2012).

An important feature of the C-plug is the presence of several basic amino acids that make intra-domain interactions and inter-domain contacts with several transmembrane domains (Ma et al., 2012). Although there is no significant sequence similarity between the C-plug of GadC and the AtHAK5 autoinhibitory domain, it is a remarkable coincidence that this AtHAK5 domain is also rich in positively charged amino acids (Supplemental Figure 4). As described for the GadC C-plug, the AtHAK5 autoinhibitory domain could establish interactions with other domains of the transporter to block the K⁺ pathway of AtHAK5. Interestingly, these positively charged residues are conserved in the majority of the Arabidopsis KT/HAK/KUP proteins (Supplemental Figure 4), suggesting that the presence of an autoinhibitory domain in the C-terminus is a general feature of this transporter family.

A similar regulatory mechanism controlled by a CIPK-type kinase has been described for the CPA1 Na⁺/H⁺ antiporter SOS1 from Arabidopsis (Quintero et al., 2011; Núñez-Ramírez et al., 2012). An autoinhibitory domain located in the cytoplasmic C-terminus is phosphorylated by the kinase SOS2 (AtCIPK24), relieving the interaction of the autoinhibitory domain with a SOS1 domain essential for its activity and allowing the efflux of Na⁺. In the case of AtHAK5, AtCIPK23 does not phosphorylate the C-terminal tail (Ragel et al., 2015) but the N-terminus at S35 (Figure 4).

Serine at position 35 seems to play a pivotal role in AtHAK5 regulation. Mutation of serine 35 to alanine precludes phosphorylation of AtHAK5 (Figure 4) and the activation by AtCIPK23/AtCBL1 (Figures 5 and 9). These observations could be explained if activation by AtCIPK23/AtCBL1 would require phosphorylation of S35 to, through the C-terminal activation domain, fully activate the transporter. Maximal activity of WT AtHAK5 can be reached by not only AtCIPK23/AtCBL1-mediated phosphorylation of S35 (Figures 3, 4, and 7) but also deleting the AtHAK5 C-terminus from position 571 (Figures 3 and 8). Thus, it seems likely that phosphorylation of S35, through the C-terminal activation domain, leads to the release of the autoinhibitory domain and to maximal activation of AtHAK5.

Phosphorylation at residues located in the hydrophilic cytosolic extensions is a common theme in the regulation of several families of eukaryotic APC transporters (Hartmann and Nothwang, 2015; Razavi et al., 2018; Bianchi et al., 2019), as for example the family of Cation-Chloride Cotransporters (CCC; Alessi et al., 2014; Hartmann and Nothwang, 2015). Interestingly, it was demonstrated that phosphorylation of the N-terminal region of the NKCC1 dimer induced a strong conformational change in the C-terminal tails that moved apart relative to one another. This structural change can affect the organization of the transmembrane region, activating ion transport (Monette and Forbush, 2012).

Another example of regulation of an APC transporter by phosphorylation of a cytosolic tail, that also involved molecular motion, comes from the human dopamine transporter hDAT. Phosphorylation of the N-terminus of hDAT activates amphetamine-induced dopamine efflux (Khoshbouei et al., 2004). Using molecular dynamics it was shown that the N-terminus of hDAT interacted with several intracellular loops and with the C-terminal region. More importantly, a phosphomimic N-terminal domain showed stronger binding to the C-terminus compared to the WT domain (Razavi et al., 2018). These examples clearly point to a structural transition of the transporter from an inactive to an active state triggered by phosphorylation of the N-terminus.

Taking into consideration the above examples and the results from this work, we suggest the following model to explain the activation of AtHAK5 by AtCIPK23 (Figure 10 and Supplemental Figure 5). Upon phosphorylation by AtCIPK23, the N-terminal region of AtHAK5 increases its mobility and contacts the C-terminal activation domain. This interaction is translated into a conformational change that promotes the release of the autoinhibitory domain from the path of K⁺, allowing the transport activity of AtHAK5. Conversely, dephosphorylation of AtHAK5 would be needed to turn-off the system. In Arabidopsis, the voltage-dependent K⁺ channel AKT1 is activated by AtCIPK23 phosphorylation and deactivated by the PP2C AIP1 phosphatase (Lee et al., 2007). Given that CIPK-CBL phosphorylation is counteracted by PP2C phosphatases (Lee et al., 2007; Léran et al., 2015), it is likely that a PP2C protein can also dephosphorylate AtHAK5. This model helps to explain the results obtained, but other possibilities cannot be ruled out. Moreover, in addition to the AtCIPK23/AtCBL1 kinase complex, the activity of AtHAK5 is likely controlled
by other regulators. For instance, residues Y31 and T43 are phosphorylated by unknown kinase(s) (Zhang et al., 2013). Recently, it was found that other members of the AtCIPK family upregulate the transport capacity of AtHAK5 expressed in yeast (Lara et al., 2020). Whether these or other protein kinases phosphorylate Y31 or T43 deserves further investigation.

Conclusions

A mutational approach analyzing the HAK/KUP/KT AtHAK5 K⁺ transporter identified domains involved in transport of K⁺ and in AtCIPK23/AtCBL1-mediated activity regulation. This study shows that residues G67, Y70, G71, D72, D201, and E312 are essential for K⁺ transport. These residues can be arranged into two groups. On the one hand, residues D72, D201, and E312, located in transmembrane segments TM1, TM3, and TM6, respectively, and homologous to residues D23, E116, and E229 of the APC K⁺ transporter KimA, which have been recently shown to be involved in K⁺ transport. On the other, residues G67, Y70, G71, and D72 within the GVVYG motif of Shaker-like K⁺ channels. Thus, it is likely that the first group of residues is involved in K⁺ transport and the second one in shaping the selective filter for K⁺.

Regarding regulation of AtHAK5, the phosphorylation of S35 and the autoinhibitory and activation domains located in the C-terminus interplay to activate the transporter. Phosphorylation of S35 by AtCIPK23 leads to activation of AtHAK5 by, in concurrence with the activation domain, releasing the autoinhibitory domain from the K⁺ pathway. In spite of the important role that HAK/KUP/KT transporters play in K⁺ nutrition in plants, information regarding their functional domains is scarce. The study presented here sheds some light on this matter and may help in developing tools for improving K⁺ nutrition of plants.

Materials and Methods

Homology modeling of AtHAK5

The structure of the transmembrane domains of AtHAK5 was built using the homology modeling approach with the Discovery Studio version v20 (Biovia). Search for homologous structures in the PDB database identified KimA (PDB identifier 6S3K; Tascón et al., 2020) as the best template. A multiple sequence alignment of KimA with the corresponding sequences of AtHAK5 and the bacterial homologue EcKUP was generated with PROMALS 3D server (http://promals3d.prodata.swmed.edu/promals3d/promals3d.php; Supplemental Figure 1). Insertions and deletions were shifted manually to protein loop regions whenever possible. The modeled structure of AtHAK5 ranged from F63 to E537 (transmembrane domains 1–12) and did not include the N-terminus and C-terminus and part of the TM2-TM3 linker (S134–S180) for the following reasons: the N-terminus is absent in the KimA structure, the C-terminus of KimA and AtHAK5 showed a lower sequence conservation than TM segments, and a large segment of the AtHAK5 C-terminus (K647–K726) was not conserved in KimA and the segment of the TM2-TM3 linker ranging from S134 to S180 of AtHAK5 was not conserved in KimA. Refined structures were obtained with Modeler and
Profiles 3D tools (Discovery Studio). Ramachandran plot indicated the absence of weird backbone conformations in the AtHAK5 model.

Yeast strains and growth experiments
The Saccharomyces cerevisiae 9.3 yeast strain (MATa, ena1ΔHis3:ena4Δ, leu2, ura3-1, trp1-1, ade2-1, trk1Δ, trk2:pCK64; Bañuelos et al., 1995), deficient in the endogenous K+ uptake systems TRK1 and TRK2, was used for genetic complementation with AtHAK5 and mutant derivatives. Yeast transformants were obtained as indicated elsewhere (Elble, 1992). Yeast cells were grown at 28°C in synthetic dextrose (SD) media (Sherman, 1991) supplemented with the appropriate requirements for transformants selection. For growth complementation assays, the minimal arginine phosphate (AP) medium (Rodríguez-Navarro and Ramos, 1984), nominally free of Na+ and K+, was supplemented with KCl at the concentrations indicated in each experiment. For growth tests, overnight cultures were brought to 1 OD550 and then 5–10 μl drops of decimal dilutions of the cell suspensions were spotted onto plates with solid AP media. Plates were incubated at 28°C for 3–4 d.

Plasmid constructs
AtHAK5 WT complementary DNA (cDNA) was cloned into pDR195 yeast expression plasmid under the control of the PMA1 (Plasma Membrane ATPase 1) promoter (Rubio et al., 2000). For AtHAK5 detection in immunoblots, the HA was inserted into AtHAK5 between the third and fourth amino acid residues by performing the bridge-overlap-extension method (Mehta and Singh, 1999) using the primers indicated in Supplemental Table 1. The insertion point of the HA-tag in AtHAK5 was chosen according to a similar HA insertion described for the HAK1 transporter of Neurospora crassa, which did not affect transporter function (Rivetta et al., 2013).

The single-point mutations of AtHAK5 were obtained by polymerase chain reaction (PCR)-based site-directed mutagenesis of WT AtHAK5:HA cloned into pDR195 plasmid by following the QuickChange site-directed mutagenesis method. Sequential deletions of the AtHAK5 C-terminus were obtained following two strategies. The first strategy was used to delete the entire AtHAK5 C-terminus from residue 539 (AtHAK5::Δ539) and consisted of amplification by PCR of the AtHAK5:HA WT cDNA fragment with a forward primer that inserted a NotI restriction site upstream of the ATG codon, and a reverse primer that inserted a STOP codon plus a SpeI restriction site after amino acid residue 539. The amplified cDNA fragment was digested with NotI and SpeI and cloned into pDR195 plasmid. The second strategy was used to obtain the rest of the AtHAK5 C-terminal deletions and consisted of amplification by PCR of an AtHAK5:HA WT cDNA fragment with a forward primer amplifying from residue 536, upstream of the unique BgIII site present in AtHAK5 cDNA, and a reverse primer located at the point of each deletion and that inserted a STOP codon followed by a SpeI restriction site. The PCR products were then digested with BgIII and SpeI and cloned into the previously obtained AtHAK5::Δ539 construct in plasmid pDR195. The internal deletion of AtHAK5 between positions 571 and 633 was obtained by following the QuickChange site-directed mutagenesis method. Primers used for constructions and site-directed mutagenesis are listed in Supplemental Table 1.

The full-length cDNAs of AtCIPK23 and AtCBL1 (Ragel et al., 2015) were cloned together into the yeast expression vector p414GPD (Mumberg et al., 1995). AtCIPK23 cDNA was cloned under the control of GPD (glucose-6-phosphate 1-dehydrogenase) promoter present in p414GPD plasmid. AtCBL1 cDNA was first cloned into plasmid pYPGE15 (Brunelli and Pall, 1993) under control of the PGK1 (Phosphoglycerate Kinase 1) promoter, and then the expression cassette containing the PGK1 promoter, the AtCBL1 cDNA, and the CYC1 terminator was moved to the previous construct containing AtCIPK23 in p414GPD.

Expression and purification of fusion proteins
A constitutively active AtCIPK23 kinase with the T190D mutation in the activation loop and a deletion of the C-terminal autoinhibitory domain (CIPK23(T190D,Δ331)) was used for in vitro phosphorylation assays (Chaves-Sanjuan et al., 2014). The translational fusion GST-AtCIPK23(T190D,Δ331) was purified from yeast as described previously (Chaves-Sanjuan et al., 2014). Glutathione-S-transferase (GST) fusion proteins AtHAK5(1–48), HAK5(49–94), AtHAK5(30–49), AtHAK5(1–94), and AtHAK5 S35A(1–94) were constructed by PCR amplification using the primers indicated in Supplemental Table 1. Primers incorporated restriction sites at the S’ and 3’ ends of the amplicon, thereby allowing in-frame insertions into vectors pGEX4T1 or pGEX4T2 (GE Healthcare). GST fusion constructs were transformed into E. coli Rosetta cells (Merck). A 3-ml overnight Luria-Bertani (LB) culture was transferred to a fresh 200 ml of 2xYTA (tryptone 16 g/l, yeast extract 10 g/l, and NaCl 5 g/l) and further cultured at 37°C until the OD600 reached ca. 0.8. Recombinant protein expression was induced by 1 mM isopropyl β-D-thiogalactopyranoside for 4 h. Cells were harvested by centrifugation and resuspended in ice-cold PBS buffer pH 7.5, containing a mixture of protease inhibitors (Sigma Aldrich P2714). Lysozyme (1 mg/ml) was added to the cell suspension and incubated on ice with gentle shaking before sonication. The lysate was clarified by centrifugation, and the recombinant proteins were affinity-purified by chromatography on glutathione-Sepharose 4B (GE Healthcare). SDS-PAGE analysis was used to evaluate protein integrity and purity of each preparation. Gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich).

Phosphorylation assays
Substrate proteins (~100 ng) were subjected to phosphorylation by the AtCIPK23(T190D,Δ331) protein kinase (~100 ng) in 30 μl of buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM DTT). Reactions were started by adding ATP.
(0.2 mM with 1 μCi of [γ-32P]ATP), incubated at 30°C for 30 min, and stopped with 10 μl of 4× SDS/PAGE sample buffer. Aliquots were then resolved by SDS/PAGE and the gel was exposed to X-ray films.

**Rb⁺ uptake experiments in yeast**

Yeast cells transformed with the empty pDR195 and p414GPD plasmids or expressing WT AtHAK5 or its mutants in the absence or the presence of AtCIPK23/AtCB1 were grown overnight on AP media supplemented with 5 mM K⁺ at 28°C with shaking. Exponentially growing cells were collected by centrifugation and transferred to AP medium without K⁺ and incubated at 28°C with shaking for 4 h for starving the cells of K⁺. Then, cells were harvested by centrifugation and transferred to AP media supplemented with 100 μM Rb⁺. Samples of these yeast suspensions were taken at different time points, the cells pelleted by centrifugation, washed with cold double distilled water, and suspended in 0.1 M HCl. Extracted Rb⁺ in the supernatant of each sample was determined by atomic emission spectrometry with a Perkin Elmer AAnalyst 400 spectrometer. Rb⁺ uptake rates were determined from the internal Rb⁺ accumulated in yeast cells per dry weight and time units.

**Immunoblot analysis**

To confirm expression of the different AtHAK5 versions in yeast, total membranes were obtained from overnight cultures grown in selective SD media supplemented with 100 mM K⁺ as described (Serrano, 1988). Proteins were quantified by the Bradford method (Bradford, 1976), separated by SDS–PAGE (10% acrylamide gel), transferred to PVDF membrane (Amersham Hybond-PGE Healthcare) by using Trans-Blot SD Semi-Dry System (Bio-Rad), and immunoblotted with a primary monoclonal antibody against the HA-tag (Sigma-Aldrich) at 1/5,000 dilution and them with a horse-radish peroxidase (HRP)–conjugated anti-rabbit secondary antibody at 1/10,000 dilution. The protein ladder used was PageRuler Plus Prestained Protein (Thermo Scientific). For substrate detection, SuperSignal West Pico PLUS (Thermo Scientific) was used and HRP chemiluminescence was detected by using an Amersham Imager 600 (GE Healthcare).

**Statistical Analyses**

The Statistix V8 software (Analytical Software, Tallahassee, FL, USA) was used for analysis of variance. Differences in means were established by using the LSD test (P < 0.05).

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF129478 (AtHAK5), NM_102766 (AtCIPK23), and AF076251 (AtCB1).

**Supplemental Data**

**Supplemental Figure S1.** Amino acid sequence alignment of *Bacillus subtilis* K⁺ transporter KimA, *Escherichia coli* K⁺ uptake protein, and the K⁺ transporter AtHAK5 from *Arabidopsis thaliana*.

**Supplemental Figure S2.** Immunoblot analysis of microsomal fractions from yeast expressing wildtype AtHAKS and different mutant versions.

**Supplemental Figure S3.** Alignment of the N-terminal region of Arabidopsis HAK/KUP/KT transporters.

**Supplemental Figure S4.** Alignment of the putative auto-inhibitory domains of Arabidopsis HAK/KUP/KT transporters.

**Supplemental Figure S5.** Graphical representations of the activated or inhibited states of the different C-terminal deletion and S35 mutants of AtHAK5.

**Supplemental Table S1.** Primers used.

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