AtCHX13 Is a Plasma Membrane K+ Transporter

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Potassium (K⁺) homeostasis is essential for diverse cellular processes, although how various cation transporters collaborate to maintain a suitable K⁺ required for growth and development is poorly understood. The Arabidopsis (Arabidopsis thaliana) genome contains numerous cation:proton antiporters (CHX), which may mediate K⁺ transport; however, the vast majority of these transporters remain uncharacterized. Here, we show that AtCHX13 (At2g30240) has a role in K⁺ acquisition. AtCHX13 suppressed the sensitivity of yeast (Saccharomyces cerevisiae) mutant cells defective in K⁺ uptake. Uptake experiments using 86Rb⁺ as a tracer for K⁺ demonstrated that AtCHX13 mediated high-affinity K⁺ uptake in yeast and in plant cells with a Kₘ of 136 and 196 μM, respectively. Functional green fluorescent protein-tagged versions localized to the plasma membrane of both yeast and plant. Seedlings of null chx13 mutants were sensitive to K⁺ deficiency conditions, whereas overexpression of AtCHX13 reduced the sensitivity to K⁺ deficiency. Collectively, these results suggest that AtCHX13 mediates relatively high-affinity K⁺ uptake, although the mode of transport is unclear at present. AtCHX13 expression is induced in roots during K⁺-deficient conditions. These results indicate that one role of AtCHX13 is to promote K⁺ uptake into plants when K⁺ is limiting in the environment.

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Potassium (K⁺) plays an essential role in plant growth and development, affecting nutrition, membrane potential, enzyme function, and the homeostasis of many other ions (Schroeder et al., 1994; Lebaudy et al., 2007). K⁺ uptake by plants exhibits a biphasic kinetic. Molecular studies in combination with membrane patch-clamp and radioisotope-flux assays suggest the presence of low-affinity voltage-gated K⁺ channels, such as KAT1 (Lebaudy et al., 2007), high- and low-affinity H⁺-coupled K⁺ transporters (Rodriguez-Navarro and Rubio, 2006), as well as dual-affinity transporters like KUP1 (Fu and Luan, 1998; Kim et al., 1998). Perturbations in specific K⁺ transporters cause alterations in leaf K⁺ acquisition, root growth, and cell expansion (Gaymard et al., 1998; Rigas et al., 2001; Elumalai et al., 2002). However, many other transporters likely play important roles in plant K⁺ nutrition and homeostasis.

The complete Arabidopsis (Arabidopsis thaliana) genome has revealed additional genes encoding cation transporter homologs (Maser et al., 2001). A database search of polytopic membrane proteins identified a list of 1,120 putative open reading frames (ORFs) encoding proteins homologous with classified transporters (Bock et al., 2006). A majority of these uncharacterized ORFs are predicted to function as secondary active transporters or H⁺-coupled cotransporters (Maser et al., 2001; Ward, 2001). Among the genes encoding putative H⁺-coupled transporters in Arabidopsis, approximately 44 genes encode proteins similar to Na⁺/H⁺ exchangers. Phylogenetic analysis indicates these Arabidopsis monovalent cation:proton transporters can be divided into three families: CPA1 (NHX, eight members), NhaD (two members), and CPA2 (including CHX, 28 members; KEA, six members; Sze et al., 2004). Several CPA1 family members have been characterized extensively. For example, AtNHX1 localizes to the vacuolar membrane and can transport both Na⁺ and K⁺ and is also involved in pH regulation (Fukada-Tanaka et al., 2000; Venema et al., 2002; Pardo et al., 2006).
Members of the CPA2 family are poorly defined. The plant CHX proteins have 10 to 12 membrane-spanning domains and a carboxyl tail of variable length. Phylogenetic analysis indicates the CHX family can be separated into five subclades (Sze et al., 2004). Several members of subclade IV have been partially characterized, including AtCHX17 (At4g23700), AtCHX20 (At3g35720), AtCHX21 (At2g37910), and AtCHX23 (At1g05580; Cellier et al., 2004; Song et al., 2004; Sze et al., 2004; Hall et al., 2006). Many of the CHX transporters are preferentially or specifically expressed as pollen matures (Sze et al., 2004) and several are expressed in vegetative tissues. However, no pollen defects in atchx mutants have been described possibly due to functional redundancy. Mutant studies infer that AtCHX17, most closely related to yeast (Saccharomyces cerevisiae) KHA1, is an endomembrane transporter that plays a role in K+ homeostasis (Cellier et al., 2004; Maresova and Sychrova, 2006). AtCHX20 is also an endomembrane-localized transporter that plays a critical role in osmoregulation specifically in guard cells (Padmanaban et al., 2007). AtCHX21 resides on the plasma membrane and appears to have a role in regulating xylem Na+ concentrations and Na+ accumulation in the leaf when the plant is under salt stress (Hall et al., 2006). The function of AtCHX23 is less clear. One study concluded this transporter affects chloroplast function and plant salt tolerance, perhaps through its role in regulation of stomatal pH (Sze et al., 2004); however, another study suggests a role in pollen function (Sze et al., 2004; H. Sze, unpublished data). Heterologous expression in yeast was used to show that AtCHX17 can suppress yeast endosomal defects in K+ transport (Maresova and Sychrova, 2006), whereas AtCHX20 promotes yeast growth at low K+ when the pH is alkaline (Padmanaban et al., 2007), suggesting a role of CHX17 in K+ homeostasis and of CHX20 in K+ and pH homeostasis. However, none of the previous studies analyzed directly the transport function of CHX.

Here, we have taken multiple approaches to ascertain the function of AtCHX13, a member of subclade III (Sze et al., 2004), which has not yet been studied (to our knowledge). First, we describe the growth characteristics of yeast strains expressing the transporter and provide detailed analysis of the transport kinetics of AtCHX13 in yeast. Second, we analyze the intracellular localization of AtCHX13 and the influence of various stresses on AtCHX13 expression. Third, we show that perturbing AtCHX13 in planta causes alterations in growth and K+ (86Rb+) uptake. Collectively, these studies demonstrate that AtCHX13 localizes to the plasma membrane and has a role in mediating high-affinity K+ uptake.

RESULTS

AtCHX13 Belongs to a Distinct Clade of the CHX Family

In a family of 28 CHX members phylogenetically separated into five subclades, AtCHX13, AtCHX14, along with AtCHX26 and AtCHX27, compose subclade III (Sze et al., 2004). None of these subclade III transporters has been experimentally characterized. Interestingly, rice (Oryza sativa) CHX transporters related to subclade III have not yet been identified (Sze et al., 2004), suggesting monocots lack these transporters. To study the function of AtCHX13, reverse transcription (RT)-PCR was used to clone the AtCHX13 (At2g30240) cDNA. Previous studies revealed that AtCHX13 is highly expressed in pollen grains (Sze et al., 2004). Therefore, total RNA extracted from Arabidopsis pollen grains was used to amplify the AtCHX13 coding sequences using gene-specific primers. The AtCHX13 cDNA consists of 2,496 nucleotides and a predicted polypeptide containing 831 amino acids (EF571901). The closely related AtCHX14 cDNA comprises 2,490 nucleotides, which could encode a protein with 829 amino acids (EF571900; Supplemental Figure S1A; data not shown). The AtCHX13 cDNA and deduced protein revealed substantial similarities to other AtCHX transporters: AtCHX17 and AtCHX23 (AY926473 and AY926477, respectively; Supplemental Fig. S1A). AtCHX13 shows the highest identity (71%) and similarity (84%) with AtCHX14, confirming they are products of an ancient chromosomal segmental duplication (Sze et al., 2004). In contrast, AtCHX13 shares 31% identity and 52% similarity with AtCHX17, 29% identity and 50% similarity with AtCHX23. The deduced AtCHX13 protein contains 10 predicted transmembrane domains, (Supplemental Fig. S1B). AtCHX13 does not contain a predicted organelle-targeting sequence (data not shown) and the C-terminal region did not show substantial similarity to proteins of known functions (data not shown). Computational analysis (using the TMHMM2 program) clearly indicated AtCHX13 has similar topology with the other AtCHX transporters (Supplemental Fig. S1B).

Function of AtCHX13 in K+ Acquisition in Yeast

We detected a growth change in a yeast mutant (LMM04) expressing AtCHX13. LMM04 lacks several functional K+ transporters, including TRK1, TRK2, TOK, and the endomembrane KHA1 is very sensitive to low K+. LMM04 can only grow in medium with high levels of exogenous K+ (Maresova and Sychrova, 2005). We confirmed that the yeast strains grew normally in medium supplemented with 200 mm KCl, whereas vector controls were not able to grow in the medium with low K+ (Fig. 1A). Expression of AtKAT1, coding an inward-rectifying Arabidopsis K+ channel (Nakamura et al., 1997), suppressed the hypersensitivity of LMM04 to lower K+ (Fig. 1A). Surprisingly, yeast mutant cells expressing AtCHX13 grew on medium containing low (5–20 mm) K+ in a similar manner to AtKAT1-expressing cells suggesting that AtCHX13 has a role in acquiring K+ (Fig. 1A).

To determine whether AtCHX13 could directly alter K+ content (accumulation) in yeast, LMM04 cells expressing vector, AtCHX13, and KAT1 were grown in synthetic medium (SC) with 5 mm KCl and then...
subjected to ion analysis using inductively coupled plasma-mass spectroscopy. The resulting accumulation profile of several elements (calcium [Ca], K, magnesium [Mg], phosphorus [P], sodium [Na]) was determined in these yeast strains (Lahner et al., 2003). As shown in Figure 1B, K⁺ content in AtKAT1- and AtCHX13-expressing cells was significantly higher than the vector controls. Interestingly, P levels were also significantly higher in the AtKAT1- and AtCHX13-expressing cells, whereas the other ions were not different than the controls. Thus, an increase in yeast growth caused by AtCHX13 is accompanied by K⁺ accumulation.

To further characterize AtCHX13, we expressed the transporter in yeast strains lacking only functional TRK1 and TRK2 (Supplemental Fig. S2). AtCHX13 and AtKAT1 restored growth of mutants (trk1 trk2) at low K⁺ (1–5 mM) when compared to vector controls. For both LMM04 (Fig. 1) and trk1 trk2 (Supplemental Fig. S2), the difference between controls and both AtKAT1- and AtCHX13-expressing cells was most pronounced at pH 4.5.

**AtCHX13 Mediates High-Affinity K⁺ Uptake**

To further test AtCHX13 function in K⁺ uptake, we expressed the full-length cDNA in the yeast mutant strain LMM04 (where the phenotype was most robust) and measured K⁺ uptake using radioactive rubidium (⁸⁶Rb) as a tracer. For simplicity, we assumed that ⁸⁶Rb mimics K⁺ influx and later directly tested this assumption (see below). We analyzed ⁸⁶Rb uptake as a function of time in LMM04 vector controls and cells expressing AtCHX13 or the K⁺ channel AtKAT1. To identify experimental conditions under which the initial rate of K⁺ uptake could be measured, time course analysis of K⁺ uptake was performed at 0.02 mM (Fig. 2A) and 20 mM (Fig. 2B) external K⁺. Under both conditions, uptake was linear for at least 5 min and both AtCHX13- and AtKAT1-expressing cells had significantly higher K⁺ uptake rates compared to vector controls. Yeast cells expressing AtCHX13 had higher K⁺ uptake at low (0.02 mM) K⁺ concentrations compared to AtKAT1-expressing cells, whereas at 20 mM K⁺, AtKAT1-expressing cells demonstrated increased uptake compared to AtCHX13-expressing cells.

Kinetic analysis was performed for K⁺ uptake by yeast cells expressing AtCHX13 or AtKAT1. Initial uptake rates (within the first 5 min of K⁺ addition) were plotted as a function of external K⁺ concentration (Fig. 2C). K⁺ uptake by AtCHX13-expressing yeast was near maximum at below 1 mM, indicating high-affinity K⁺ uptake. Uptake in AtKAT1-expressing yeast did not show saturation up to 5 mM K⁺, indicating a lower affinity for K⁺ (Fig. 2C). After subtracting the K⁺ uptake rate from vector controls, reciprocal plots for AtCHX13-expressing yeast cells showed a Kₘ for K⁺ of 136.4 μM and Vₘₐₓ of 14.4 nmol h⁻¹ 10⁻⁷ cells (Fig. 2D). Given that AtKAT1-mediated K⁺ uptake did not show saturation in the substrate range measured, we were unable to estimate a Kₘ for AtKAT1.

To establish that Rb⁺ uptake by AtCHX13-expressing cells is an accurate indicator of K⁺ uptake kinetics, we measured Rb⁺ uptake at 5 min in assay solutions containing 0.02, 2.0, and 20 mM of RbCl and KCl. The uptake rates were virtually identical in the K⁺- and Rb⁺-containing uptake solutions (data not shown), an indicator that Rb⁺ flux is mimicking K⁺ uptake.

AtCHX13-mediated K⁺ uptake assays at 0.02 mM K⁺ was blocked by cesium (Cs⁺) and Na⁺ (Fig. 2E). Cs⁺ inhibited the high-affinity uptake of K⁺ significantly at concentrations above 1 mM. Similarly, K⁺ uptake in AtCHX13-expressing yeast cells was inhibited when greater than 1 mM NaCl was present (Fig. 2E). These results suggest that Na⁺ and Cs⁺ block K⁺ transport by competing for the same cation-binding site. Cs⁺ inhibits K⁺ uptake through most K⁺ channels and some other transporters (Hedrich and Schroeder, 1989; Tester, 1990). Early studies demonstrated that K⁺ uptake by plant roots is inhibited by millimolar concentrations of NaCl (for review, see Epstein, 1972).

To probe the mode of AtCHX13-mediated K⁺ uptake in yeast, we tested the effect of pH and carbonyl cyanide m-chlorophenylhydrazone (CCCP; Fig. 2F). AtCHX13-mediated uptake at 20 μM external K⁺ was higher at pH 4.3 relative to pH 7.5. Furthermore,
CCCP, a protonophore, reduced K⁺ uptake. Glc had little to no effect, indicating that energy from carbon metabolites was not limiting. These results suggest that K⁺ uptake is dependent on a pH gradient (acid outside).

**AtCHX13-GFP Localized to the Plasma Membrane**

To determine the subcellular localization, a fusion of AtCHX13 with GFP at the C terminus (AtCHX13-GFP) was constructed. The function and localization of AtCHX13-GFP was tested in yeast (Supplemental Fig. S3). The addition of GFP did not abolish the function of AtCHX13, although the fusion protein showed weaker ability to restore yeast growth than CHX13 or KAT1. In yeast, AtCHX13-GFP was predominantly localized peripherally, consistent with plasma membrane localization (Fig. 3A) and in contrast to the uniform cytoplasmic labeling in cells expressing GFP (Fig. 3B). In plant cells, AtCHX13-GFP was transiently expressed under the control of the cauliflower mosaic virus 35S promoter in tobacco (Nicotiana tabacum) and onion (Allium cepa) epidermal cells (Fig. 3, D and E). When imaged by confocal microscopy, the localization pattern of AtCHX13-GFP was markedly different from that of soluble GFP. A maximal projection image of several confocal optical sections of cells expressing soluble GFP revealed an extensive network of cytoplasmic strands, which is characteristic of cytoplasmic localization (Fig. 3C). On the other hand, AtCHX13-GFP-expressing cells displayed intense fluorescence confined to the cell periphery. Maximal projection images of a series of confocal optical sections revealed uniform GFP labeling along the cell surface and the absence of any fluorescent strands. These features are strongly indicative of the plasma membrane localization of AtCHX13 (Fig. 3, D and E). To exclude the possibility that AtCHX13-GFP localized to the cell wall, onion cells were plasmolyzed by treatment with 1 M Suc. The fluorescence in the plasmolyzed cells detached from the cell wall, confirming that the AtCHX13-GFP signal was not from the cell wall or the apoplastic space between the cells (Fig. 3E). Our plasmolysis experiment also allowed us to distinguish between plasma membrane and tonoplast labeling. In fully differen...
ated cells, such as the onion inner epidermal cells used here, a large central vacuole typically pervades a large portion of the cell leaving only a thin layer of cytoplasm compressed toward the edge of the cell. This often makes it difficult to distinguish whether GFP signal originates from the plasma membrane, tonoplast, or cytoplasm. We therefore imaged regions of the cell where we could readily visualize the cytoplasm. In a plasmolyzed onion cell expressing 35S::GFP, a slightly thickened region of the cytoplasm could be seen using bright-field optics. A corresponding fluorescence image showed that GFP signal was
uniformly distributed within this cytoplasmic domain (Fig. 3D). In contrast, AtCHX13-GFP-expressing cells showed intense fluorescence originating from a thin layer along the outer edge of the cytoplasm, confirming plasma membrane, but not tonoplast, localization (Fig. 3E).

AtCHX13 Is Expressed in Seedlings and in Response to K⁺ Fluctuations

Microarray and preliminary promoter-driven GUS results suggest that AtCHX13, like 13 other AtCHX genes, is expressed during pollen development (https://www.genevestigator.ethz.ch; Sze et al., 2004). To more systematically characterize AtCHX13 expression, we monitored expression using RT-PCR and AtCHX13 promoter-driven GUS activity. Using northern analysis, AtCHX13 showed little or no expression in seedlings given a variety of ionic stresses, including increased and decreased levels of Na⁺, K⁺, and Ca²⁺. Using RT-PCR, we confirmed microarray results that AtCHX13 was expressed weakly in several plant tissues, including roots (Fig. 4A). Using RT-PCR to determine expression in seedlings, we showed that AtCHX13 expression could be detected 1 d after plants were depleted of K⁺ (Fig. 4B). When plants were exposed to higher levels of K⁺ (100 mM), AtCHX13 expression could be detected in seedlings after 1 d, but expression appeared to dissipate at day 2 and beyond.

To verify the expression results, promoter-driven GUS activity was determined. Due to low expression levels in vegetative tissues, AtCHX13::GUS activity could only be reproducibly measured in pollen when plants were grown on standard medium (J. Zhao and K. Hirschi, unpublished data; Sze et al., 2004). When K⁺ was limiting, AtCHX13::GUS root expression increased compared to lines grown in normal medium (10 mM K⁺; Fig. 4C). AtCHX13 promoter-GUS expression and RT-PCR data indicated AtCHX13 expression is enhanced in response to decreased K⁺ conditions.

Altered Expression of AtCHX13 in Plants

To study the function of AtCHX13 in plants, we obtained two independent T-DNA insertion lines for atchx13 (Fig. 5A). Homozygous lines were identified and the positions of the T-DNA insertions were confirmed by sequencing (Fig. 5A). Using RT-PCR, no AtCHX13 transcripts could be reverse transcribed from total RNA isolated from the mutant flowers (Fig. 5B). The atchx13 lines showed no obvious morphological or growth defects compared to control plants under standard growth conditions (data not shown).

Additional attempts to alter AtCHX13 expression were made by constitutive expression of the ORF in Arabidopsis (in the Columbia [Col-0] background). In the 35S::AtCHX13 lines, PCR analysis showed augmented basal level of native gene expression (Fig. 5C). Despite the increased amount of AtCHX13 transcripts in these lines, no change in plant growth or development was detected when the plants were grown in standard conditions (data not shown).

Phenotypes of atchx13 Lines

The maintenance of K⁺ homeostasis is important for stress responses (Marschner, 1995). The observations that the expression of AtCHX13 was induced in seedlings grown under low K⁺ conditions and that AtCHX13 expression in yeast mediated K⁺ transport suggested that AtCHX13 may function in K⁺ uptake during K⁺ deficiency. Under standard growth conditions (0.5× Murashige and Skoog medium containing about 10 mM K⁺) atchx13 mutants and 35S::AtCHX13 seedlings grew similarly to control lines (Fig. 6A; Supplemental Fig. S4A). However, when these lines were germinated and grown on low K⁺ medium at varying pH values, atchx13 lines displayed obvious

![Figure 4](https://www.plantphysiol.org/)

**Figure 4.** AtCHX13 expression. A. Vegetative and reproductive organs. Total RNA was isolated from roots (Rt), leaves (Le), and stems (St; from 3-week-old plants), flowers (Fl), and siliques (Si; from 4-week-old plants), reverse transcribed, and subjected to RT-PCR. ACTIN1 was used as internal standard. Images shown represent results obtained from three independent amplification reactions. B. RT-PCR analysis of AtCHX13 expression when seedlings were grown on high and low K⁺. RT-PCR analysis of AtCHX13 expression. One-week-old Col-0 seedlings were transferred to 0.5× Murashige and Skoog medium supplemented with 100 mM KCl or to modified 0.5× Murashige and Skoog with low levels of K⁺ (the agar provides the medium with approximately 0.4 mM K⁺). The seedlings were collected at specified time points as indicated for RNA isolation. The samples were then analyzed as described in A. ACTIN1 was used as internal control. C. AtCHX13::GUS lines were grown on 0.5× Murashige and Skoog plate for 9 d and then transferred to 0.5× Murashige and Skoog containing either no K⁺ or with 10 mM K for 2 d prior to GUS staining. [K⁺] was removed (0 mM K) by replacing KNO₃, KH₂PO₄, and KI with BTP-NO₃, NaH₂PO₄, and NaI, respectively.

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growth phenotypes when compared to controls. Specifically, the atchx13 lines grew more slowly and appeared to have more leaf chlorosis and bleaching (Fig. 6, B–D; Supplemental Fig. S4A). The sensitivity of atchx13 lines to limited K⁺ is obvious at low pH (4.3); however, this sensitivity is not pH dependent as some sensitivity is also observed at pH 7.5. Measurements of total chlorophyll and fresh-weight analysis in these growth conditions (Fig. 6, A–F).

86Rb uptake kinetics of 35S::AtCHX13, and wild-type (Col-0) seedlings were directly analyzed by measuring time- and concentration-dependent 86Rb⁺ uptake into roots of K⁺-starved plants. The initial uptake of 86Rb⁺ was linear in all lines up to 20 min, with uptake being dramatically higher for the 35S::AtCHX13-expressing lines than control mutant lines (Fig. 7). Concentration-dependent 86Rb⁺ uptake was subsequently measured at 10-min intervals at different external K⁺ concentrations. Uptake rates for 86Rb⁺ revealed a significant difference between vector control and 35S::AtCHX13 lines at low K⁺ concentrations (0.02 mM K⁺; Fig. 7, A and B). The atchx13 mutants showed uptake at 0.02 mM K⁺ lower than controls (data not shown). At high K⁺ concentrations (20 mM), the difference in uptake kinetics could not be resolved between vector control and the lines with enhanced AtCHX13 expression (Fig. 7B). The influx at 20 mM K⁺ was similar in all lines tested. In atchx13 lines, 86Rb⁺ uptake was slightly reduced compared to controls when K⁺ was limiting (0.02 mM K⁺), but was similar to controls at higher K⁺ levels (Supplemental Fig. S5).

We measured 86Rb⁺ uptake in 35S::AtCHX13 lines under different pH conditions and in the presence of the protonophore CCCP (Fig. 7E). In a manner similar to the yeast cells expressing AtCHX13, optimal uptake was measured at acidic pH conditions and the protonophore inhibited K⁺ uptake in these whole-plant experiments. Results suggest K⁺ uptake is dependent on a pH or electrochemical gradient.

**DISCUSSION**

The ability of plants to grow and develop under myriad nutrient and environmental conditions appears to depend on multiple transporters to sustain K⁺ homeostasis. In spite of numerous pathways used by plants for K⁺ uptake, including channels and cotransporters, we demonstrate here that a distinct member of the CHX gene family has a role in supporting growth under K⁺-deficient conditions.

**AtCHX13 Function**

Unlike CHX20 and CHX17, which have been localized to endomembranes (Maresova and Sychorva, 2006; Padmanaban et al., 2007) in plants and in yeast, we demonstrate here that AtCHX13 localizes to both yeast and plant plasma membranes. Several lines of evidence support its role in facilitating K⁺ uptake at the plasma membrane: (1) AtCHX13 can suppress yeast mutants defective in K⁺ uptake (Figs. 1 and 3; Supplemental Fig. S2); (2) AtCHX13-expressing yeast showed increased K⁺ (86Rb⁺) uptake and K⁺ content compared to vector controls (Fig. 2); (3) in Arabidopsis, AtCHX13 expression was responsive to the K⁺-deficient conditions (Fig. 4); (4) T-DNA insertion mutants defective in AtCHX13 expression demonstrate perturbed growth in response to K⁺ starvation;
and (5) plants with increased AtCHX13 expression showed increased rates of $^{86}$Rb uptake relative to plants harboring vector only (Fig. 7).

K$^+$ uptake in plant roots displays biphasic kinetics (Epstein, 1972; Marschner, 1995). High-affinity uptake occurs in the low [K$^+$] range (1–200 $\mu$M), whereas low-affinity uptake takes place in the high [K$^+$] range (1–250 mM). The high-affinity system, with the tight coupling of the proton motive force, can function in transporting K$^+$ against its electrochemical potential gradient. K$^+$ ($^{86}$Rb) uptake into yeast ($K_m = 136.4$ $\mu$M) or plants ($K_m = 196$ $\mu$M) with AtCHX13 expression suggest a high-affinity K$^+$ transporter because uptake above the background was increased at 20 $\mu$M K$^+$, but was not altered at 20 mM K$^+$. The $K_m$ determined for K$^+$ uptake mediated by AtCHX13 in yeast and plants is similar. Minor differences may be a function of membrane potential, pH, or the presence/absence of modifying proteins (Hedrich and Schroeder, 1989; Kochian et al., 1993; Fu and Luan, 1998). In both expression systems, AtCHX13 appears to mediate high-affinity K$^+$ uptake.

At present, the mode of K$^+$ uptake is unclear. AtCHXs characterized so far are thought to mediate K$^+$ uptake into endomembrane compartments via a cation/proton exchange mechanism (Cellier et al., 2004; Padmanaban et al., 2007). To our knowledge, AtCHX13 is the first characterized plasma membrane-localized CHX that facilitates uptake of K$^+$ with high affinity. AtCHX21 has also been localized to the PM; however, the transport activity of this protein has not been tested (Hall et al., 2006). AtCHX3-mediated K$^+$ uptake is enhanced by a proton electrochemical gradient (acidic outside; Figs. 1, 2, and 7), suggesting the protein may operate as a K$^+$/H$^+$ symporter instead of an antiporter (K$^+$ influx for H$^+$ efflux). However, we were unable to measure currents in oocytes expressing either AtCHX13 or the closely related AtCHX14 (data not shown) in initial tests. The possibility of AtCHX13 behaving like an influx channel is also considered. The sequence of CHX shares little homology to known K$^+$ channels, and specific inhibitors of voltage-gated K$^+$ channels, such as TEA and Ba$^{2+}$, inhibit AtKAT1 function in yeast cells (Anderson et al., 1992), but did not perturb yeast growth in AtCHX13-expressing cells (data not shown). Yet AtCHX13, like KAT1, restored growth to yeast mutants defective in K$^+$ uptake pathways. AtCHX13 mediated relatively high-affinity K$^+$ uptake, similar to some KUP/HAK/KT K$^+$ transporters (Very and Sentenac, 2003). It is possible that AtCHX13 protein has multiple catalytic modes at
different K⁺ concentrations and varied pH, like another member of the CPA2 family from bacteria, Kef C (Booth et al., 1996). Future studies of a purified and active CHX in a reconstituted system are needed to establish the mode of transport.

Role of AtCHX13 in K⁺ Nutrition

*atchx13* seedlings displayed growth inhibition when grown under K⁺-deficient conditions (pH 4.3; Fig. 6; Supplemental Fig. S6). Our seedling expression data confirmed expression of the transporter during K⁺-limiting conditions. These results support the idea that AtCHX13 is important for K⁺ acquisition when this nutrient is limited. Consistent with this idea, plants overexpressing *AtCHX13* are more tolerant to low K⁺ than wild-type controls.

*AtCHX13* is preferentially expressed in mature pollen grains and in elongating pollen tubes (Sze et al., 2004), but also in the roots. However, in vitro pollen tube elongation of *atchx13* lines was similar to wild type. Preliminary studies with *atchx13/14* double mutants also failed to reveal obvious differences in pollen tube growth (data not shown). Yet *atchx13* seedlings grew poorly on K⁺-depleted medium compared to wild type and displayed reduced K⁺ uptake at low K⁺ concentrations (Supplemental Fig. S5). Wild-type roots showed little or no detectable *AtCHX13* promoter::GUS activity in roots, although K⁺ starvation induced an increase in GUS activity particularly in the root tip (Fig. 4C). Expression of *AtCHX13* in wild-type roots as shown by RT-PCR (Fig. 5B) is confirmed by *AtCHX13* transcript detected in lateral root cap cells by ATH1 microarray (Birnbaum et al., 2003). Assuming *AtCHX13* function is limited to the root tip of the vegetative plant body, these results would suggest that *AtCHX13* could play a signaling role critical for transmitting the low K⁺ signal to the rest of the plant. The root tip (including the root cap cells) can serve as a sensor of the environment (Svistoonoff et al., 2007), and *AtCHX13* activity may be involved in transducing environmental signals. Mutant seedlings unable to communicate the K⁺ levels in the milieu may not elicit appropriate responses and thus seedlings fail to tolerate the K⁺ nutrient stress. As root tip cells express other members of the CHX family (Birnbaum et al., 2003; Padmanaban et al., 2007), it is intriguing to speculate the high-affinity K⁺ uptake and the plasma membrane location of AtCHX13 give root cells the specific features needed to sense and respond to environmental stresses.
SUMMARY

AtCHX13 is the first functionally characterized CHX transporter to be localized to the plasma membrane. Using both yeast and plant expression systems, AtCHX13 facilitated high-affinity K⁺ uptake when K⁺ was limiting.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) Col-0 seeds were used as wild type (control). atchx13 T-DNA insertion mutant lines in the Col-0 ecotype were obtained by screening SALK and SAIL lines (http://signal.salk.edu). Seeds from these lines were obtained from the Arabidopsis Biological Resource Center. Two atchx13 homozygous lines were screened from T-DNA insertion lines, SALK_095075 (atchx13-1) and SALK_023605 (atchx13-2). In both lines, the T-DNA was inserted in the last exon of the AtCHX13 ORF. PCR was used to confirm the lines contained the proper T-DNA insertion by using T-DNA primers and AtCHX13 gene-specific primers, 5'-ATGGAGGCTTCCAGATGGTGCACCTAGT-3' (for SALK_023605 lines) or 5'-GAGTTTACCGAGATCAATGGTAGTCTT-3' (for SALK_095075 lines).

Arabidopsis seeds were surface sterilized and sown on 0.5% Murashige and Skoog plates. K⁺-deficient 0.5 Murashige and Skoog medium and K⁺-containing plates (50 and 100 m M K⁺). The resulting cDNA fragment was cloned into yeast (Saccharomyces cerevisiae) expression vector pUGpd, using the GPD promoter to express the transporter to be localized to the plasma membrane.

Analyzing Growth, Biomass, and Chlorophyll Content

Arabidopsis Col-0 seeds were used as wild type. Col-0, atchx13-1, atchx13-2, and SALK_095075 atchx13-1 seeds were germinated on normal 0.5% Murashige and Skoog medium and incubated in a growth chamber at 16-h-light/8-h-dark cycles at 22 °C. Plant phenotypes were recorded after 3 to 4 weeks. Total chlorophyll content was extracted from seedlings with 90% acetone, and chlorophyll a/b was measured as described previously (Porra et al., 1989). Seedlings were removed and blotted dry with tissue prior to determining biomass. Biomass of plant seedlings was expressed as fresh weight per 20 seedlings.

Plasmid Construction

To isolate AtCHX13 cDNA, total RNA was isolated from mature pollen grains of Arabidopsis Col-0 plants by the guanidine/acid-phenol method (Sze et al., 2004). The first-strand cDNA was synthesized using reverse transcriptase. Primers X13Cf (5'-ATGGAGGCTTCCAGATGGTGCACCTAGT-3') and reverse primer P2, 5'-GACGGCTGGTATGCCAGGATGTTG-3' were used to amplify the AtCHX13 cDNA by PCR (25 cycles, 94°C 30 s, 55°C 30 s, and 72°C 90 s). The forward and reverse primers contain attB1 and attB2 sequences for Gateway recombination cloning. Gel-purified PCR products were recombined with pDONR221 using BP clonase, according to the manufacturer’s instructions (Invitrogen). Resulting clones were sequenced using forward and reverse M13 primers and gene-specific primers X13s1 (5'-GTGGTGAGGAAGGGGTTGACGTA-3') and reverse primer P1, 5'-GACGGCTGGTATGCCAGGATGTTG-3'. The cloned sequence containing the ORF was named as entry clone pECHX13. The resulting cDNA fragment was cloned into yeast (Saccharomyces cerevisiae) expression vector pUGpd, using the GPD promoter to express the GFP-tagged AtCHX13, and was made using PCR. The AtCHX13 cDNA was amplified by using the following primers for PCR: 5’-GGTTACGACTAGT-GAGTTTACCGAGATCAATG-3’ (attB1 and attB2 sequences for Gateway recombination cloning. Gel-purified weight per 20 seedlings.

Determining biomass. Biomass of plant seedlings was expressed as fresh weight per 20 seedlings.

Gene Expression Analysis by Northern Blot, RT-PCR, and Promoter-Driven GUS Activity

For RNA analysis, Col-0 seeds were germinated in soil (Metro-Mix 360) and grown under continuous light. Two-week-old seedlings were washed with water and then floated in the metal-containing medium for 16 h. RNA was extracted from tissues using an RNeasy kit (Qiagen). Ten micrograms of total RNA was used for northern-blot analysis as described previously. For RT-PCR analysis, 4-week-old Col-0 seedlings were used. For K⁺ stress treatment, Col-0 seedlings were grown on 0.5% Murashige and Skoog for 1 week under 16-h-light/8-h-dark cycles, then transferred to K⁺-deficient plates (0 or 2 m M K⁺) and K⁺-containing plates (50 m M K⁺ and 100 m M K⁺). Whole seedlings were harvested daily to monitor gene expression. Total RNA was isolated from root, leaf, flower, and silique tissues of Arabidopsis using an RNeasy kit (Qiagen). First-strand cDNA was synthesized with a Superscript II reverse transcriptase kit using oligo(dT)_12-18 as primer (Invitrogen), according to the manufacturer’s instructions. PCR was performed with the following program: 94°C for 2 min to denature DNA, followed by 30 cycles of 94°C (30 s), 58°C (30 s), and 72°C (60 s), followed by 72°C for 10-min extension. ACTIN1 was used as control. ACTIN1: forward primer, 5’-GTGTCGACTCTGGAGATGTTG-3’ and reverse primer, 5’-CGGGACATCGAGAAGGTG-3’. Gene Expression Analysis by Northern Blot, RT-PCR, and Promoter-Driven GUS Activity

Constructs for Plant Expression

Plastid transient expression constructs were generated by PCR amplification using the yeast AtCHX13-GFP plasmid DNA as a template. AtCHX13-GFP was amplified with a forward primer, 5’-CGGGACCATCGAGAAGGTG-3’ and a reverse primer, 5’-CGGGACATCGAGAAGGTG-3’. The PCR product was sequenced and ligated into the yeast pUGpd-GFP vector at Spel and NolI sites. The resulting constructs contained the AtCHX13 reading frame fused to the 5’ of the GFP ORF. The clone was confirmed by sequencing and introduced into yeast.

Particle Bombardment of Plant Cells and Confocal Microscopy

Five micrograms of plasmid DNA containing the 35S::GFP, 35S::AtCHX13-GFP was mixed with 20 μl of an aqueous suspension containing 1.6-μm gold particles. The gold DNA suspensions were vortexed in the presence of CaCl₂ and spermidine and incubated on ice. After centrifugation, the plasmid-coated gold particles were washed and resuspended in ethanol. The gold was spread onto plastic carrier discs for biolistic bombardment of tobacco and onion epidermal cells using a Bio-Rad 1000/HE particle delivery system. After 12 to 15 h, GFP from the epidermal cells of tobacco leaves and onion were imaged with a Leica TCS SP2 AOBs laser confocal scanning microscope (Leica Microsystems). GFP was excited with the 488-nm line of the argon laser and emission was detected at 520 nm.
Transport K⁺ (⁸⁶Rb) Uptake into Plant

To characterize the K⁺-transport activities of AtCHX13, ⁸⁶Rb tracer experiment was performed (Wu et al., 1996; Rigas et al., 2001). The ⁸⁶RbCl salt with a specific activity of 7.85 mCi/mg was purchased from Perkin-Elmer (Life and Analytical Sciences). Plants were grown hydroponically for 7 d in sterile flasks on B5 medium, 2% Suc, and 0.05% MES, pH 5.7. Seedlings were harvested and preincubated for 2 d in K⁺-free Murashige and Skoog medium. ⁸⁶Rb⁺ uptake assays were conducted in K⁺-free Murashige and Skoog medium buffered with 5 mM MES to pH 5.0, supplemented with 0.5 μCi ⁸⁶Rb and various concentrations of KCl (0.02 or 20 mM). Samples (about six seedlings) were taken at 10, 20, 30, 60, 80, 100, and 120 min, respectively, washed with 3 × 10⁻³ mL of K⁺-free Murashige and Skoog medium, and then placed for 20 min in 40 mL of ice-cold K⁺-free Murashige and Skoog medium. Subsequently, the seedlings were blotted on filter paper, weighed, and activity measured using a scintillation counter.

For kinetic analysis, seedlings were incubated in various external K⁺ concentrations (10, 20, 50, 100, 200, 300, 500 μM) for 10 min. Different pH values of uptake buffer containing 2% Suc were obtained by adjusting MES- Tris combinations. Uptake lasted for 10 min and then samples were washed as described above for radioactivity counting.

Yeast Strains, Medium, and Growth Conditions

Yeast strains LMM04 (ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mtl0 ena1::HIS3-ena4 nha1::LEU2 trk1::LEU2 trk2::HIS3 kha1:: kanMX:tok1a) and 59m15 (trk1::LEU2 trk2::HIS3) were used to characterize AtCHX13 (Madrid et al., 1998; Maroseva and Syčrová, 2005). LMM04 or 59m15 cells expressing AtCHX13, AtKAT1, or empty vector were grown in SC-Ura medium containing 100 mM KCl at 30°C overnight. Five-fold serial dilutions were prepared from the saturated yeast cultures and 3-Dilutions were made on SC-Ura plates with or without various concentrations of KCl under different pH conditions (Maroseva and Syčrová, 2005). The pH of SC medium was adjusted with 5 mM MES for pH 4.5 and 6.0 and 5 mM Tris-Cl buffer for pH 7.5. Plates were incubated at 30°C for 4 d.

Yeast cation analysis was performed as described previously (Eide et al., 2005; Mei et al., 2007). Yeast LMM04 cells expressing AtCHX13, AtKAT1, and empty vector were grown overnight in 5 mL SC-Ura medium supplemented with 100 mM KCl. After rinsing and diluting with K⁺-free SC-Ura medium, 100 μL of yeast cultures were incubated in 5 mL fresh SC-Ura medium supplemented with 5 mM K⁺ and grown at 30°C overnight. About 2.5 mL of the yeast cultures were collected by vacuum filtration using isopore membrane filters (1.2-μm pore size; Fisher Scientific). Cells were washed three times with 1 mL of 0.1 M ethylenediaminetetraacetic acid disodium salt solution, pH 8.0, followed by three washes with 1 mL of deionized water. The filters were dried in a 70°C oven for 4 h for inductively coupled plasma-mass spectrometry analysis (Lahner et al., 2003).

For observation of AtCHX13-GFP signals, the yeast harboring the AtCHX13-GFP fusion were grown in SC-His-Leu medium overnight. The yeast cells were viewed under laser-scanning confocal microscope (Olympus Fluoview, FV 500; Olympus Optical). An argon laser beam was used for excitation at 488 nm and GFP visualization with emission at 525 nm.

Transport in Yeast

To characterize the K⁺ transport activities of AtCHX13 in yeast, K⁺ uptake using ⁸⁶Rb as tracer was performed (Fu and Luan, 1998; Rubino et al., 1999). LMM04 yeast cells expressing vector, AtCHX13, and AtKAT1 were grown to midlog phase in the Ura-deficient SC medium containing 50 mM KCl. Cells were harvested and washed and K⁺ starved in low-salt Arg-P medium (Rodriguez-Navarro and Ramos, 1984) for 5 h before the uptake assay. For the time course study, 5 × 10⁶ cells were added to 50 mL of the uptake solution (pH 4.5) containing 0.5 μCi ⁸⁶Rb/mL and 0.02 and 20 mM KCl in culture flasks and gently shaken. A fraction of the cells was harvested at the indicated times. For kinetic studies, 10² cells were used in each uptake sample in a 1-mL solution containing 0.5 μCi ⁸⁶Rb and various concentrations of KCl. The ⁸⁶Rb radioactivity taken up into the cells was measured by a liquid scintillation counter. For comparison whether other conditions reduced K⁺ uptake, 0.0, 0.1, 0.2, 0.5, 1, 5, 10, 20, or 100 mM NaCl or CoCl₂ was included in the uptake assay reaction containing 0.02 μM KCl. For comparison of K⁺ and K⁺ affinity, ⁸⁶Rb uptake in the standard reaction plus nonradioactive K⁺ or Rb⁺ at 0.02, 2.0, and 20 mM was conducted. The different pH of the uptake medium containing 10 mM Glc was adjusted using 5 mM MES or Tris. Effect of Glc on AtCHX13-mediated K⁺ uptake was tested by comparison of uptake in 10 mM Glc with that after depletion of 10 mM Glc from uptake buffer under pH 4.5. Protonophore CCCP was added to the yeast cells in uptake medium (pH 4.5) 5 min prior to K⁺ and ⁸⁶Rb tracer addition. After 5 min of uptake, samples were filtered and washed with 10 mM RbCl for scintillation counting. All uptake experiments were performed in duplicate with three independent replicates (unless otherwise noted).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EF571901.

Supplemental Data

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

Supplemental Figure S1. AtCHX13 is similar to other Arabidopsis CHX transporters.

Supplemental Figure S2. Functional expression of AtCHX3 in yeast mutant 59m15.

Supplemental Figure S3. GFP-tagged AtCHX13 functions in yeast suppression assays.

Supplemental Figure S4. Phenotyping attch3 mutants and overexpression transgenic lines.

Supplemental Figure S5. K⁺ (⁸⁶Rb⁺) uptake in seedlings of attch3 mutants.

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