RESEARCH PAPER

The high affinity K⁺ transporter AtHAK5 plays a physiological role in planta at very low K⁺ concentrations and provides a caesium uptake pathway in Arabidopsis

Zhi Qi¹, Corrina R. Hampton², Ryoung Shin¹, Bronwyn J. Barkla³, Philip J. White⁴ and Daniel P. Schachtman¹,*

¹ Donald Danforth Plant Science Center, 975 N. Warson Road, St Louis, MO 63132, USA
² Warwick HRI, Warwick CV35 9EF, UK
³ Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, México
⁴ Scottish Crop Research Institute, Dundee DD2 5DA, UK

Received 12 October 2007; Revised 27 November 2007; Accepted 28 November 2007

Abstract

Caesium (Cs⁺) is a potentially toxic mineral element that is released into the environment and taken up by plants. Although Cs⁺ is chemically similar to potassium (K⁺), and much is known about K⁺ transport mechanisms, it is not clear through which K⁺ transport mechanisms Cs⁺ is taken up by plant roots. In this study, the role of AtHAK5 in high affinity K⁺ and Cs⁺ uptake was characterized. It is demonstrated that AtHAK5 is localized to the plasma membrane under conditions of K⁺ deprivation, when it is expressed. Growth analysis showed that AtHAK5 plays a role during severe K⁺ deprivation. Under K⁺-deficient conditions in the presence of Cs⁺, Arabidopsis seedlings lacking AtHAK5 had increased inhibition of root growth and lower Cs⁺ accumulation, and significantly higher leaf chlorophyll concentrations than wild type. These data indicate that, in addition to transporting K⁺ in planta, AtHAK5 also transports Cs⁺. Further experiments showed that AtHAK5 mediated Cs⁺ uptake into yeast cells and that, although the K⁺ deficiency-induced expression of AtHAK5 was inhibited by low concentrations of NH₄⁺ in planta, Cs⁺ uptake by yeast was stimulated by low concentrations of NH₄⁺. Interestingly, the growth of the Arabidopsis atakt1-1 mutant was more sensitive to Cs⁺ than the wild type. This may be explained, in part, by increased expression of AtHAK5 in the atakt1-1 mutant. It is concluded that AtHAK5 is a root plasma membrane uptake mechanism for K⁺ and Cs⁺ under conditions of low K⁺ availability.

Key words: Caesium, plasma membrane, potassium, uptake.

Introduction

Caesium (Cs⁺) is a group I alkali metal with chemical properties similar to potassium (K⁺). It is found naturally as the stable isotope ¹³³Cs. Caesium is not required by plants and although Cs⁺ can perturb cellular biochemistry by competing with K⁺, it is rarely present at toxic concentrations in soil solutions (White and Broadley, 2000). Nevertheless, two anthropogenic radioisotopes of Cs⁺ (¹³⁴Cs and ¹³⁷Cs) produced in nuclear reactors and thermonuclear explosions are of environmental concern (White and Broadley, 2000). These radioisotopes emit harmful β and γ radiation during their decay, have relatively long half-lives (2.06 and 30.17 years, respectively) and are incorporated rapidly into biological systems (White and Broadley, 2000). Efforts to reduce the entry of radiocaesium into the food chain through phytoremediation technologies and/or the development of ‘safer’ crops that accumulate less radiocaesium are,
therefore, a priority for contaminated land (White and Broadley, 2000; White et al., 2003).

Various studies on the fate of Cs\(^+\) in a variety of ecosystems have been conducted (Avery, 1996; Delvaux et al., 2000a, b). Where Cs\(^+\) is present in the soil solution, plant roots take it up through K\(^+\) uptake pathways because of the chemical similarity between the two cations (Delvaux et al., 2000b; White and Broadley, 2000; Zhu and Smolders, 2000). Plants can be used to extract Cs\(^+\) from contaminated sites, but these plants if eaten by animals are a potential source of Cs\(^+\) in the food chain (White et al., 2003). To increase or reduce plant Cs\(^+\) accumulation in a targeted fashion, it will be necessary to identify and characterize the precise mechanisms of Cs\(^+\) influx.

Early studies found that plant roots could accumulate Cs\(^+\) and it was proposed that K\(^+\), Rb\(^+\), and Cs\(^+\) shared the same uptake mechanisms: high affinity mechanisms at micromolar range and low affinity mechanisms in the millimolar range (Collander, 1941; Epstein and Hagen, 1952). In the past decade, significant progress has been made in identifying proteins responsible for both high and low affinity K\(^+\) uptake in *Arabidopsis* (Maser et al., 2001; Very and Sentenac, 2003; Ashley et al., 2006). The wealth of insertional mutants available in *Arabidopsis* (Krysan et al., 1999; Alonso et al., 2003) and the knowledge of the molecular identities of K\(^+\) transporters provide a platform for elucidating the Cs\(^+\) uptake mechanisms in plants (Hampton et al., 2005).

Multiple mechanisms for K\(^+\) uptake have been characterized in plants, but it is not known which of these transport mechanisms provide routes for Cs\(^+\) uptake. The *Arabidopsis* inward-rectifying K\(^+\) channel AKT1 is a major pathway for K\(^+\) uptake into plant root cells (Hirsch et al., 1998; Spalding et al., 1999; Reintanz et al., 2002; Gierth et al., 2005; Xu et al., 2006). The transport of K\(^+\) through the AKT1 channel expressed in yeast is inhibited by Cs\(^+\) (Bertl et al., 1997), but the *Arabidopsis* *akt1* mutant does not have reduced Cs\(^+\) influx or accumulation (Broadley et al., 2001). These data suggest that AKT1 is a Cs\(^+\)-sensitive K\(^+\) channel, but probably not a main Cs\(^+\) uptake pathway.

Based on theoretical models and pharmacological studies, voltage-insensitive cation channels (VICCs) are thought to mediate most Cs\(^+\) influx into root cells when plants are K\(^+\) replete (White and Broadley, 2000; Hampton et al., 2005). In *Arabidopsis*, VICCs are encoded by homologues of animal ionotropic glutamate receptors (AtGLRs) and cyclic nucleotide gated channels (AtCNGCs) (Demidchik et al., 2002; White et al., 2002). AtGLRs are suggested to be glutamate-gated non-selective cation channels that conduct Ca\(^{2+}\), K\(^+\), and Cs\(^+\) (Dennison and Spalding, 2000; Demidchik et al., 2004; Qi et al., 2006). Expression of two genes encoding AtGLRs (AtGLR1.2 and AtGLR1.3) significantly increases in roots of plants grown at high Cs\(^+\) concentrations (Hampton et al., 2004). Some AtCNGCs conduct K\(^+\), Ca\(^{2+}\), and Cs\(^+\) in heterologous expression systems (Leng et al., 1999, 2002). *Arabidopsis* mutants lacking individual AtCNGCs have either higher or lower Cs\(^+\) accumulation in shoots than the wild type (Hampton et al., 2005). It has been suggested that the absence of individual AtCNGCs might alter the expression of genes encoding other Cs\(^+\) transporters, which contributes to the higher Cs\(^+\) accumulation in some mutants (Hampton et al., 2005).

When plants are grown in media containing low K\(^+\), members of high affinity K\(^+\) transporter family KUP/HAK/KT (Maser et al., 2001) have been suggested to function as Cs\(^+\) transporters in *Arabidopsis* (White and Broadley, 2000). AtHAK5 (At4g13420) appears to be a promising candidate for a Cs\(^+\) transporter (Rubio et al., 2000), and the gene encoding AtHAK5 is the only member of this family whose expression is consistently up-regulated in K\(^+\)-starved *Arabidopsis* (Ahn et al., 2004; Shin and Schachtman, 2004; Gierth et al., 2005). Furthermore, both high affinity K\(^+\) and Cs\(^+\) uptake in *Arabidopsis* are induced by K\(^+\) starvation and inhibited by NH\(_4\)\(^+\) (Cao et al., 1993; Hampton et al., 2004; Shin and Schachtman, 2004). Although AtHAK5 has been shown to be a high affinity K\(^+\) transporter in kinetic studies, and KUP transporters are known to be inhibited by NH\(_4\)\(^+\), a physiological role for AtHAK5 has not been demonstrated in planta. In this study, several questions were asked related to the function of AtHAK5. First, it was asked whether AtHAK5 is localized to the plasma membrane and whether it is required for root elongation under low K\(^+\) conditions. Then the question of whether AtHAK5 contributes to high affinity Cs\(^+\) uptake and accumulation in *Arabidopsis* was explored. It is also reported that AtHAK5 is up-regulated in an *akt1* mutant that shows increased Cs\(^+\)-sensitive root elongation. Our data provide the basis for a proposed model regarding the role of AtHAK5 and AKT1 in plant sensitivity to environmental Cs\(^+\).

### Materials and methods

#### Plant materials and growth conditions

The atakh5 T-DNA insertion lines used in this study were obtained from the SALK collection and homozygous lines were identified as outlined on the SIGNAL website at http://signal.salk.edu/tdnaprimers.2.html (Alonso et al., 2003). *Arabidopsis* line SALK_005604 was designated as atakh5-2 to be consistent with the previous study (Gierth et al., 2005). Our analysis indicated that atakh5-2 was a null allele. *Arabidopsis* line SALK_130604 was designated as atakh5-3. The T-DNA insertion was located in exon 6 in *atahk5-2* and in exon 4 in *atahk5-3*.

*Arabidopsis* seeds of Wassilewskija (Ws2; N1601), Columbia-0 (Col-0; N1092), and *atahkl-1* (Hirsch et al., 1998) were used in this study. For *in vitro* plate assays *Arabidopsis* seeds were sterilized with 75% ethanol and 18–20 seeds were sown on the surface of...
0.8% agarose (SeaKem LE Agarose, Cambrex Bio Science). Seeds were directly sown and germinated on the different media used in the low potassium and high caesium experiments. After 2–3 d at 4 °C, plates were placed in a growth chamber with a 16/8 h day/night cycle and maintained at 22 °C. Plates were oriented vertically for measuring root elongation and for GUS staining or horizontally for examining cotyledon development, total chlorophyll and Cs⁺ content. The growth medium contained 0.5 mM H₂PO₄, 2 mM Ca(NO₃)₂, 0.75 mM MgSO₄, 74 μM Fe-EDTA, 5 μM H₂BO₃, 1 μM MnCl₂, 2 μM ZnSO₄, 0.1 μM CuSO₄, 0.075 μM NH₄MoO₄, and 1% sucrose. K⁺ and Cs⁺ concentrations were adjusted with KCl or CsCl. pH was adjusted to 5.8 with Ca(OH)₂.

Construction of plasmids and transformed plants

Transgenic plants expressing AtHAK5 under the control of the FMV promoter and AtHAK5 native promoter were generated. AtHAK5 cDNA was amplified without stop codon using AtHAK5 forward primer with PstI (CTGAGATGGAGTGGAGGAACATCAAA-TAG) and AtHAK5 reverse primer with NcoI (GGATCCTAAGTCATGGGTGATGGAGG). The FMV promoter (Sanger et al., 1990) and AtHAK5 promoter were then cloned into the pCAMBIA 1380 vector (CAMBIA, Canberra, Australia). The AtHAK5 promoter was amplified using a forward primer with XhoI (CTCGAGGATCACTAAAGTTGAGGAGGAGA) and a reverse primer with HindIII (AACGTTTCTT-TTATTTTTTTTTGTTGTTG). The FMV promoter (Sanger et al., 1990) and AtHAK5 promoter were then cloned into the pCAMBIA 1380 vector. For the localization assay, the –1365 bp to 0 AtHAK5 promoter was cloned into the pCAMBIA 1380 vector (CAMBIA, Canberra, Australia). The Agrobacterium tumefaciens binary vector with a GUS reporter gene as described in Collier et al., 1990 was used to produce K⁺-replete plants or an MS solution containing 0.5 mM K⁺ to produce K⁺-starved plants. Caesium uptake experiments were performed on intact plants as described by Hampton et al. (2004). Briefly, polycarbonate discs supporting Arabidopsis plants were transformed by the Agrobacterium tumefaciens strain GV3101. Arabidopsis plants were transformed by the Agrobacterium-mediated floral dip method (Clough and Bent, 1998).

GUS staining

To localize GUS activity, plants 5 d after germination were stained with 1 mM X-Gluc solution (Vicente-Agullo et al., 2004) and were destained as described by Malamy and Benfey (1997). The pictures were taken using a Nikon SMZ1500 dissecting microscope.

Membrane isolation and purification

To determine the subcellular localization of AtHAK5 protein, a construct expressing a fusion of the AtHAK5 protein and an N-terminal haemagglutinin (HA) epitope tag driven by the AtHAK5 promoter was created. The construct was then transformed into Arabidopsis wild type (Col-0). Microsomal fractions were isolated from transgenic plants which were grown in liquid culture on a shaker for 3 weeks and subsequently starved of K⁺ for 2 d at 22 °C with 16 h daylight at 200 μmol m⁻² s⁻¹. Microsomes were layered onto a continuous sucrose gradient (5% to 50%) and centrifuged at 35 000 rpm for 24 h at 4 °C using a Beckman SW 41 Ti high-speed rotor. Thirty-two 1 ml fractions were collected from the gradient according to the method of Barkla et al. (1999). The linearity of the sucrose gradients was measured with a refractometer. Protein (100 μg) from each fraction was precipitated in 1:1 (v/v) ethanol:acetone by incubation overnight at –20 °C and then centrifuged at 10 000 g for 20 min at 4 °C. Pellets were resuspended with SDS sample buffer and 20 μg of protein was electrophoresed on 5%/10% acrylamide SDS-PAGE gels. SDS-PAGE-separated proteins were transferred onto hydrophobic polyvinylidene difluoride (PVDF) membrane (Amersham Bioscience, Piscataway, NJ, USA). Western blot analyses were performed as previously described (Vera-Estrella et al., 2004). An antibody was used against the HA tag (COVANCE, Berkeley, USA) for detecting AtHAK5, a calreticulin antibody (Nelson et al., 1997) for ER localization, a SOS1 antibody (Qiu et al., 2003) for plasma membrane localization and a PPhase antibody (Kim et al., 1994) for tonoplast localization.

Total chlorophyll and tissue Cs⁺ content measurement

Total chlorophyll was extracted using ice-cold 80% acetone (Aron, 1949) and the absorbance at 645 nm (A₆₄₅) and 663 nm (A₆₆₃) was measured. Total chlorophyll per fresh weight tissue (μg mg⁻¹) was calculated using the equation: 20.2(A₆₄₅)+8.02(A₆₆₃) (Aron, 1949).

For tissue Cs⁺ content measurements, whole seedlings were collected and washed with water and dried at 60 °C for 3 d. After weighing, the dried tissue was extracted with 0.5 M HCl at 37 °C for 2 d (Hunt, 1982). Cs⁺ content was measured using an EDL (Electrodeless Discharge Lamp) with a Perkin Elmer Analyst 300 atomic absorption spectrometer.

Cs⁺ uptake in seedlings

Older plants than those used for plate assays were used for Cs⁺ influx experiments, Arabidopsis seeds were sown on perforated polycarbonate discs over agar containing the complete MS basal salt mix. Seven days after sowing, plants were transferred to a hydroponics system situated in a Saxcil growth cabinet (Hampton et al., 2004). In the hydroponics system, plants were supported on polycarbonate discs over 450 ml of aerated nutrient solution containing complete MS basal salt mix for 7 d. Plants were then transferred for a further 7 d to either MS solution with 2 mM K⁺ to produce K⁺-replete plants or an MS solution containing 0.5 μM K⁺ to produce K⁺-starved plants. Caesium influx experiments were performed on intact plants as described by Hampton et al. (2004).

Briefly, polycarbonate discs supporting Arabidopsis were placed over 455 ml of an aerated, 1 'single salt' solution containing 50 μM CsCl. After 20 min, plants were transferred to 450 ml of a solution containing 50 μM CsCl plus 1 mM CaCl₂ for 2 min to remove 134Cs from the root apoplast. Plant roots were blotted with tissue paper, and roots of individual plants were harvested, washed, and their Cs contents estimated from 134Cs γ-emissions determined using a gamma counter. Since the rate of Cs⁺ accumulation by plants was constant over a 20 min period (CR Hampton and PJ White, unpublished data), these measurements approximate unidirectional influxes.

Analysis of shoot Cs⁺ concentration

For the analysis of shoot Cs⁺ concentration in Ws2 and atakt1-1, seeds of Ws2, and atakt1-1 were grown on 0.8% agar in polycarbonate boxes in a growth room at 24 °C as described by Hampton et al. (2004). Agar contained 1% (w/v) sucrose and a basal salt mix at 10% of the full-strength Murashige and Skoog formulation. The effect of Cs⁺ concentration in the agar ([Cs⁺]ₐₐ₉) on [Cs⁺]shoot was determined in the presence of 2 mM K⁺. The [Cs⁺]ₐₐ₉ was raised to 0.3, 1, 10, 100, 178, 300, 562, 794, 1000, 1778, 3162, and 10 000 μM using CsCl. The radioisotope 134Cs (Radioisotope Centre Polatom, S´wierk, Poland) was used to quantify [Cs⁺]shoot and the agar spiked with 134Cs. Boxes were placed in a growth room set to 24 °C with 16 h light per day. Illumination was provided by a bank of 100 W 8 fluorescent tubes (Philips, Eindhoven, Netherlands) giving a photon flux density of 45 μmol photons m⁻² s⁻¹ at plant height. Shoots were harvested 21 d after sowing for the analysis of [Cs⁺]shoot. Six shoots of each
acquisition were bulked and their $^{134}$Cs content was determined by counting $\gamma$-emissions for 900s per sample on a well-type automatic gamma counter (Wallac 1480 Wizard, Perkin-Elmer Life Sciences, Turku, Finland).

**Yeast Cs⁺ uptake**

A previous study (Rubio *et al.*, 2000) demonstrated that changing the leucine to histidine at position 776 of AtHAK5 was required for efficient activity of AtHAK5 to complement a yeast mutant deficient in K⁺ uptake. To introduce the mutation in the AtHAK5 protein, a corresponding point mutation was introduced in the AtHAK5 cDNA with a PCR-based oligonucleotide-directed method with mutagenic primers 5'-TCAAGGCTCAGTGG-3' and 5'-CCTAGGAACTGCAATGTTGTGC-3'. The sequence of the amplified DNA was verified and then the cDNA with the point mutation was cloned into the pYES2 vector at the BamHI and XhoI sites. Also a C-terminal 5× HA tagged version of HAK5 L776H was tested for complementation. The pYES2-AtHAK5 containing the base change was transformed into the K⁺ uptake-deficient yeast cells CY162 (Δtrk1, Δtrk2) (Ko and Gaber, 1991).

For Cs⁺ uptake assays, yeast cells were grown overnight at 30 °C in arginine phosphate media (AP) supplemented with 100 mM K⁺, 2% sucrose and 2% galactose. Yeast cells with density OD₆₀₀ 1.0–1.2 were pelleted, washed twice with Milli-Q H₂O and resuspended in AP media without K⁺ and grown at 30 °C for 6 h. The yeast cells were then resuspended in uptake buffer containing 1% sucrose, 1% galactose, and 10 mM MES at pH 6.0 with Ca(OH)₂. Aliquots of yeast cells (5 ml) were taken in 10 min intervals for a 30 min period after CsCl was added. The yeast cells were immediately filtered through 0.8 µm nitrocellulose membranes and washed with 5 ml of cold 20 mM CaCl₂ solution. The filters were incubated in a 0.5 M HCl solution at 37 °C for 36 h, and Cs⁺ was determined by atomic absorption spectrometry (Perkin Elmer AAnalyst 3000). For studying the effect of NH₄⁺ on the Cs⁺ uptake, the yeast cells were pretreated with NH₄Cl for 30 min before Cs⁺ was added to the uptake buffer.

**Transcriptional profiling and quantitative PCR**

Seeds of Ws2 and atakl-1 were sown on perforated polycarbonate discs and placed over agar containing the complete MS basal salt mix. After 14 d the polycarbonate discs were transferred to discs and placed over agar containing the complete MS basal salt mix. After 14 d the polycarbonate discs were transferred to a hydroponics system situated in a Saxcil growth cabinet (Hampton mix. After 14 d the polycarbonate discs were transferred to discs and placed over agar containing the complete MS basal salt mix. Seeds of Ws2 and atakl-1 were sown on perforated polycarbonate discs and placed over agar containing the complete MS basal salt mix. After 14 d the polycarbonate discs were transferred to discs and placed over agar containing the complete MS basal salt mix.

**Results**

**AtHAK5 localizes to the plasma membrane and is required for root elongation under low K⁺ conditions**

AtHAK5 is expressed in root cells under low K⁺ conditions and contributes to high affinity K⁺ uptake (Ahn *et al.*, 2004; Shin and Schachtman, 2004; Gierth *et al.*, 2005). To test the hypothesis that AtHAK5 is localized in the plasma membrane, transgenic plants containing native promoter-driven AtHAK5 cDNA with a 3× haemagglutinin epitope tag (HA) were created. After growing these plants in a nutrient-replete medium for 3 weeks, they were starved of K⁺ for 1 d or transferred to nutrient-replete medium as a control. Membranes from the roots of these plants were separated on a continuous sucrose gradient and probed with various antibodies. Calreticulin was used as the ER marker, SOS1 as a plasma membrane marker, and the PPase as the tonoplast marker. AtHAK5 was detected in membrane fractions corresponding to the endoplasmic reticulum (ER), plasma membrane (PM), and vacuolar membrane (Fig. 1). Under potassium-replete conditions, the AtHAK5 protein was not found in the plasma membrane (Fig. 1A) whereas under potassium-deficient conditions a fraction of the total protein was in the plasma membrane fraction (Fig. 1B). Protein also remained in the ER and may have been localized to the vacuolar membrane which may be due to the presence of the HA tag at the c-terminus.

To determine the physiological significance of AtHAK5 in K⁺ nutrition, root elongation in wild-type plants and in two athak5 mutants possessing null alleles of AtHAK5 was measured. The SeaKem agarose used in these studies was previously determined to contain trace amounts of K⁺, which was estimated to be 1–3 µM. After 6 d of growth, both athak5 mutants had significantly shorter roots than those of the wild type when grown in the absence of added K⁺ or with very low levels (1 µM K⁺) of K⁺ (Fig. 2). However, with higher concentrations of K⁺ (10 µM) in the growth medium, there were no differences in root length between wild-type plants and the athak5 mutants (Fig. 2).

**Cs⁺ sensitivity of athak5**

In previous work, a yeast strain expressing AtHAK5 was shown to possess the ability to take up Cs⁺ (Rubio *et al.*, 2000). Therefore, it was determined whether athak5 mutants differed from wild-type plants in their response to rhizosphere Cs⁺. The root elongation of wild-type plants and two mutants lacking AtHAK5 was examined on vertical plates containing media with low (100 µM) and high (1.75 mM) K⁺ concentrations. In the presence of 100 µM K⁺, roots of both athak5 mutants were significantly shorter than those of wild-type plants when the Cs⁺ concentration in the
medium was greater than or equal to 200 μM (Fig. 3A).

Thus, the expression of AtHAK5 enhanced the Cs⁺ tolerance of Arabidopsis grown at low K⁺ concentrations. When grown in media containing 1.75 mM K⁺, there were no differences in root elongation between Arabidopsis lacking AtHAK5 and wild-type plants (Fig. 3B).

During the measurement of root elongation, it was noticed that the cotyledon of the knockout lines remained green whereas those of the wild type were bleached. To examine cotyledon development, seeds of Col-0 and athak5-3 were germinated on the same media used for the root elongation assay except that the plates were horizontally placed. When seeds of Col-0 and the athak5-3 mutant were germinated on plates containing 100 μM KCl, both lines had a similar appearance. However, when plants were grown on 300 μM Cs⁺, the cotyledons of wild-type plants bleached after 6 d and appeared to contain very little chlorophyll (Fig. 4A), whereas the cotyledons of athak5-3 remained green for 12 d (not shown). Total chlorophyll content of wild-type plants and two athak5 mutants was measured 6 d after germination. When grown in the absence of Cs⁺, chlorophyll content did not differ among the lines (Fig. 4B). However, the chlorophyll content of both wild-type and athak5 plants was significantly reduced in the presence of 300 μM Cs⁺, although the mutants contained significantly more chlorophyll than wild-type plants. Analysis of the plants grown
in the presence of 300 μM Cs⁺ showed that the athak5 mutants contained significantly less Cs⁺ than wild-type plants (Fig. 4C). Since the uptake of Cs⁺ has been associated with a decrease in chlorophyll concentration in Arabidopsis leaves (Le Lay et al., 2006), both these data also suggest that AtHAK5 mediates the uptake of Cs⁺.

**Effect of NH₄⁺ on AtHAK5-mediated Cs⁺ uptake in yeast and K⁺ starvation-induced AtHAK5 expression in planta**

Cs⁺ influx is inhibited by NH₄⁺ in K⁺-starved Arabidopsis (Hampton et al., 2004). To test the hypothesis that AtHAK5 mediates NH₄⁺-inhibited Cs⁺ influx, the cDNA of a modified version of AtHAK5, with enhanced transport activity (Rubio et al., 2000), was transformed into a mutant yeast strain (CY162) defective in K⁺ uptake and unable to grow on media containing low K⁺ concentrations. The yeast system is useful in these types of studies because the expression of the transporter is less subject to other control factors that may be imposed by the plant
because gene expression is induced by galactose. Expression of the modified AtHAK5 cDNA enabled this yeast mutant to grow on media containing low K⁺ concentrations, as previously reported (Rubio et al., 2000). Consistent with the observations of Rubio et al. (2000), yeast expressing AtHAK5 also displayed concentration-dependent Cs⁺ uptake (data not shown). Yeast containing the empty vector was also tested and the levels of Cs⁺ uptake were undetectable with or without added ammonium in the short-term (data not shown). Cs⁺ influx was examined at a series of NH₄⁺ concentrations. At 100 μM NH₄⁺, Cs⁺ influx was significantly higher than without added NH₄⁺, whereas concentrations of NH₄⁺ at or above 2 mM inhibited Cs⁺ influx (Fig. 5A).

These results (Fig. 5A), in combination with previous studies (Ashley et al., 2006), show that millimolar concentrations of NH₄⁺ reduce Cs⁺ and K⁺ uptake through AtHAK5. In planta the reductions in uptake through AtHAK5 by NH₄⁺ may be due to a direct blockade of the transporter or they could also be due to the reduced expression of the transporter in the presence of high NH₄⁺.

To test whether NH₄⁺ alters AtHAK5 expression, the effect of NH₄⁺ was examined at a series of concentrations on K⁺ deprivation-induced AtHAK5::GUS (β-glucuronidase) expression. The seedlings grown on medium without K⁺ showed strong GUS staining (Fig. 5B) which was not detectable in plants grown on K⁺-replete medium (data not shown). The addition of 100 μM NH₄⁺ was sufficient to almost completely suppress the K⁺ deprivation-induced AtHAK5::GUS staining. At higher NH₄⁺ concentrations, no AtHAK5::GUS staining was detected (Fig. 5B) even under K⁺-deprived conditions. The response of athak5 to NH₄⁺ was also examined on media with low (100 μM) and high (1.75 mM) K⁺ concentrations containing a range of NH₄⁺ concentrations. No differences were found between the athak5 insertions lines and the wild type in terms of root elongation, fresh weight, and growth rate (data not shown).

Cs⁺ influx to Arabidopsis wild-type and athak5 plants

The plate assays above demonstrated that AtHAK5 confers K⁺ uptake at a very low concentration range and that the AtHAK5-mediated Cs⁺ uptake is not the dominating Cs⁺ influx pathway since the athak5 mutant could not survive on media with high Cs⁺ concentration (300 μM) longer than 12 d. To determine the extent that AtHAK5 contributes to Cs⁺ uptake by more mature Arabidopsis plants in vivo, Cs⁺ influx was compared in wild type and athak5 knock-out lines grown in a hydroponics system.

Cs⁺ influx was determined with either K⁺-replete intact plants (previously grown in the presence of 2 mM K⁺) or with plants that had been starved of K⁺ (previously grown in the presence of 0.5 μM K⁺). When plants had been grown in a solution containing 2 mM K⁺, no significant difference in Cs⁺ influx in wild-type plants and athak5 mutants was observed (Table 1). Potassium deprivation increased Cs⁺ influx to wild type by 35% and, interestingly, also increased Cs⁺ influx to the athak5 mutants, but by a lesser degree (between 18–21%) (Table 1). However, there were no significant differences in Cs⁺ influx in wild-type plants and athak5 mutants under the K⁺-deprivation condition (0.5 μM).

AtHAK5 is up-regulated in atakt1, which shows Cs⁺-sensitive root elongation

AtAKT1 is the dominant K⁺ uptake channel in the root cells of Arabidopsis (Hirsch et al., 1998; Spalding et al., 1999). To test if AtAKT1 is the main Cs⁺ entry pathway in the plant, Cs⁺ influx was determined with either K⁺-replete intact plants (previously grown in the presence of 2 mM K⁺) or with plants that had been starved of K⁺.

Table 1. Cs⁺ influx into roots from a hydroponic solution containing 50 μM CsCl in Arabidopsis accessions Ws2, Col-0, akt1-1, athak5-2, and athak5-3

Plants were initially grown in hydroponic nutrient solutions containing either 2 mM or 0.5 μM K⁺. All data are expressed as mean ±SE (n=3).

<table>
<thead>
<tr>
<th>Accession</th>
<th>Cs influx (μmol g⁻¹ FW root h⁻¹)</th>
<th>[K] = 2 mM</th>
<th>[K] = 0.5 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ws2</td>
<td>0.251±0.004</td>
<td>0.313±0.010</td>
<td></td>
</tr>
<tr>
<td>ak1</td>
<td>0.275±0.032</td>
<td>0.343±0.006</td>
<td></td>
</tr>
<tr>
<td>Col-0</td>
<td>0.215±0.013</td>
<td>0.291±0.009</td>
<td></td>
</tr>
<tr>
<td>athak5-2</td>
<td>0.205±0.004</td>
<td>0.243±0.015</td>
<td></td>
</tr>
<tr>
<td>athak5-3</td>
<td>0.223±0.009</td>
<td>0.270±0.002</td>
<td></td>
</tr>
</tbody>
</table>
(previously grown in the presence of 0.5 μM K⁺). No significant difference in Cs⁺ influx in the roots of wild-type plants (Ws2) and atakt1 mutant was observed (Table 1), although Cs⁺ influx of atakt1 under both conditions was 10% higher than those of wild type. This demonstrates that AtAKT1 is not a main Cs⁺ uptake pathway. To confirm this conclusion further, the Cs⁺ content was measured in the shoots of wild type (Ws2) and the atakt1 mutant grown on agar containing various Cs⁺ concentrations. Parallel regression analysis indicated that although Cs⁺ concentration in shoots of atakt1 tended to be higher than in shoots of wild-type plants (Fig. 6), this was not significant at \( P < 0.05 \).

To test the Cs⁺ sensitivity of atakt1, the growth of atakt1 roots was examined on agar containing either 100 μM (Fig. 7A) or 1.75 mM KCl (Fig. 7B) and a range of Cs⁺ concentrations. The root length of atakt1 was less than the wild type at 100 μM K⁺ at all Cs⁺ concentrations tested (Fig. 7A). With higher concentrations of K⁺ the root elongation of akt1 was inhibited more by Cs⁺ than in the wild type between 400–800 μM Cs⁺ (Fig. 7B).

To investigate the mechanism of increased sensitivity of atakt1 to Cs⁺, the expression of the whole genome was compared between the roots of atakt1 and wild-type (Ws2) plants using the Arabidopsis ATH1 Genome Array, which represents approximately 24 000 genes. The complete set of the microarray data is available from the microarray database of Nottingham Arabidopsis Stock Centre (NASC) with experimental reference number 76 (http://affymetrix.arabidopsis.info/narrays/experiment-browse.pl). After excluding those genes with signal values lower than 50, a comparison showed that only four genes were up-regulated and four genes down-regulated at 2-fold or higher levels (Table 2). Surprisingly, the most up-regulated gene in atakt1 was AtHAK5, with a 2.86-fold increase and a \( P \)-value of 0.0178. This result was verified by quantitative PCR which indicated that AtHAK5 exhibited a 25-fold greater expression in roots of atakt1-1 compared with the wild-type plants. However, biological variation in AtHAK5 expression in both atakt1-1 and Ws2 was large, which led to a non-statistically significance difference (\( t \) test, \( P = 0.073 \)).

### Discussion

There are 13 genes encoding KT/KUP/HAK transporters in Arabidopsis. The KT/KUP/HAK family of transporters was originally identified in bacteria (Schleyer and Bakker, 1993) and later in the soil-borne fungus Schwanniomyces occidentalis (Bañuelos et al., 1995). In fungi, the KT/HAK/KUP transporters mediate high affinity K⁺ uptake, whereas in E. coli they mediate low affinity K⁺ uptake (Uozumi, 2001). Some of the plant KT/HAK/KUPs have been shown to have both a high affinity and a low affinity for Cs⁺.
Table 2. The most differentially expressed genes in wild type (Ws2) as compared to atak1-1 plants based on results from microarray experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>At4g13420</td>
<td>3.0</td>
<td>AtHAK5</td>
</tr>
<tr>
<td>At5g26130</td>
<td>2.5</td>
<td>Putative pathogenesis-related protein</td>
</tr>
<tr>
<td>At5g10040</td>
<td>2.5</td>
<td>Unknown</td>
</tr>
<tr>
<td>At3g29970</td>
<td>2.2</td>
<td>Germination protein-related</td>
</tr>
<tr>
<td>At3g27950</td>
<td>0.5</td>
<td>Putative early nodule-specific protein</td>
</tr>
<tr>
<td>At1g34310</td>
<td>0.5</td>
<td>ARF12, AUXIN RESPONSE FACTOR 12</td>
</tr>
<tr>
<td>At3g10720</td>
<td>0.5</td>
<td>Putative pectin esterase</td>
</tr>
<tr>
<td>At5g43360</td>
<td>0.5</td>
<td>Phosphate transporter</td>
</tr>
</tbody>
</table>

The fold change is the transcript abundance in atak1, relative to the wild type.

---

for K⁺ (Santa-Maria et al., 1997; Fu and Luan, 1998; Kim et al., 1998; Rubio et al., 2000; Senn et al., 2001). Characterization of two Arabidopsis mutants with defects in AtKT/HAK/KUP genes revealed that the AtKT/HAK/ KUPs are involved in cell expansion (Foreman and Dolan, 2001; Rigas et al., 2001; Elumalai et al., 2002). At least one AtKT/HAK/KUP transporter, AtHAK5, has been shown to mediate high affinity K⁺ uptake (Rubio et al., 2000; Gierth et al., 2005). The role of this transporter in planta is unclear, except for data showing that the gene encoding this transporter is up-regulated in response to potassium deprivation (Ahn et al., 2004; Armengaud et al., 2004; Gierth et al., 2005) and that the K⁺ content of a athak5 knockout was slightly decreased compared to the wild type when grown with 40 μM K⁺ (Gierth et al., 2005).

Two important unresolved questions were identified about the function of AtHAK5 in Arabidopsis that were addressed in this manuscript. In our study, biochemical methods were used to determine in which membrane AtHAK5 resides and the root elongation on low K⁺ concentrations was characterized to determine whether AtHAK5 contributes to plant growth. It has been proposed that the members of the KT/HAK/KUP family function in different subcellular locations (Senn et al., 2001). The only available data on the membrane localization of KT/HAK/KUP transporters are from a rice homologue, OsHAK10. Based on visualization of an OsHAK10-GFP fusion protein, it was concluded that OsHAK10/KUP10 was localized to the vacuolar membrane (Bañuelos et al., 2002). In this report it is shown that an AtHAK5-HA fusion protein is located in the plasma membrane which supports a role for AtHAK5 in high affinity K⁺ uptake (Gierth et al., 2005). To determine why much of AtHAK5 remained in the endoplasmic reticulum fraction, we checked if the HA tagged AtHAK5 protein was functional in yeast. The yeast strain CY162 that is defective in K⁺ uptake (Schachtman and Schroeder, 1994) was transformed with the c-terminal HA tagged cDNA containing the L776H substitution. The tagged AtHAK5 cDNA did not complement the yeast mutant on low K⁺ media (not shown). In yeast, the tagged protein was either not targeted to the plasma membrane or was unable to function because it was misfolded. However, it appears that some of the tagged protein in planta was induced by K⁺ deprivation to relocate from ER to the plasma membrane. A slight increase in uptake in the lines overexpressing the tagged protein (not shown) could also be measured. The reason for the relocation of the tagged protein under potassium-deprived conditions is unknown at this time and could be due to the increased abundance of AtHAK5 protein triggered by the expression of the endogenous gene or due to some regulatory mechanism associated with protein trafficking. The cycling or trafficking of transport proteins between membranes under conditions of stress has been shown recently for plant aquaporins (Vera-Estrella et al., 2004) and an Arabidopsis boron transporter AtBOR1 (Takano et al., 2005). Similar to what has been observed, AtBOR1 accumulates at the plasma membrane under conditions of boron deficiency but is relocated to internal membranes when boron is supplied.

Measurement of root elongation showed that this high affinity transporter plays a significant role in the growth of Arabidopsis at very low potassium concentrations. Root growth was less in athak5 mutants than in wild-type plants when 1 μM K⁺ or less was added in the growth medium. This is consistent with studies on a HAK transporter in pepper that was shown to function below concentrations of 10 μM K⁺ (Martinez-Cordero et al., 2005). These low K⁺ levels might be required to reveal a phenotype in part because of the contaminating amounts of K⁺ in agarose. SeaKem agarose was used which was found to have very low concentrations of K⁺, approximately 1–3 μM. From these results it appears that multiple mechanisms contribute to potassium uptake at low concentrations and that AtHAK5 is one mechanism of primary importance at very low potassium concentrations.

The increased expression of AtHAK5 during K⁺ starvation and the demonstration that AtHAK5 transports Cs⁺ indicate that AtHAK5 may contribute to Cs⁺ influx under K⁺-deficient conditions (Rubio et al., 2000; White and Broadley, 2000; Hampton et al., 2004). Cs⁺, however, does not affect the expression of AtHAK5, as revealed by genome-wide expression analyses (Hampton et al., 2004; Sahr et al., 2005). To determine the role AtHAK5 plays in Cs⁺ transport, the growth and Cs⁺ uptake of two null mutants compared to the wild type was studied. At low K⁺ concentrations in the growth medium with added Cs⁺, it was shown that wild-type plants expressing AtHAK5...
have greater root elongation than plants lacking AtHAK5. Although AtHAK5 is a plasma membrane Cs+ transporter in roots, knocking out AtHAK5 was not sufficient to alleviate the detrimental effect of Cs+ on root elongation, but it did slow the loss of chlorophyll as compared to wild type. The increased sensitivity to Cs+ of the plants lacking AtHAK5, as determined by root growth, may be due to the blockade by Cs+ of other K+ uptake pathways such as AKT1 (Bertl et al., 1997; Schachtman, 2000). Under these conditions AtHAK5 may remain an important pathway for K+ uptake at low concentrations when other pathways are blocked. At higher concentrations of K+ there were no differences between the wild type and the athak5 knockouts with added Cs+. Under these high K+ conditions other low affinity K+ uptake pathways could provide plants with K+ (Very and Sentenac, 2003). Based on our short-term uptake measurements there may be other Cs+ influx pathways since we only measured a 20% decrease in Cs+ influx in the athak5 knock-out which was not statistically different from the wild type (Table 1). However, in longer term experiments (Fig. 4), about half of the Cs+ accumulation could be attributed to AtHAK5 suggesting it may be a major pathway under certain conditions.

Ammonium inhibition of Cs+ influx was previously demonstrated for Arabidopsis grown under K+-deficient conditions (Hampton et al., 2004). In pepper, NH4+ competitively inhibits CaHAK1 and also reduces the expression of this transporter in roots (Martinez-Cordero et al., 2005). In Arabidopsis the inhibitory effects of NH4+ on AtHAK5 have not been characterized and could be due to the inhibition of K+ transporter function or a NH4+ induced repression of AtHAK5 expression. Therefore, we tested how the expression of AtHAK5 responds to NH4+ in planta and whether this transporter is blocked by NH4+ in yeast cells expressing AtHAK5. In yeast, it was found that 2 mM NH4+ strongly inhibited the AtHAK5-mediated Cs+ uptake and the expression of AtHAK5 decreased in planta when ammonium was added to the growth medium. In addition to the inhibition of AtHAK5-mediated Cs+ uptake at high ammonium concentrations, it was also observed that low concentrations of NH4+ (100 μM NH4+) stimulated K+ uptake. The effects of low concentrations of NH4+ on Cs+ uptake were similar to the increased uptake of K+ at low Na+ concentrations (Spalding et al., 1999). Both NH4+ and Na+ penetrate K+ transporters slowly (Bertl et al., 1997) and might bind and modulate the function of these transport proteins to facilitate Cs+ or K+ influx at low concentration. However, the effects of 100 μM NH4+ and higher concentrations on AtHAK5 activity may be of limited significance, since soils rarely contain higher than 100 μM NH4+.

Both AtHAK5 and AtAKT1 contribute to K+ uptake under low K+ conditions (Hirsch et al., 1998; Spalding et al., 1999; Gierth et al., 2005, Xu et al., 2006). Plants lacking one of these transporters will have a greater dependence on the remaining transporter. Plants lacking AtAKT1 are more sensitive to NH4+ (Spalding et al., 1999), Na+ (Qi and Spalding, 2004), and Cs+ (Fig. 2A) which could be due to the sensitivity of AtHAK5 to NH4+, Na+, and Cs+. Similarly, the shorter root phenotype of athak5 under Cs+ stress could be explained by the sensitivity of AtAKT1 to Cs+. As support for this hypothesis, AKT1-mediated K+ currents in yeast cells are strongly inhibited by Cs+ but much less by NH4+ (Bertl et al., 1997), and AtHAK5-mediated Cs+ uptake in yeast is inhibited by NH4+ (Fig. 5B) which is consistent with previous findings that Cs+ influx of Arabidopsis under K+-deficient condition was inhibited by NH4+ (Hampton et al., 2004).

**Effect of Cs on the akt1 knock-out**

Microarray analysis showed that one of the major changes in gene expression in the akt1-1 null mutant is an up-regulation of AtHAK5. The consistent increase in Cs+ influx and accumulation in akt1 may therefore be due to the up-regulated expression of AtHAK5. The increased expression of AtHAK5 in akt1-1 may also confer the Cs+-sensitive growth phenotype of this mutant. However, the increased uptake of Cs+ in akt1-1 was only 10% (not significantly different from the wild type) and, therefore, it seems unlikely that this increased Cs+ influx is the sole cause of the Cs+-sensitive phenotype of akt1-1. We speculate that it is more likely that the Cs+-sensitive growth phenotype of akt1 is due to the impaired K+ nutritional status of plants lacking the K+ transporter AKT1.

The extraction of radioaesium from contaminated soils using plants has been proposed as a phytoremediation technology. However, since it is likely that there are no specific Cs+ transporters in plants, K+ uptake pathways must be targeted to accelerate Cs+ accumulation. Cs+ in soil could be extracted with plants overexpressing certain Cs+/K+ transporters. However, although AtHAK5 contributes significantly to Cs+ uptake by roots of K+-deficient plants, it is not a promising target for phytoremediation purposes because plants overexpressing AtHAK5 had only a marginally greater rate of Cs+ influx and accumulation than wild-type plants (data not shown). Another strategy, to utilize agricultural soils contaminated by radioaesium, is to grow crops that accumulate less radioaesium by preventing Cs+ from entering plant root cells. To do so, the selectivity of the complement of Cs+/K+ transporters must be modified such that K+ can be accumulated but not Cs+. When grown in media containing low K+ concentrations, Arabidopsis lacking AtHAK5 show reduced rates of Cs+ influx (Table 1) and accumulation (Fig. 4C), but they also show reduced growth rates (Fig. 2) presumably because they lack sufficient K+. Similarly, Arabidopsis plants lacking certain AtCNGCs have reduced radioaesium
accumulation rates, but these mutants exhibit other detrimental phenotypes including reduced growth (Hampton et al., 2005). It may, therefore, be necessary to manipulate the cationic selectivity of individual transporters. Proof of concept has been demonstrated for the KAT1 K⁺-channel protein (Anderson et al., 1992; Schachtman et al., 1992), which is expressed predominantly in guard cells (Nakamura et al., 1995). Mutagenesis of the channel reduced Cs⁺ uptake which increased resistance to Cs⁺ toxicity (Ichida and Schroeder, 1996; Ichida et al., 1997, 1999).

Acknowledgements

Thanks to JK Zhu for the SOS antibody and P Rea for the PPase antibody. DPS and BJB thank NSF INT-0305414 and CONACYT No. 49735 for funding their collaboration. CRH and PJW were supported by the Biotechnology and Biological Sciences Research Council (BBSRC, UK) and the Scottish Executive Environment and Rural Affairs Department (SEERAD, UK). We thank Helen Bowen (Warwick HRI) for her contribution to the work presented here.

References


Schleyer M, Bakker EP. 1993. Nucleotide sequence and 3’-end deletion studies indicate that the K⁺-uptake protein Kup from Escherichia coli is composed of a hydrophobic core linked to a large and partially essential hydrophilic C-terminus. Journal of Bacteriology 175, 6925–6931.


