The Potassium Transporter *AtHAK5* Functions in K^+ Deprivation-Induced High-Affinity K^+ Uptake and *AKT1* K^+ Channel Contribution to K^+ Uptake Kinetics in Arabidopsis Roots[1][w]

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Potassium is an important macronutrient and the most abundant cation in plants. Because soil mineral conditions can vary, plants must be able to adjust to different nutrient availabilities. Here, we used Affymetrix Genechip microarrays to identify genes responsive to potassium (K^+) deprivation in roots of mature Arabidopsis (*Arabidopsis thaliana*) plants. Unexpectedly, only a few genes were changed in their expression level after 6, 48, and 96 h of K^+ starvation even though root K^+ content was reduced by approximately 60%. *AtHAK5*, a potassium transporter gene from the KUP/HAK/KT family, was most consistently and strongly up-regulated in its expression level across 48-h, 96-h, and 7-d K^+ deprivation experiments. *AtHAK5* promoter-β-glucuronidase and -green fluorescent protein fusions showed *AtHAK5* promoter activity in the epidermis and vasculature of K^+ deprived roots. Rb^+ uptake kinetics in roots of *athak5* T-DNA insertion mutants and wild-type plants demonstrated the absence of a major part of an inducible high-affinity Rb^+/K^+ (K_m approximately 15–24 μM) transport system in *athak5* plants. In comparative analyses, uptake kinetics of the K^+ channel mutant *akt1-1* showed that *akt1-1* roots are mainly impaired in a major transport mechanism, with an apparent affinity of approximately 0.9 mM $K^+(Rb^+)$. Data show adaptation of apparent K^+ affinities of Arabidopsis roots when individual K^+ transporter genes are disrupted. In addition, the limited transcriptome-wide response to K^+ starvation indicates that posttranscriptional mechanisms may play important roles in root adaptation to K^+ availability in Arabidopsis. The results demonstrate an in vivo function for *AtHAK5* in the inducible high-affinity K^+ uptake system in Arabidopsis roots.

Potassium is the most abundant cation in plants and has important functions as a major osmolyte in vacuoles, in turgor-driven movements, as a cofactor for enzymes, and for maintaining the plasma membrane potential. Root potassium uptake has been described in classical experiments as a biphasic process (Epstein et al., 1963) with mechanism I, also referred to as the high-affinity system, showing apparent affinities of approximately 20 μM Rb^+. Mechanism II, the low-affinity system (K_m in the millimolar range), provides an increasing contribution from >200 μM to millimolar external K^+ concentrations. Early analyses of the K^+ uptake systems in plants have served as models for many other nutrients that also show more than one kinetic uptake phase (Epstein and Rains, 1965; Epstein, 1966; Welch and Epstein, 1968; Kochian and Lucas, 1982; Siddiqi and Glass, 1983; Kochian et al., 1985; Siddiqi et al., 1990).

In Arabidopsis (*Arabidopsis thaliana*), several molecular components have been shown to contribute to potassium uptake into plant roots. *AKT1*, a shaker-family potassium channel, is expressed in root tissues and mediates potassium uptake over a wide range of external potassium concentrations (Sentenac et al., 1992; Lagarde et al., 1996; Hirsch et al., 1998; Spalding et al., 1999). Genetic disruption of *AKT1* reduces K^+ uptake into Arabidopsis roots and impairs seedling growth on low K^+ media in the presence of NH_4^+ (Hirsch et al., 1998) and to a lesser extent also in the absence of NH_4^+ (Spalding et al., 1999). The α-subunits of *AtKC1*, another shaker-like potassium channel expressed in root hair cells and in the root endodermis, do not form a functional channel but rather seem to influence the transport properties of other root potassium channels like *AKT1* (Reintanz et al., 2002).

Members of the KUP/HAK/KT family of potassium transporters in Arabidopsis were suggested to be involved in high-affinity K^+ uptake because several gene family members transport K^+ in the low micromolar range as demonstrated by heterologous expression in yeast (Santa-Maria et al., 1997; Fu and Luan,

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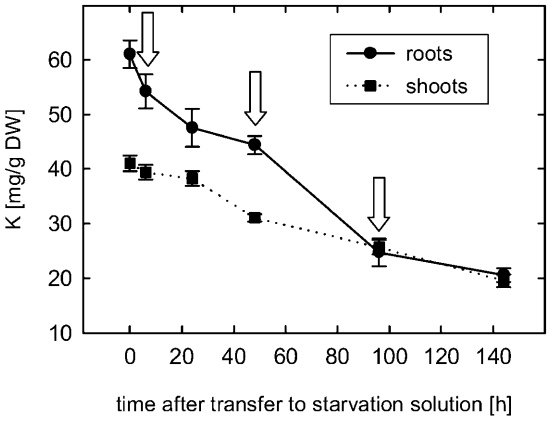

Figure 1. Potassium content in roots and shoots of hydroponically grown Arabidopsis after up to 6 d of K^+ starvation. Arrows indicate time points when root RNA samples were harvested for microarray analyses (see Fig. 2). $n = 5$ to 6 independent root or shoot samples per data point ± SE.

1998; Rubio et al., 2000) or by radiotracer uptake experiments in transgenic plant cells (Kim et al., 1998) and roots (Rigas et al., 2001). However, heterologous expression of the *AtKUP2* (=*AtKT2*) cDNA in mutant yeast cells lacking native K^+ transporters could only rescue growth on millimolar K^+ concentrations (Quintero and Blatt, 1997). Several KUP/HAK/KT transporters, including *AtKUP3* (Fu and Luan, 1998; Kim et al., 1998) and *AtHAK5* (Rubio et al., 2000), have been shown to be expressed in Arabidopsis roots and hence may contribute to potassium uptake. In addition, monitoring the expression of all members of the KUP/HAK/KT family by reverse transcription (RT)-PCR recently suggested that most of these genes are expressed in roots and that *AtHAK5* expression is responsive to K^+ deprivation (Ahn et al., 2004). T-DNA insertion mutants in *AtKUP4* lack the ability to extend root hairs and exhibit decreased $^{86}Rb^+$ uptake into mutant plants (Rigas et al., 2001). However, experimental evidence for the proposed K^+ uptake functions of other KUP/HAK/KT K^+ transporters in roots has not yet been obtained. A function in K^+ dependent cell expansion of growing tissues was suggested for *ATKUP2*, based on analyses of a semidominant mutation in the *ATKUP2* gene (Elumalai et al., 2002). Detailed kinetic analyses in plant roots of Rb^+ uptake rates as a function of external Rb^+ concentrations have not yet been reported for K^+ channel and KUP/HAK/KT mutants, thus limiting comparisons of gene functions to classical physiological components of $K^+(Rb^+)$ uptake (Epstein et al., 1963; Kochian and Lucas, 1982; Siddiqi and Glass, 1983).

Since potassium is one of the most important macronutrients, plants must be able to adjust their uptake systems rapidly to a varying supply to maintain growth and development. In this respect, high-affinity potassium uptake becomes particularly important for plants growing in soils with limited potassium supply. Here, we describe the response of Arabidopsis roots to potassium starvation based on transcriptome analyses, K^+ and Rb^+ uptake measurements, and genetic disruption of the K^+ starvation-induced K^+ transporter *AtHAK5*.

RESULTS

To investigate the response of Arabidopsis roots to potassium starvation, a hydroponic system was used that allowed analyses of individual plants. Plants were grown for 21 d in a K^+ replete (1.75 mM) medium and subsequently the roots were washed and the plants transferred to nominally potassium-free medium. The potassium content in root and shoot tissue had already started to decrease after 6 h of growth on potassium free solution and continued to decline with progressing starvation time (Fig. 1), with root K^+ content being reduced by 60% after 96 h of K^+ starvation. K^+ concentrations were initially higher in roots than in shoots, whereas after ≥96 h of starvation they were similar (Fig. 1).

Removal of K^+ from the growth medium causes rapid changes in the membrane potential of root cells (Rubio et al., 1996; Hirsch et al., 1998; Wang et al., 1998). To investigate the plant's response to K^+ starvation at the molecular level, we performed microarray analyses of total root mRNA. The transcriptome-wide changes in expression of individual genes in

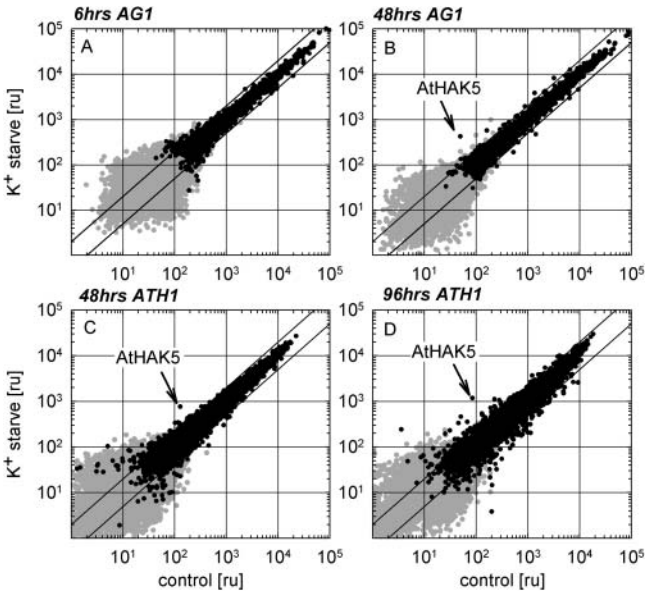

Figure 2. Expression overview for Affymetrix Genechip experiments (A and B, AG1-Genechip; C and D, ATH1-Genechips) of control plants (*x* axis) and plants subjected to K^+ starvation for the indicated periods of time (*y* axis). Diagonals indicate a 2-fold change in gene expression for visual reference. Data points in A and B represent the mean signal intensity from two independent experiments. Genes that were assigned a present call (P) by the MAS 5.0 algorithm in both control or both starvation replicates are represented by black symbols, and genes that did not meet these criteria are represented by gray symbols. ru, Relative units.

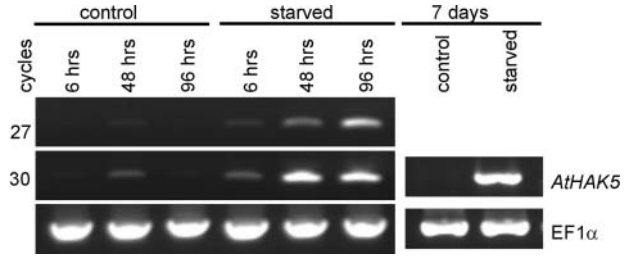

Figure 3. RT-PCR experiments confirm induction of *AtHAK5* mRNA by K^+ starvation. PCR was performed using cDNA isolated from roots of wild-type (Col-0) plants transferred to K^+-free nutrient solution for the times indicated. Elongation factor 1α (EF1α) was used as a control.

response to different periods of potassium starvation are shown in Figure 2. Expression levels were detected by hybridizing labeled cRNA from roots to Affymetrix Microarray Chips spotted with oligonucleotides representing approximately 8,300 genes (AG1-Genechip). In addition, experiments after 48 and 96 h of starvation were performed using the ATH1 Genechip (approximately 23,000 genes).

Remarkably, no dramatic changes were observed. Genes that were reliably expressed were analyzed using the statistical comparison algorithm of the MAS 5.0 software. Interestingly, less than 1% of the genes showed a significant change in expression levels compared to nonstarved roots after 6 h (21 genes) and 48 h (83 genes) of potassium starvation in both of the replicate experiments (Supplemental Tables III and V). None of those genes was changed over 2-fold in its transcript abundance after 6 h of starvation and only 1 gene (*AtHAK5*) was changed over 2-fold after 48 h of starvation in both experiments (Supplemental Tables III and V). For the 48 h starvation time point, the comparison of results obtained with AG1 and ATH1 chips showed the same general observation of only a small number of mRNAs that showed significant and over 2-fold changed expression levels (Fig. 2, B and C; Supplemental Tables V and VII).

The gene most strongly and most consistently affected in all experiments for 48- and 96-h K^+ starvation was the potassium transporter *AtHAK5* (arrows in Fig. 2), a member of the KUP/HAK/KT family. Interestingly, analysis of all of the K^+ transporter and K^+ channel genes represented on the chips showed that *AtHAK5* was the only K^+ translocator responsive to low external K^+ concentrations under the imposed conditions (Supplemental Table I). Under starvation conditions, *AtHAK5* was among the most highly expressed K^+ transporter genes in roots, which include *AtHAK5*, *AtHAK6*, *AtHAK8*, *AKT1*, and *KAT3* (Supplemental Table I). Note that other growth conditions and developmental stages can cause induction of other transporters. For example, *AtKUP3* mRNA levels are elevated in seedling roots after 2 to 3 weeks of growth on 40 μM K^+ (Kim et al., 1998; A. Alex, P. Mäser, and J.I. Schroeder, unpublished data).

The increased transcript level of the *AtHAK5* gene in response to potassium starvation was confirmed by semiquantitative RT-PCR for the time points tested on Affymetrix chips and also for a longer starvation period of 7 d, showing a continuing induction of *AtHAK5* transcripts (Fig. 3).

The *AtHAK5* expression pattern was analyzed by generating transgenic plants expressing both the β-glucuronidase (GUS) and green fluorescent protein (GFP) reporter genes under the control of the promoter region of the *AtHAK5* gene (2 kb upstream of ATG). Analysis of GUS staining in roots of K^+-starved plants revealed *AtHAK5* promoter activity in the cortex and stele of mature roots and even stronger staining in lateral roots (Fig. 4A). Very low or no activity was detectable in root tips (Fig. 4, A and B). Seven days after resupply of K^+ to roots of the same plants, GUS staining in mature and lateral roots was barely detectable and only visible in the root vasculature (Fig. 4B). The pCAMBIA1303 cassette allowed GUS activity and GFP synthesis to be controlled by the *AtHAK5* promoter at the same time. Analysis of GFP localization in

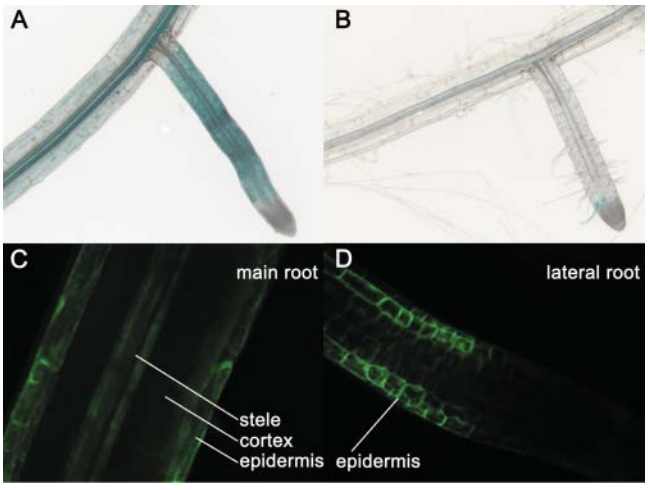

Figure 4. Roots of transgenic plants expressing the GUS and GFP reporter gene driven by the *AtHAK5* promoter. A and B, Roots of plants expressing the GUS reporter gene under the control of the *AtHAK5* promoter. GUS activity is strongest in the roots of K^+-starved plants (A) and decreases after resupply of potassium to the nutrient solution in roots of the same plants (B). C and D, Confocal microscopy reveals that the *AtHAK5* promoter activity (GFP localization) in starved roots is highest in the epidermis of main (C) and lateral roots (D) and in the stele of main roots (C).

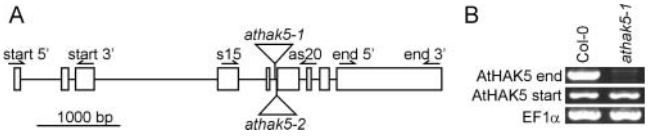

Figure 5. Isolation of two homozygous T-DNA insertion mutants in the *AtHAK5* gene. A, The cartoon shows the positions of the two T-DNA insertion lines, which were verified by sequencing of the left border PCR products. B, RT-PCR using cDNA from roots of wild-type (Col-0) and *athak5-1* plants starved for 4 d. Primer pairs anneal to cDNA as indicated in A. *AtHAK5* mRNA is absent downstream of the insertion site in *athak5-1* plants.

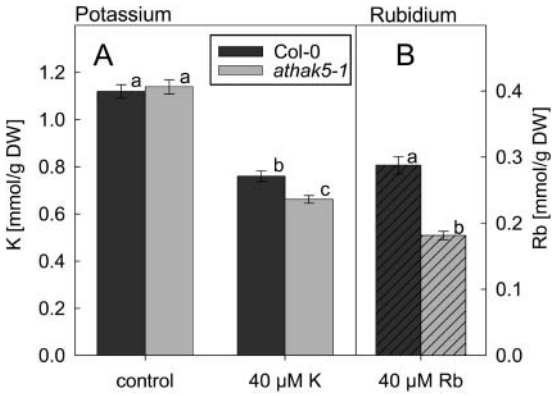

Figure 6. K^+ and Rb^+ content in Col-0 and *athak5-1* plants 4 d after transfer of mature plants to nutrient solutions supplemented with either (A) 1.75 mM K^+ (control solution), (A) 40 μM K^+, or (B) 40 μM Rb^+. Different letters indicate significantly different values ($P < 0.01$). $n = 9$ independent root samples/bar, mean ± SE.

transgenic K^+-starved roots by confocal microscopy gave a more detailed view of *AtHAK5* promoter activity (Fig. 4, C and D). In optical cross sections through K^+-starved main roots, GFP was detectable in the root epidermis and in the stele (Fig. 4C), whereas in lateral roots GFP was only visible in the epidermis (Fig. 4D).

To characterize the contribution of *AtHAK5* to plant potassium homeostasis, *AtHAK5* T-DNA insertion mutants were identified from the sequence-indexed SIGNAL database (http://signal.salk.edu/cgi-bin/tdnaexpress), and two homozygous lines, *athak5-1* (Salk_014177) and *athak5-2* (Salk_05604), were isolated (Fig. 5A). In both homozygous lines, the T-DNA insertion is located in the fifth intron, at positions 3,143 (*athak5-1*) and 3,159 (*athak5-2*; Fig. 5A). The insertion sites were verified and two independent lines were confirmed by sequencing the left border PCR products with primers used to identify the T-DNA mutants from the seed population supplied by the Arabidopsis Biological Resource Center (ABRC). RT-PCR using mRNA from K^+-starved roots did not detect any full-length *AtHAK5* transcript but only a truncated mRNA (Fig. 5B). The resulting protein would be disrupted after the third of 13 predicted transmembrane regions, making it unlikely to produce a functional transporter even if translated.

When mature, 3-week-old *athak5-1* plants were subjected to 4 d of potassium deprivation at 40 μM K^+, plants showed a slightly though significantly lower potassium content compared to wild-type Columbia (Col-0) plants (Fig. 6A; $P < 0.01$). When K^+ was replaced with 40 μM Rb^+ (used as a pulse-chase substitute for K^+) in the starvation media, the difference in tissue Rb^+ content between wild-type (Col-0) and *athak5-1* plants was clearly resolved (Fig. 6B; $P < 0.01$), demonstrating reduced $K^+(Rb^+)$ uptake in *athak5-1* plants. As expected, $Rb^+(K^+)$ uptake did not drop to 0 in *athak5-1* plants given the many K^+ transporter and K^+ channel genes expressed in roots (Supplemental Table I).

Rubidium uptake kinetics of *athak5* and wild-type (Col-0) plants were directly analyzed by measuring time- and concentration-dependent $^{86}Rb^+$ uptake into roots of K^+-starved plants. The initial uptake of $^{86}Rb^+$ was linear in wild-type and mutant roots for at least 25 min, with uptake being dramatically higher for Col-0 than for both *athak5* mutant alleles (Fig. 7A). Concentration-dependent $^{86}Rb^+$ uptake was subsequently measured for 20 min at a range of external Rb^+ concentrations. Uptake rates for $^{86}Rb^+$ revealed a significant difference between wild-type (Col-0) and both *athak5* alleles at low Rb^+ concentrations (Fig. 7B). At high Rb^+ concentrations greater than 500 μM, a difference in uptake kinetics could not be resolved between wild-type and *athak5* alleles (Fig. 7C). Uptake by wild-type (Col-0) roots at external concentrations below 200 μM revealed that the high-affinity component was greatly reduced in *athak5* plants (Fig. 7B).

As it has been demonstrated that the potassium channel gene *AKT1* contributes to root K^+ uptake, $^{86}Rb^+$ uptake kinetics were also analyzed for the mutant *akt1-1* and the corresponding wild-type (ecotype Wassilewskija [WS]) plants (Fig. 7, E and F). The effect of the *akt1-1* mutation on $^{86}Rb^+$ uptake is strongest at concentrations ≥500 μM, largely reducing Rb^+ uptake in the low-affinity range (Fig. 7E). At concentrations ≤200 μM, the Rb^+ uptake rates in *akt1-1* plants were smaller than in wild-type (WS) plants at some, but not consistently at all, concentrations (Fig. 7F). The $^{86}Rb^+$ uptake rate differences between wild-type (WS) and *akt1-1* plants could be described by Michaelis-Menten kinetics, indicating an apparent affinity for the component missing in *akt1* roots of approximately 0.9 to 1 mM (Fig. 8).

In wild-type (Col-0) roots, Rb^+ uptake rates were best described by Michaelis-Menten uptake kinetics, indicating two major phases of Rb^+ transport with K_m values of 24 μM for mechanism I and 4 mM for mechanism II, similar to the Rb^+ affinities found in barley (*Hordeum vulgare*) roots (Epstein et al., 1963; Table I). The high-affinity (K_m approximately 24 μM Rb^+) component disappeared in the two *athak5* disruption lines (Table I). Michaelis-Menten kinetics using 2 major phases also gave better curve fitting results for wild-type (WS) and *akt1-1* roots (R^2 closer to 1; Table I). Curve-fitting results for data extracted from Figure 1 in Epstein et al. (1963) are also included in Table I, showing that both for K^+-starved barley roots (Epstein et al., 1963) and for K^+-starved wild-type Arabidopsis roots, two uptake phases describe the data better than a single phase. In contrast, Rb^+ uptake kinetics in *athak5* plants can be described as dominated by only 1 component with an intermediate affinity (approximately 350 μM; Table I). Based on subtraction of *athak5* Rb^+ uptake from wild-type Col-0 measurements, the component missing in roots of *athak5* null mutants has an estimated K_m of approximately 14 μM and a V_{max} of approximately 2.4 μmol gFW^{-1} h^{-1} (Fig. 7D). These data suggest that the *AtHAK5* gene encodes an important component

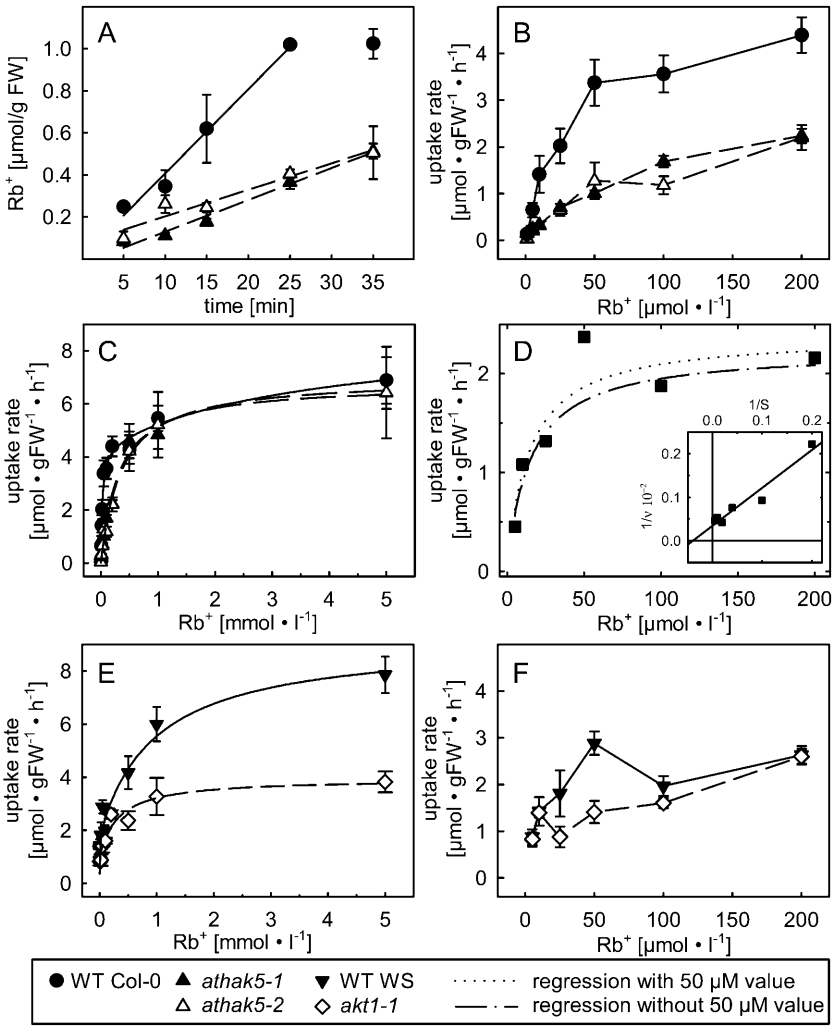

Figure 7. A, Time-dependent $^{86}Rb^+$ uptake in roots of wild-type (Col-0, black circles), *athak5-1* (black triangles), and *athak5-2* (white triangles) plants after 4 d of K^+ starvation; Rb^+ concentration in the uptake solution was 100 μM. $n = 4$ root samples/data point. Mean ± SE. B and C, Concentration-dependent $^{86}Rb^+$ uptake in roots of wild-type (Col-0) and intact *athak5-1* and *athak5-2* plants after 4 d of K^+ starvation. B shows the concentration range from 0 to 200 μM (high-affinity) and C the range from 0 to 5 mM. Curves in C are plotted using the results from Table I (two-term Michaelis-Menten fit for Col-0, one-term for *athak5 null* mutants). $n = 8$ to 12 independent root samples (Col-0, *athak5-1*) and $n = 6$ from 3 experiments (*athak5-2*). Mean ± SE. D, Michaelis-Menten kinetics for *AtHAK5* calculated by fitting a curve to the difference in uptake between wild-type (Col-0) and *athak5-1* roots. Inset shows Lineweaver-Burk plot of AtHAK5 uptake kinetic. Fits to the data including and excluding the relatively large Rb^+ uptake rate at 50 μM are shown as labeled and result in nearly identical values for K_m of 14.1 and 14.5 μM and V_{max} of 2.4 and 2.2 μmol gFW^{-1} h^{-1}, respectively. E and F, Concentration-dependent $^{86}Rb^+$ uptake in roots of intact wild-type (WS, black inverted triangles) and *akt1-1* (white diamonds) plants after 4 d of K^+ starvation. E shows the complete range from 0 to 5 mM and F the concentration range from 0 to 200 μM. Curves in E are plotted using the results from Table I (two-term Michaelis-Menten fit). $n = 6$ independent root samples. Mean ± SE.

that functions in K^+ starvation-induced high-affinity K^+ uptake in roots.

DISCUSSION

Limited Genome-Wide K^+ Starvation Effect on mRNA Levels

Being rooted in one place, plants need to concentrate nutrients from the soil and to adapt to environmental changes. Here, we analyzed the response of Arabidopsis to potassium deprivation by starving mature plants in nominally K^+-free hydroponic medium. The lack of available K^+ to Arabidopsis roots was detectable through a decrease in root K^+ content as early as 6 h after the start of the starvation period (Fig. 1). A decrease in tissue [K^+] has recently been observed in mature roots after 1 d of starvation (Ahn et al., 2004). The initial decline in root K^+ content might be explained by continued translocation of K^+ to the shoot, which showed a relatively more stable K^+ tissue level for the first 24 h of the starvation experiment (Fig. 1).

Interestingly, the rapidly decreasing root K^+ tissue content resulted in a significant and 2-fold change in transcript abundance of only few of the genes represented on the Genechips analyzed here after 6, 48, and 96 h of K^+ starvation (Fig. 2; Supplemental Tables III, V, VII, and IX). This is surprising with regard to the many essential functions of K^+ in plant metabolism and the high demand of plants for this ion. Rapid and massive changes in gene expression levels on a genomic scale have been reported when other major plant nutrients including nitrogen or phosphorus were supplied in a different ionic form (Wang et al., 2003) or withheld from the media (Hammond et al., 2003). Our results are in agreement with gene expression data of plant membrane transporters in response to K^+ deprivation by Maathuis et al. (2003), who found only few transporter genes affected, among them the stelar potassium outward rectifier channel, SKOR. Our analyses expand this conclusion to nontransporter-encoding genes genome wide and demonstrate a limited transcriptional response to K^+ deprivation in Arabidopsis roots of mature plants under the imposed condi-

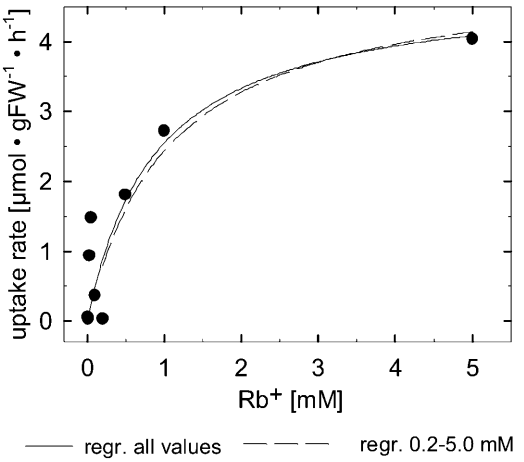

Figure 8. Rb^+ uptake rates for *AKT1*. The difference in Rb^+ uptake rates between wild-type (WS) and *akt1-1* roots is shown. Fits of Michaelis-Menten kinetics to the data including and excluding the Rb^+ uptake rates from 5 to 100 μM are shown as labeled and result in similar values for the K_m of 0.88 ($R^2 = 0.81$) and 1.1 mM ($R^2 = 0.91$) and V_{max} of 4.8 and 5.0 μmol gFW^{-1} h^{-1}, respectively.

tions. Recently, it has been shown that many genes involved in reactive oxygen metabolism are changed in their expression levels after 6 h of K^+ deprivation (Shin and Schachtman, 2004). However, in our experiments, these genes were not significantly and consistently affected by K^+ starvation after 6 h (Supplemental Table III), indicating that differences in experimental conditions may affect the changes in transcript levels. After 48 h, 2 of these genes (ACC oxidase, At2g19590 and RHD2, At5g51060) showed a 1.4- to 1.5-fold and significant induction in AG1 experiments but not in ATH1 experiments (Supplemental Tables V and VII). A very recent study using spotted microarrays reported a large number of transcripts responsive to varying K^+ supply, under other experimental conditions in plate grown plants cultivated on low K^+ containing agar (Type A, Sigma; 299 transcripts in roots at a false discovery rate of 1%) and a portion of those genes were linked to the phytohormone jasmonic acid (Armengaud et al., 2004).

Implications for K^+ Sensing

K^+ channels and K^+ transporters allow plant cells to respond instantaneously to K^+ concentration changes by means of membrane potential changes (Grabov, 1990; Schroeder et al., 1994; Rubio et al., 1996; Hirsch et al., 1998; Spalding et al., 1999). The relatively limited changes in K^+ starvation-affected transcript levels found here and in a transporter study (Maathuis et al., 2003) indicate that Arabidopsis does not use this external K^+ supply sensing ability for direct regulation of broad transcriptional responses but has a means of monitoring the intracellular K^+ status. Together, these findings support a hypothesis in which posttranslational, in addition to transcriptional, responses may contribute to Arabidopsis root short-term adaptation to K^+ starvation.

AtHAK5 mRNA Induced by K^+ Starvation

The K^+-starvation induction of the *AtHAK5* mRNA levels observed in this study is in agreement with recently reported results (Ahn et al., 2004; Armengaud et al., 2004; Shin and Schachtman, 2004). Importantly, in this study, *AtHAK5* was the most consistently and strongly up-regulated mRNA after 48 and 96 h of starvation in microarray experiments (Fig. 2) and also after 7 d of withholding K^+ from the nutrient solution (Fig. 3). Shin and Schachtman (2004) found the strongest *AtHAK5* induction after 6 h of K^+ starvation, whereas in this study, *AtHAK5* mRNA levels continued to increase after 48 and 96 h of K^+ of starvation (Supplemental Tables II and III).

However, a repression of the *AtHAK5* transcript was observed in plate-grown seedling roots when K^+ was withheld from the growth media (Rubio et al., 2000). This differs from the results of this study and recent data of Ahn et al. (2004), and differences may depend on experimental conditions. The closest homologs of *AtHAK5* in barley (*HvHAK1*; Santa-Maria et al., 1997) and tomato (Wang et al., 2002) also show greatly enhanced mRNA levels in response to K^+ deprivation.

Expression Pattern of *AtHAK5*

The *AtHAK5* gene is a member of the KUP/HAK/KT family of K^+ transporters, comprised of 13 members in Arabidopsis (Mäser et al., 2001; Very and Sentenac, 2003). High-affinity K^+ transport activity has been demonstrated for this gene by functional complementation of a *trk1 trk2* yeast mutant (Rubio et al., 2000) but not yet in plant roots in vivo. The expression pattern of *AtHAK5* in Arabidopsis was examined in roots of transgenic plants expressing GUS and GFP reporter genes driven by the *AtHAK5* promoter (Fig. 4). *AtHAK5* promoter activity was found in the epidermis of main and lateral roots and with less intensity in the vasculature of main roots (Fig. 4). This expression pattern is consistent with a function of *AtHAK5* in K^+ uptake into and also translocation from roots. The reduction of GUS staining in roots of the same plants after resupply of K^+ to the nutrient media showed that the *AtHAK5* promoter is active when K^+ supply is low.

AtHAK5 Functions in High-Affinity K^+ Uptake in Vivo

The function of *AtHAK5* in roots has not been analyzed to date. Furthermore, detailed kinetic Rb^+ uptake isotherms in intact roots of K^+ transporter and K^+ channel mutants have not yet been reported with exception of *AKT1* at 3 Rb^+ concentrations (Hirsch et al., 1998). To analyze whether *AtHAK5* functions in root K^+ uptake, we isolated homozygous *athak5* T-DNA insertion lines (Fig. 5, A and B). Mutant *athak5*

Table I. *Michaelis-Menten curve fitting results for $^{86}Rb^+$ uptake kinetics in roots of K^+-starved wild-type (Col-0, WS) and athak5 and akt1-1 null plants (Fig. 7C)*

Results for data extracted from Figure 1 in Epstein et al. (1963) are included for comparison. Curve fitting was performed with SigmaPlot 8.0 (SPSS, Chicago) software using either a one- or two-term Michaelis-Menten equation.

	R^2	K_m1	$V_{max}1$	K_m2	$V_{max}2$	Test
		μM	μmol gFW^{-1} h^{-1}	μM	μmol gFW^{-1} h^{-1}	
Epstein et al. (1963)	0.997	19.7	10.8	9,920	14.8	Two-term
	0.857	147.8	17.9			One-term
Wild type (Col-0)	0.988	24.1	4.4	3,991	4.5	Two-term
	0.931	48.9	5.8			One-term
athak5-1	0.990	0.3	0.2	345	6.7	Two-term
	0.988	312.9	6.7			One-term
athak5-2	0.990	362.9	3.4	363	3.5	Two-term
	0.990	362.9	7.0			One-term
Wild type (WS)	0.968	4.2	1.9	985	7.3	Two-term
	0.826	269.2	7.7			One-term
akt1-1	0.927	0.8	1.0	337	2.9	Two-term
	0.769	68.8	3.3			One-term

plants accumulated significantly less K^+ and Rb^+ over a 96-h starvation period (Fig. 6). This shows that *athak5* plants are partly impaired in K^+/Rb^+ accumulation from low external K^+ concentrations and also that other constitutively expressed transporters must be active, contributing to the K^+/Rb^+ uptake at low external supply.

Experiments characterizing $^{86}Rb^+$ transport in roots of intact plants revealed that a high-affinity component was strongly reduced in *athak5* mutants but not completely absent (Fig. 7, B and D). In addition, Michaelis-Menten analyses indicated a change in the apparent K_m of a major Rb^+ transport component from 24 μM in wild-type (Col-0) roots to 300 to 360 μM in *athak5* roots (Table I). These results demonstrate an important physiological contribution of *AtHAK5* to high-affinity Rb^+/K^+ uptake at limiting external K^+ availability.

Constitutively expressed root K^+ transporters include the K^+ channel AKT1, which is expressed in the root epidermis (Lagarde et al., 1996), and *AtKC1* (also named *AtKAT3* or *AtAKT4*), expressed in root hairs and trichomes (Reintanz et al., 2002; Pilot et al., 2003). On low potassium media, seedlings of a T-DNA insertion mutant in the *AKT1* gene showed reduced growth in the absence of ammonium and were unable to grow in the presence of high ammonium concentrations (Hirsch et al., 1998), demonstrating a contribution of AKT1 to K^+ transport at micromolar K^+ concentrations and suggesting the existence of an ammonium-sensitive component of high-affinity K^+ uptake (Spalding et al., 1999). In agreement with this, earlier studies investigating the influence of ammonium nutrition on root development and ion uptake reported ammonium sensitivity of $Rb^+(K^+)$ uptake at micromolar Rb^+ concentrations in Arabidopsis wild-type roots (Cao et al., 1993) and rice roots (Wang et al., 1996). However, in K^+-starved roots of mature Arabidopsis plants and in the absence of ammonium, the AKT1 protein seems to contribute to K^+ uptake mainly at an intermediate to low apparent affinity with the V_{max} being strongly reduced in *akt1-1* roots (Figs. 7E and 8; Table I). In addition, analyses of $^{86}Rb^+$ uptake in *akt1-1* roots revealed a partial effect at micromolar K^+ concentrations.

Subtraction of $^{86}Rb^+$ uptake kinetics in roots of K^+ transporter mutants from the respective wild-type reflect the change in transport properties on the whole root level including tuning of kinetic characteristics of other transporters present in mutant roots. For example, K^+ channels and transporters affect the membrane potential, which in turn affects uptake via other transporters. Taking this into account, the *athak5* mutation has a stronger effect on the high-affinity mechanism, whereas the *akt1* mutation affects the low-affinity component most strongly (Figs. 7D and 8).

For AtHAK5, the permeability of Rb^+ and K^+ has been found to be very similar (Rubio et al., 2000), whereas the permeability of Rb^+ over K^+ has not been determined for AKT1. However, a substantial Rb^+ permeability has been reported for other shaker-type inward-rectifying potassium channels in Arabidopsis (KAT1; Schachtman et al., 1992; Uozumi et al., 1995; Marten et al., 1996) and animals (Ding and Horn, 2002), indicating that $^{86}Rb^+$ kinetics from *athak5* and *akt1* plants can provide relevant information. In addition, a permeability ratio of Rb^+ over K^+ of 0.65 was found in Arabidopsis root cell protoplasts for inward rectifying K^+ channels (Maathuis and Sanders, 1995). Taken together, these data support a model in which inward K^+ channels show lower affinity K^+ uptake kinetics. However, in addition K^+ channels can provide a backup mechanism for uptake from micromolar external K^+ concentrations, particularly when other depolarizing high-affinity transporters are blocked or impaired (Schroeder and Fang, 1991; Hirsch et al.,

1998; Spalding et al., 1999). In the presence of NH_4^+, the apparent affinity of AKT1-mediated K^+ uptake (Hirsch et al., 1998) may shift to a higher affinity, and detailed Rb^+ uptake analyses are needed to test this hypothesis. Interestingly, for all of the *athak5-1*, *athak5-2*, and *akt1-1* mutants, the apparent affinity of the low-affinity component shifted from the millimolar range in wild type to approximately 350 μM Rb^+ in these mutants (Table I). These results further underline our finding that the apparent K^+ affinities of roots (i.e. of individual K^+ transport proteins) adapt if important transporters are impaired or knocked out. Hyperpolarization of the plasma membrane could, for example, partially compensate for the lack of important K^+ transporters (Schroeder et al., 1994; Spalding et al., 1999) such as AtHAK5.

For *HvHAK1*, the barley homolog of *AtHAK5*, ammonium sensitivity has been demonstrated by yeast mutant complementation studies (Santa-Maria et al., 2000), indicating that transporters of the KUP/HAK/KT family contribute to the ammonium-sensitive component. Other candidate genes that might account for the constitutive high-affinity Rb^+ uptake in *athak5* plants are *AtHAK6*, *AtHAK8*, *KAT3*, and *AKT1* all of which were highly expressed in Arabidopsis roots (Supplemental Table I).

In conclusion, we show here that K^+ deprivation causes only a limited transcriptional response in Arabidopsis roots and that *AtHAK5* functions as a major K^+ starvation-induced high-affinity (mechanism I) K^+ uptake transporter in Arabidopsis roots. Furthermore, detailed kinetic $^{86}Rb^+$ flux analyses provide molecular genetic, in planta, support for the classical model in which major contributions of high-affinity and low-affinity uptake components can be mechanistically and genetically separated. Furthermore, apparent root $K^+(Rb^+)$ uptake affinities adapt to transporter impairment.

MATERIALS AND METHODS

Plant Culture

Seeds of Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 were surface sterilized, washed, and stored in water for 4 d at 4°C. Seeds were then germinated on foam plugs inserted into Magenta box rafts (Sigma, St. Louis) and grown in hydroponic medium (100 mL) under a 16/8 h day/night cycle for 4 weeks. The hydroponic solution contained: 1.0 mM $Ca(NO_3)_2$; 0.5 mM KH_2PO_4; 1.25 mM KNO_3; 0.5 mM $MgSO_4$; 20 μM FeNaEDTA; 0.5 μM $CuSO_4$; 0.5 μM $MnSO_4$; 10 μM H_3BO_3; 0.05 μM Na_2MoO_4; 0.25 μM NaCl; and 0.5 μM $ZnSO_4$. In the potassium-free solution, KNO_3 and KH_2PO_4 were substituted by HNO_3 and H_3PO_4 and the pH was adjusted with *N*-methyl-glucamine to 5.5. The solution was renewed after 1, 2, and 3 weeks, and on the second day preceding the start of the starvation period. At the beginning of the starvation period, plants were washed for 5 min in deionized water and then transferred to either potassium-free or control medium. During the experiment, the solutions were exchanged every 24 h. Plants were always harvested 8 h after daybreak.

For determination of Rb^+ tissue content and $^{86}Rb^+$ uptake kinetics, plants were cultivated in the same media on a slightly modified hydroponic system consisting of plastic containers holding 4 L of aerated nutrient solution. Containers were covered with plastic lids supporting nine plants with roots growing through foam plugs into the hydroponic solution.

Microarrays

For all experiments, Affymetrix Genechips were used (Affymetrix, Santa Clara, CA); AG1 chips represented approximately 8,300 genes for 6 and 48 h of starvation and ATH1 chips for 48 and 96 h of starvation. For AG1 Genechip analyses, annotation was used as described in Ghassemian et al. (2001).

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). Two independently isolated mRNA samples were pooled for each Genechip hybridization experiment. AG1 experiments at 6 and 48 h of K^+ starvation were carried out in duplicate (total of 8 hybridizations). For ATH1-chips, a total of 4 hybridizations were performed. cRNA labeling and hybridizations were performed by the DNA Microarray Facilities at University of California, Irvine, and University of California, San Diego, according to the Affymetrix manual. Expression data sets will be deposited at The Arabidopsis Information Resource (TAIR; http://www.arabidosis.org).

Expression data were analyzed with Microarray Suite 5.0 (Affymetrix) using default settings to calculate raw data values. The detection signal value was calculated as a measure of the relative transcript abundance, and a detection *P*-value was computed for each probe set from independent control and starvation experiments and was used to evaluate whether or not a transcript was reliably expressed. Only genes that received a present call (P) and a detection *P*-value of $\leq$0.04 in both control or both starvation experiments were selected for further analysis. Comparison analysis, aimed at identifying genes whose expression levels were changed in response to different periods of potassium starvation, were performed by comparing individual treatments to their corresponding control hybridization. Only genes that were consistently increased (I) or decreased (D) in their expression level in replicate experiments and were determined to be significantly expressed by the criteria described above were considered. The $\log_2$ signal-log ratio listed in supplemental tables is a measure of the change in gene expression and can be converted into fold change according to the formula provided in the MAS 5.0 manual: signal log ratio $\geq$0, fold change = $2^{(\text{signal log ratio})}$; signal log ratio $<$0, fold change = $(-1) \times 2^{-(\text{signal log ratio})}$.

ICP Analyses

Roots of plants were washed for 5 min in 0.2 mM $CaSO_4$, separated into roots, leaves, and stems, and surface dried by blotting with ash-free filter paper. The fresh weight was determined prior to drying the samples for 24 h at 75°C. An aliquot of dry matter was digested in HNO_3 (concentrated) at 95°C for 1 h. After complete digestion, the acid concentration was adjusted to 5% by dilution with double deionized water. Mineral element concentrations in the solutions were determined by ICP-OES (Perkin Elmer Optima 3000 DV, Boston) at the Scripps Institution of Oceanography (University of California, San Diego) analysis facility.

Semiquantitative RT-PCR

cDNA was synthesized from 2.5 μg of root total RNA using the Amersham-Biosciences First Strand cDNA kit (Uppsala). The three-step PCR reaction was run for a total of 30 cycles and aliquots were removed after 27 cycles. Primers used to validate the expression pattern of *AtHAK5* were S15 (5′-gctcagaagagcccatatgatca-3′) and AS20 (5′-agctcgaacactgaagtgacctaga-3′).

Primers used to check for the presence of *AtHAK5* transcript in Col-0 and *athak5* null mutants are given below. The PCR product obtained for elongation factor 1α was used as a control for RT-PCR experiments: HAK5_start_5′ (5′-ggtgaggaacatcaaatagatggtg-3′), HAK5_start_3′ (5′-ggagcgctacgagagtaatagtgt-3′), HAK5_end_5′ (5′-gcgatgatggcgatttggcaata-3′), HAK5_end_3′ (5′-gccaaccttgagaagcttcga-3′), elongation factor 1α forward (5′-ggccacgtcgattctggaaa-3′), reverse (5′-ggcttggttggagtcatctt-3′).

Isolation of *athak5* T-DNA Insertion Lines

Putative T-DNA insertion lines were identified by searching the SIGNAL database (http://signal.salk.edu/cgi-bin/tdnaexpress). Plants of the identified seed populations (obtained from the ABRC) were screened for homozygous individuals by PCR using primers annealing up and downstream of the putative insertion sites (S15, AS20). Positively identified plants were subsequently tested with PCR using the T-DNA left border primer (LBa1: 5′-tggttcacgtagtgggccatcg-3′) and a gene-specific primer. PCR products

were cloned into pGEM T-easy (Promega, Madison, WI) and sequenced to verify the insertion site.

Rb^+ and $^{86}Rb^+$ Uptake Experiments

$^{86}Rb^+$ was used as a tracer to analyze kinetic properties of $Rb^+(K^+)$ uptake, as previous studies have shown substantial Rb^+ permeabilities of KUP/HAK/KT transporters and inward K^+ channels (Schachtman et al., 1992; Sentenac et al., 1992; Gassmann and Schroeder, 1994; Maathuis and Sanders, 1995; Marten et al., 1996). Moreover, use of $^{86}Rb^+$ allows comparison to physiological studies of $K^+(Rb^+)$ uptake components in roots (Epstein et al., 1963; Kochian and Lucas, 1982, 1983; Siddiqi and Glass, 1983; Kochian et al., 1985; Hirsch et al., 1998). Note that apparent K^+ affinities are expected to be higher than apparent Rb^+ affinities (e.g. Rubio et al., 1995). Roots of 3 individual plants were exposed to 10 mL of uptake medium consisting of the potassium-free nutrient solution supplemented with RbCl. For time course experiments, $^{86}Rb^+$ (Amersham Biosciences) was added at a concentration of 0.005 μCi/nmol Rb^+ and plants were incubated for the indicated periods of time. For concentration-dependent tracer uptake experiments, $^{86}Rb^+$ was added at a concentration of 1 μCi/μmol Rb^+ for concentrations $\leq$200 μM and 0.5 μCi/μmol Rb^+ for concentrations $>$200 μM. At the end of the uptake period, plants were blotted with tissue paper and immediately washed 2 times for 2 min in 4°C washing solution (K^+-free nutrient solution supplemented with 1.75 mM nonradiolabeled RbCl). Plants were separated into roots and shoots, surface-dried, and weighed (the fresh-weight was determined). After addition of 5 mL scintillation liquid, the radioactivity was measured with a scintillation counter (LS 6500, Beckman Coulter, Fullerton, CA).

Promoter GUS-GFP Analyses

The 2-kb DNA fragment upstream of the *AtHAK5* start codon was amplified by PCR from Col-0 genomic DNA using a proofreading DNA polymerase (Pwo, Roche, Indianapolis) and cloned into the *Pst*I/*Nco*I sites of the binary vector pCAMBIA 1303 replacing the cauliflower mosaic virus 35S promoter. The 3′ *Nco*I site was introduced by a mismatch in the antisense primer (5′-cctcaccatccatGGtttgctgtgtt-3′), while the 5′ *Pst*I site was present in the native DNA sequence (5′-cttacactgctgcagcctcggctt-3′). Sequence integrity was verified by sequencing the cloned product. Arabidopsis ecotype Col-0 plants were transformed by the floral dip method (Clough and Bent, 1998) with *Agrobacterium tumefaciens* cells harboring the promoter *AtHAK5*:GUS-GFP plasmid.

Seeds of the T_1 generation were surface sterilized and the population screened for hygromycin resistant individuals on one-half strength Murashige and Skoog agar plates (Murashige and Skoog, 1962). Resistant seedlings were transferred to one-half strength Murashige and Skoog plates containing 0.5% Suc for recovery and subsequently grown on hydroponics. GUS activity was analyzed in roots and leaves of these T_1 plants 4 and 8 d after transfer to potassium-free nutrient solution. Whole roots were analyzed after 24 h of incubation in a solution containing 2 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide; 0.1 M Na_2HPO_4, pH 7.2, 0.1% (w/v) K^+ ferrocyanide, 0.1% (w/v) ferricyanide, and 0.1% (v/v) Triton.

GFP-fluorescence was monitored by spinning-disc confocal microscopy (QLC100 confocal scanning unit from Solamere Technology Group, Salt Lake City attached to a NIKON Eclipse TE 2000-U bright field microscope; Tokyo) in intact roots using an argon laser (500 M Select, Laserphysics, West Jordan, UT, excitation wavelength filter at 488 nm and emission filter 500–550 nm). Images were captured by a CCD-camera (CoolSnap-HQ, Photometrics, Tucson, AZ) using Metamorph software (Universal Imaging, Downington, PA).

Upon request, all novel material 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 part of the material. Obtaining permission will be the responsibility of the requestor.

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