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Characterization of *AtCHX17*, a member of the cation/H^+ exchangers, CHX family, from *Arabidopsis thaliana* suggests a role in K^+ homeostasis

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Summary

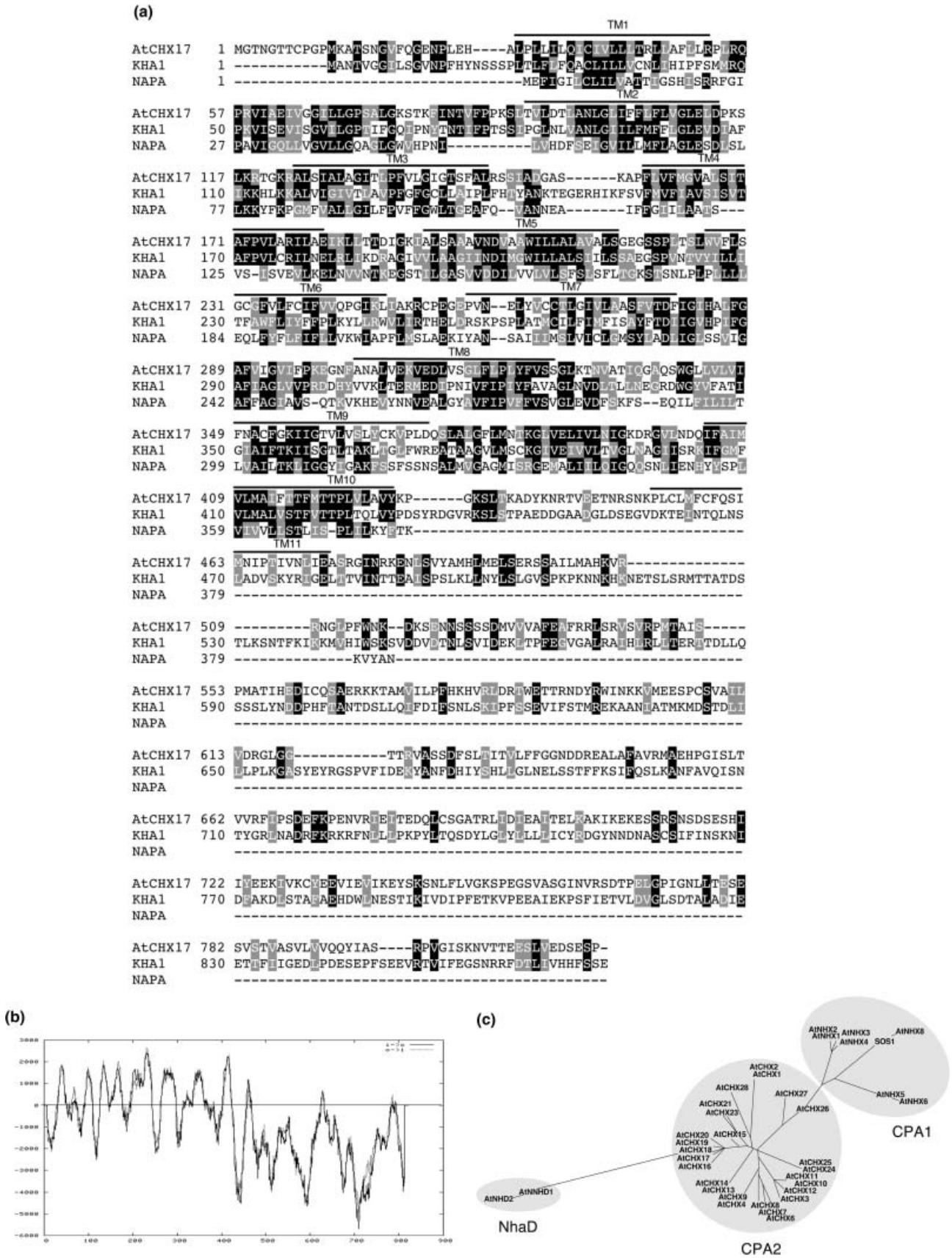
The *Arabidopsis* genome contains many sequences annotated as encoding H^+-coupled cotransporters. Among those are the members of the cation:proton antiporter-2 (CPA2) family (or CHX family), predicted to encode Na^+,K^+/H^+ antiporters. *AtCHX17*, a member of the CPA2 family, was selected for expression studies, and phenotypic analysis of knockout mutants was performed. *AtCHX17* expression was only detected in roots. The gene was strongly induced by salt stress, potassium starvation, abscisic acid (ABA) and external acidic pH. Using the β-glucuronidase reporter gene strategy and *in situ* RT-PCR experiments, we have found that *AtCHX17* was expressed preferentially in epidermal and cortical cells of the mature root zones. Knockout mutants accumulated less K^+ in roots in response to salt stress and potassium starvation compared with the wild type. These data support the hypothesis that AtCHX17 is involved in K^+ acquisition and homeostasis.

Keywords: *Arabidopsis*, salt stress, K^+-starvation, Na^+,K^+/H^+ antiporters, K^+ transport, CHX transporters.

Introduction

Plant growth and development depend on the uptake and sorting of mineral nutrients and metabolites to specific organs, cell types and intracellular compartments. Extrusion and compartmentalization of toxic ions are also required to sustain plant survival under stress conditions. Ion homeostasis relies on how plants coordinate and adapt expression and activity of nutrient transporters. The *Arabidopsis* genome contains 800 (identified by The *Arabidopsis* Genome Initiative, 2000) to 1096 (Maathuis *et al.*, 2003) predicted membrane-bound transporters. Functional ascription was reported for a range of transporters but most of them remain uncharacterized. A bulk of these unknown transporters is predicted to function as secondary active transporters or H^+-coupled cotransporters (Mäser *et al.*, 2001; Ward, 2001).

Among the genes encoding putative H^+-coupled transporters in *Arabidopsis*, 38 genes encode proteins homolog to Na^+/H^+ exchangers described in mammals or microorganisms (Mäser *et al.*, 2001). Phylogenic analysis indicated that these antiporters fall into three families: the monovalent cation:proton antiporter-1 (CPA1) family (eight members), the monovalent cation:proton antiporter-2 (CPA2) family also referred to as the CHX family (28 members) and the NhaD family (two members) (Mäser *et al.*, 2001; Saier *et al.*, 1999). In plants, Na^+/H^+ antiporters are predicted to be involved in mechanisms that are likely to be critical determinants for salt tolerance, such as salt exclusion at the plasma membrane of the root cells and/or salt compartmentalization at the tonoplast of the leaf cell vacuoles (Apse *et al.*, 1999; Shi *et al.*, 2003). Several members of the CPA1 family have been functionally characterized in yeast where they display Na^+/H^+ antiporter activity (Aharon *et al.*, 2003; Darley *et al.*, 2000; Gaxiola *et al.*, 1999; Shi *et al.*, 2002; Yokoi *et al.*, 2002). As expected, depending on their subcellular localization, they have been shown to exclude Na^+ at the plasma membrane (Qiu *et al.*, 2002, 2003) or to compartmentalize Na^+ into the vacuole (Apse *et al.*, 1999, 2003). However, AtNHX1, a member of the CPA1 family is also able to mediate K^+ transport (Apse *et al.*, 2003; Venema *et al.*, 2002; Zhang and Blumwald, 2001). CPA1 members localized at the tonoplast may also be involved in the regulation of vacuolar pH as it was predicted for the AtNHX1 homolog of morning glory (*Ipomoema nil*) (Yamaguchi *et al.*, 2001).


(a)
TM1
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KHA1
NAPA
(b)
(c)
AtNHX2
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AtNHX4
AtNHX8
SOS1
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CPA1
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AtCHX9
AtCHX4
AtCHX8
AtCHX7
AtCHX6
AtNHD2
AtNHD1
NhaD
CPA2

Thus, besides having a role in Na^+ transport, members of the CPA1 family may be involved in the regulation of K^+ and pH homeostasis. So far, no plant member of the CPA2 or NHAD family has been functionally characterized.

We present the characterization of the *AtCHX17* gene belonging to the CPA2 family. We investigated the function of *AtCHX17 in planta* by expression studies and functional characterization of knockout mutants. We have analyzed expression of *AtCHX17* in response to salt stress, K^+ starvation, changes in external pH and abscisic acid (ABA) treatment. We described the tissue localization of *AtCHX17* expression using the β-glucuronidase (GUS) reporter gene strategy and *in situ* RT-PCR experiments. The phenotype of knockout mutants was compared with the wild-type plant in response to salt stress and K^+ starvation. All together, our data suggest a role for *AtCHX17* in K^+ acquisition and homeostasis rather than a role in Na^+ transport.

Results

AtCHX17 belongs to the Na^+,K^+/H^+ antiporter family

The complete *AtCHX17* coding region was cloned and sequenced. AtCHX17 is predicted to encode a polypeptide of 820 amino acids with a molecular weight of 89 kDa. Hydrophobicity plot analysis showed that AtCHX17 has 11 predicted transmembrane domains in a highly hydrophobic N-terminal region (Figure 1a,b). AtCHX17 contains no predicted organellar-targeting sequence. Database searches revealed substantial similarities between the transmembrane region of AtCHX17 and K^+/H^+ or Na^+/H^+ putative antiporters of microbial or yeast origin. Of these, only KHA1 from *Saccharomyces cerevisiae* (Ramirez *et al.*, 1998) and NAPA from *Enterococcus hirae* (Waser *et al.*, 1992) have been functionally characterized. Over a stretch of 420 amino acids (1–420), AtCHX17 share 35% identity and 58% similarity with KHA1 from *S. cerevisiae* and 26% identity and 50% similarity with NAPA from *E. hirae* (Figure 1a). Phylogenic analysis showed that AtCHX17 clusters with other *Arabidopsis* members of the CPA2 family, but not with the CPA1 or NhaD antiporter families (Figure 1c).

AtCHX17 is induced by salt stress and K^+ starvation

A probe, corresponding to the 335 bp fragment located upstream of the stop codon of *AtCHX17*, was synthesized by RT-PCR. This probe detected a unique restriction fragment on Southern blot with *Arabidopsis thaliana* ecotype Col-0 or WS genomic DNA, and therefore was considered to be specific for *AtCHX17* (data not shown).

Variations of *AtCHX17* expression in response to salt stress was investigated by Northern blot experiments (Figure 2a). *Arabidopsis thaliana* plants grown hydroponically were transferred in nutrient solution containing 100 mM NaCl for 2, 4 or 6 days. Plant growth was inhibited in response to the treatment, but no visible damage of the plant tissues was observed. Plant response to salt stress was monitored by measuring Na^+ content in shoots (Figure 2c). As expected, sodium accumulated in shoots during the treatment. Total RNA was extracted from roots and shoots and analyzed by northern hybridization (Figure 2a). *AtCHX17* transcripts were not detected in shoots in any conditions. In roots, *AtCHX17* expression was not detected in control plants but was strongly enhanced by salt stress. The level of transcripts accumulated increased with the duration of the treatment and was correlated to salt accumulation in shoots. Such results indicate that salt-induced *AtCHX17* expression is likely to be related to progressive salt accumulation in plant rather than to the initial osmotic shock in roots due to the treatment.

Salinity stress interferes with K^+ nutrition by limiting the acquisition of K^+ by cells due to Na^+/K^+ competition (review in Hasegawa *et al.*, 2000). Therefore, the expression of *AtCHX17* was also investigated in response to K^+ starvation in the absence of Na^+. *Arabidopsis thaliana* plants cultivated in a nutrient solution containing 2 mM K^+ were rinsed 5 min in a 0.2 mM $CaSO_4$ solution and transferred in a nutrient solution without K^+ added. Only traces of K^+ (<15 μM) were measured in this solution. Plants were maintained during 5 or 2 days in the K^+-free medium then returned to a nutrient solution containing 2 mM K^+ for two additional days. Rosette growth was not completed at the beginning of the treatment and inhibition of plant growth in response to K^+ starvation was observed. A decrease in shoot K^+ content was measured indicating a severe limita-

Figure 1. AtCHX17 is similar to yeast K^+/H^+ and bacterial Na^+/H^+ antiporters.
(a) Alignment of AtCHX17 (At4g23700) with the K^+/H^+ antiporter KHA1 from *Saccharomyces cerevisiae* (Z49369) and the Na^+/H^+ antiporter NAPA from *Enterococcus hirae* (P26235). The sequences were aligned by the program CLUSTALW (http://www.ebi.ac.uk/clustalw/). Amino acids identical in at least two proteins are highlighted in black, and conservative substitutions are highlighted in gray. The 11 putative transmembrane domains (TM) were predicted by the program (http://sosui.proteome.bio.tuat.ac.jp/cgi-bin/sosui.cgi?/sosui_submit.html) and are indicated by an overline.
(b) Hydrophobicity plot of AtCHX17. The hydrophobicity values were calculated by the program TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html).
(c) Phylogenic analysis of AtCHX17 and *Arabidopsis* Na^+/H^+ antiporters homologs. Multiple sequence alignment were performed with CLUSTALW and the phylogenic tree was drawn by the program DARWIN (http://www.cbrg.inf.ethz.ch/Darwin/index.html). The AGI numbers of Na^+/H^+ antiporters homologs are as follows: AtNHX1 (At5g27150), AtNHX2 (At3g05030), AtNHX3 (At3g06370), AtNHX4 (At5g55470), AtNHX5 (At1g54370), AtNHX6 (At1g79610), SOS1 (At2g01980), SOS1b (At1g14660), AtCHX1 (At1g16380), AtCHX2 (At1g79400), AtCHX3 (At5g22900), AtCHX4 (At3g44900), AtCHX6 (At1g08140), AtCHX7 (At2g28170), AtCHX8 (At2g28180), AtCHX9 (At5g22910), AtCHX10 (At3g44930), AtCHX11 (At3g44920), AtCHX12 (At3g44910), AtCHX13 (At2g30240), AtCHX14 (1g06970), AtCHX15 (At2g13620), AtCHX16 (At1g64170), AtCHX18 (5g41610), AtCHX19 (At3g17630), AtCHX20 (At3g53720), AtCHX21 (At2g31910), AtCHX23 (At1g05580), AtCHX24 (At1g64170), AtCHX25 (At5g58460), AtCHX26 (At5g01680), AtCHX27 (At5g01690), AtNHD1 (At3g19490), AtNHD2 (At1g49810).

tion of K^+ for the plant (Figure 2c). However, K^+ resupply of K^+-starved plants restored the shoot K^+ content at a level similar to control plants indicating that the starvation effects were fully reversible (Figure 2c). Total RNA was extracted from roots and shoots and analyzed by northern hybridization. *AtCHX17* transcripts were not detected in leaves of starved plants. In roots, *AtCHX17* was strongly induced by K^+ starvation and the level of transcripts accumulated increased with the duration of the treatment (Figure 2b). The induction was reversed by the addition of K^+ (Figure 2b).

Because both treatments may result in reduction of K^+ content, the shoots and roots K^+ content of plant subjected to salt stress or K^+ starvation were compared. *Arabidopsis* plants grown as previously described were transferred either in nutrient solution containing 100 mM NaCl or in K^+-free nutrient solution. Six days after the transfer, shoots and roots were collected and washed 5 min in 0.2 mM $CaSO_4$ before ion content determination. In both shoots and roots, the decrease in K^+ content was equivalent in NaCl-treated and K^+-deprived plants (Figure 2d), suggesting that *AtCHX17* salt-induction might be a consequence of K^+ starvation.

AtCHX17 is induced at acidic external pH and in response to ABA treatment

To gain further insights toward *AtCHX17* function *in planta*, we tested whether a control by external pH might contribute to the regulation of *AtCHX17*. Plants of *A. thaliana* cultivated in hydroponic conditions were transferred to nutrient solution maintained at pH 5, 6 or 7 in the presence of organic buffers. Four days after the transfer, total RNA was extracted from roots and shoots and analyzed by northern hybridization (Figure 3a). *AtCHX17* transcripts were never detected in shoots. In roots, *AtCHX17* expression was induced by acidic external pH (pH 5). As *AtCHX17* expression was influenced by the external pH, we have monitored the external pH of the nutrient solutions during salt stress and K^+ starvation treatments. For both treatments, the pH of the nutrient solution, initially at 6, was always comprised between 6.2 and 6.4 the day of the harvest. Thus, *AtCHX17* induction in response to salt stress and K^+ starvation was not triggered by acidification of the nutrient solution.

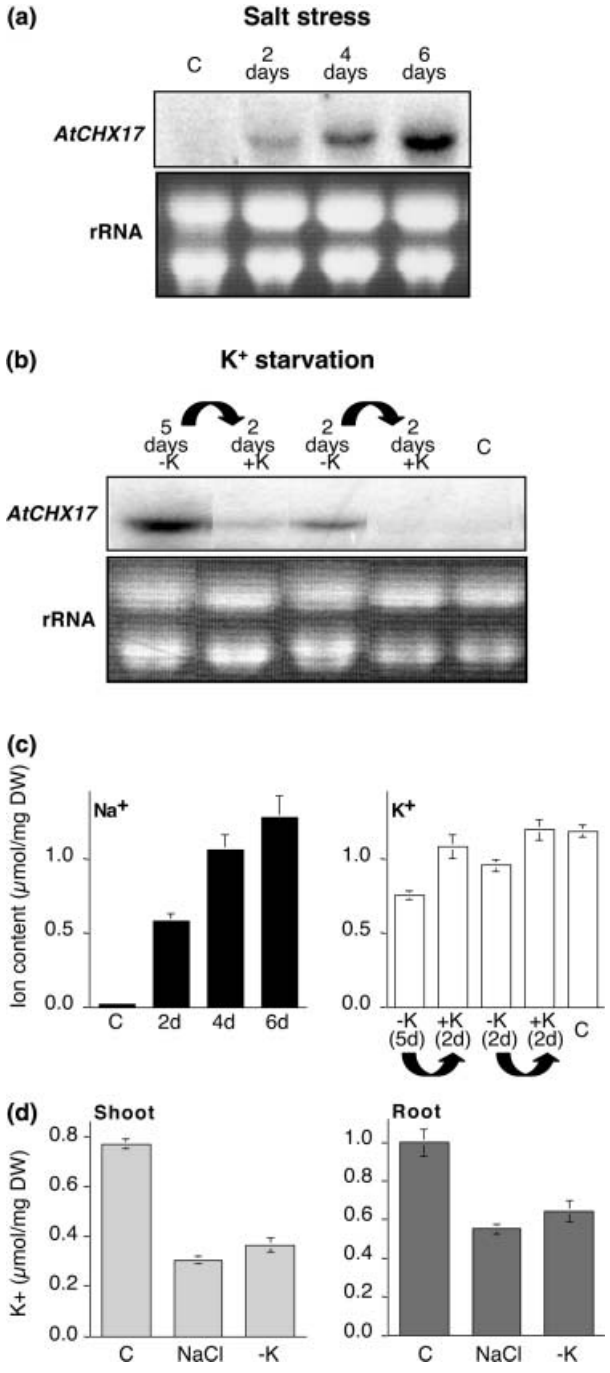

Figure 2. *AtCHX17* expression is induced by salt-stress and K^+ starvation in *Arabidopsis* roots.

(a) Salt-stress treatment. Six-week-old plants of *A. thaliana* ecotype Ws grown on hydroponic conditions were transferred on medium containing 100 mM NaCl during 2, 4 or 6 days

(b) K^+-starvation treatment. Six-week-old plants grown on hydroponic conditions in basal nutrient solution containing 2 mM K^+, were transferred to K^+-free nutrient solution for 5 or 2 days (–K) followed by a 2 days reversion (+K). Total RNA (20 µg) extracted from roots of treated or control plants (lane C) was blotted and hybridized with a ^{32}P-labeled *AtCHX17*-specific probe. Ethidium bromide-stained rRNAs is shown as a loading control. *AtCHX17* transcript size is 2.6 kb.

(c) Na^+ content in shoots of plants subjected to salt stress (black bars) and K^+ content of plants subjected to potassium starvation (white bars). Ion contents were determined by flame spectrometry from shoots of plants described in (a) and (b), respectively. Standard error is indicated ($n = 10$).

(d) Shoot and root K^+ content in response to salt stress and K^+ starvation. Six-week-old wild-type plants grown in hydroponic conditions in basal nutrient solution were transferred in nutrient solution containing 100 mM NaCl or in K^+-free nutrient solution. Six days after the transfer, shoots and roots of control or treated plants, as indicated, were collected for Na^+ and K^+ content determination by flame photometry. Data are mean ± SE ($n = 10$).

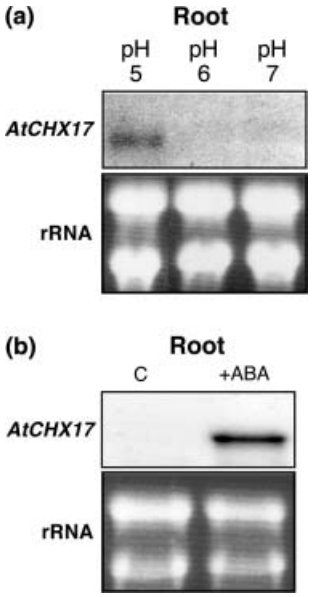

Figure 3. *AtCHX17* expression is induced at acidic external pH and in response to ABA treatment in *Arabidopsis* roots.
(a) Six-week-old plants of *A. thaliana* ecotype Ws grown on hydroponic conditions were transferred in a medium maintained at pH 5, 6 or 7. The roots and shoots were harvested 4 days after the treatment.
(b) Six-week-old plants of *A. thaliana* ecotype Ws grown on hydroponic conditions were transferred in a medium containing 50 μM of ABA during 48 h. Total RNA (20 μg), extracted from roots of treated or control (C) plants as indicated, was blotted and hybridized with a ^{32}P-labeled *AtCHX17*-specific probe. Ethidium bromide-stained rRNAs is shown as a loading control.

Because ABA is involved in the signaling pathway of many salt responsive genes (review in Zhu, 2002), the effect of ABA on *AtCHX17* expression was investigated. Plants of *A. thaliana* grown in hydroponic conditions were transferred in a nutrient solution containing 50 μM ABA. After 48 h of ABA treatment, total RNA was extracted from roots and shoots and analyzed by northern hybridization (Figure 3b).

No expression of *AtCHX17* was observed in shoots. In roots, *AtCHX17* was strongly induced by ABA treatment, suggesting that ABA is involved in *AtCHX17* regulation.

AtCHX17 is expressed in epidermal and cortical cells of mature root zone

The localization of *AtCHX17* expression was investigated using transgenic plants carrying the *Escherichia coli*-GUS gene under the control of the *AtCHX17* promoter region (2 kb). GUS activity was systematically assayed in five independent transgenic lines carrying single insertions. GUS expression was analyzed by histochemical staining of

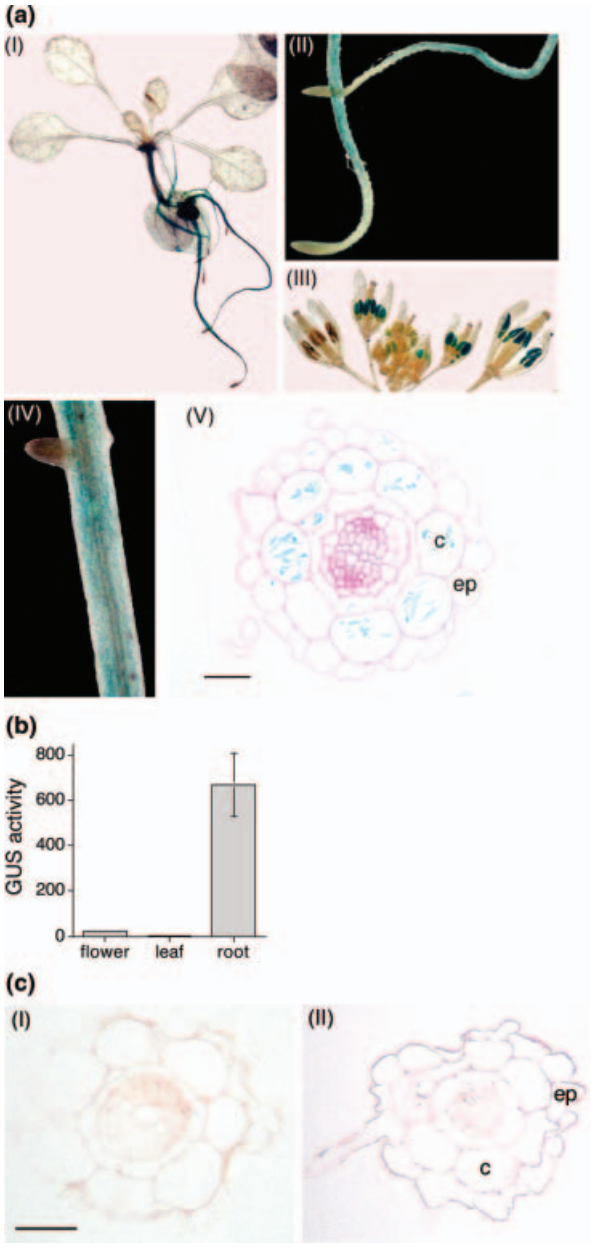

Figure 4. Tissue localization of *AtCHX17* expression.
(a) Histochemical localization of GUS activity in *AtCHX17* promoter-GUS transgenic *Arabidopsis* plants grown under various conditions and exposed to different treatments. Plants were grown *in vitro* and transferred on medium containing 100 mM NaCl (I–II) or maintained at pH 5 (V) during 5 days. Plants were grown in hydroponic conditions during 6 (IV) or 8 (III) weeks and transferred to nutrient solution containing 100 mM NaCl during 4 days. (I) 15-day-old seedling (II) close-up of the roots shown in (I); (III) flower (IV) root of mature plant (V) root cross-section. c, cortex; ep, epidermis. Scale bars = 15 μm.
(b) GUS-specific activity in flowers, leaves and roots. Plants were grown in hydroponic conditions during 6 weeks in basal nutrient solution then transferred to nutrient solution containing 100 mM NaCl during 4 days. GUS activity is given in pmol of 4-methylumbelliferone mg^{-1} protein min^{-1}. Data are expressed as means (n = 10). Bars indicate SE.
(c) *In situ* RT-PCR detection of *AtCHX17* transcripts in *Arabidopsis* roots. Plants were grown in hydroponic conditions during 6 weeks and transferred in nutrient solution containing 100 mM NaCl during 6 days. Root cross-section (a) no-RT control (b) with RT. Amplification products are revealed as brown/purple staining. c, cortex; ep, epidermis. Scale bar = 20 μm.

plants grown under various conditions (*in vitro* or hydroponically) and exposed to different treatments (salt stress, K^+ starvation, ABA and acidic pH). All the transgenic lines displayed similar patterns of GUS staining in all the conditions tested. Representative data are presented in Figure 4(a). At the vegetative stage, the GUS expression was detected along the root except the root tip. In flowering plants, in addition to the roots, GUS activity was also observed in anthers. GUS activity was quantified by fluorimetric assays in flowers, shoots and roots of NaCl-treated plants (Figure 4b). GUS activity was barely detectable in leaves, and was 30 times higher in roots than in flowers. In root cross-sections, GUS staining was mainly detected in epidermal and cortical cells (Figure 4a). For many reasons, promoter-fusion analysis may provide insufficient proof of cellular localization of gene expression (Taylor, 1997). Thus, the cellular site of *AtCHX17* expression in roots was further investigated by *in situ* RT-PCR experiments (Figure 4c). This approach confirmed the GUS investigations. *AtCHX17* transcripts were only detected in epidermal and cortical cells including root hairs (Figure 4c). We conclude that the chimeric reporter gene was therefore a good marker of *AtCHX17* transcripts localization.

Variation of *AtCHX17* promoter activity was analyzed in response to salt stress, K^+ starvation or acidic external pH. Transgenic plants containing the *AtCHX17* promoter-GUS gene were grown in hydroponic conditions in basal nutrient solution, then transferred either in nutrient solution containing 100 mM NaCl, in K^+-free solution or in nutrient solution maintained at pH 5. Four days after the transfer, GUS activity was quantified by fluorimetric assays in roots of control or treated plants (Figure 5). Surprisingly, GUS activity was detected at high level in control plant, was barely affected by K^+ starvation or acidic external pH and was only twofold higher in response to salt stress than in control plants. The *AtCHX17* promoter activity was not correlated with variations of *AtCHX17* transcript accumulation in response to salt stress, K^+ starvation and acidic pH, suggesting that *AtCHX17* regulation upon these treatments could be post-transcriptional or that regulatory sequences are missing in the *AtCHX17* promoter-GUS construct.

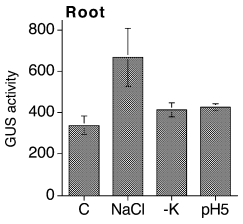

Figure 5. GUS activity in roots of *AtCHX17* promoter-GUS transgenic *Arabidopsis* plants in response to salt stress, K^+ starvation or external acidic pH.

Plants were grown in hydroponic conditions during 6 weeks in basal nutrient solution then transferred in nutrient solution containing either 100 mM NaCl, or in K^+-free solution, or in nutrient solution maintained at pH 5. Four days after the transfer, GUS activity was measured in roots of treated and control (C) plants as indicated. GUS activity is given in pmol of 4-methylumbelliferone mg^{-1} protein min^{-1}. Data are expressed as means ($n = 10$). Bars indicate SE.

Expression of AtCHX17 in yeast

The functional characterization of AtCHX17 was investigated by yeast complementation studies. The full-length coding sequence of *AtCHX17* was cloned in the yeast expression vector pYES under the control of the inducible GAL promoter and used to transform the *S. cerevisiae nhx1:ena1*, *trk1-2* and *kha1* yeast strains. The *nhx1:ena1* strain is salt-sensitive, defective in Na^+ extrusion at the plasma membrane and Na^+ compartmentalization into the vacuole (Gaxiola *et al.*, 1999). The *trk1-2* stain is defective in K^+-uptake (Ko and Gaber, 1991). The *kha1* strain overaccumulate K^+ (Ramirez *et al.*, 1998). We failed to observe any complementation of these mutants when grown on medium containing high Na^+ or various K^+ concentrations (data not shown). Because regulatory sequences/element might be missing for proper AtCHX17 targeting or functioning in yeast, such negative results do not necessarily imply that AtCHX17 is not involved in K^+ or Na^+ transport. For example, many K^+ transporters of the plant HAK family such as AtHAK5 (Rubio *et al.*, 2000), HvHAK2 (Senn *et al.*, 2001), CnHAK1 and CnHAK2 (Garciadeblas *et al.*, 2002) or OsHAK7 and OsHAK10 (Bañuelos *et al.*, 2002), failed to compensate the K^+ transport defect of the yeast *trk1-2* strain. The *Arabidopsis* inward K^+ channel SPIK was also unable to complement the *trk1-2* strain (F. Gaymard, personal communication, INRA, Montpellier, France), but has been demonstrated to mediate K^+ uptake *in planta* (Mouline *et al.*, 2002).

Molecular analysis of a knockout mutant carrying chx17-1 allele

A T-DNA insertion line within the gene *AtCHX17* (sequence-indexed line Salk-002039) was identified in the SIGnal database (http://signal.salk.edu/cgi-bin/tdnaexpress). Primers specific for *AtCHX17* (P1 and P2) were designed to amplify the junctions of the *CHX17*::T-DNA insertion and to select a homozygous line using a PCR-based screen. Genomic DNA isolated from Salk-002039 were subjected to two rounds of PCR. The first round was designed to identify the T-DNA-tagged *chx17* allele using the primer P1 located in *AtCHX17* and the primer LBb1 located in the left border (LB) to amplify a 770 bp *CHX17*::T-DNA chimeric DNA fragment (Figure 6a). The second PCR used primer pairs P1 and P2 to identify the wild-type allele, amplifying a 1.1 kb region of genomic DNA (Figure 6a). A homozygous line, at this point designed *chx17-1*, that failed to amplify the wild-type allele but did amplify the T-DNA-tagged *chx17* was selected and selfed for further studies. The progeny was subjected to the same PCR-based screen to

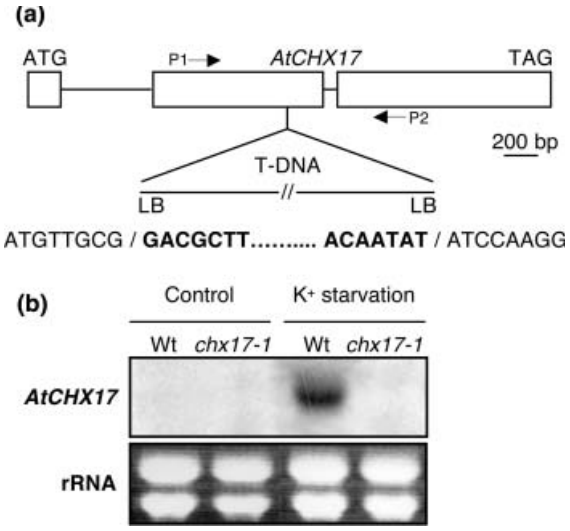

Figure 6. Characterization of the plant line with a T-DNA insertion in *AtCHX17*.
(a) Localization of the T-DNA insert. The diagram illustrates the site of insertion of the T-DNA in the second exon and the gene/T-DNA junction sequences. The location of primer (P1 and P2) binding sites used for selecting tagged and non-tagged *AtCHX17* alleles is indicated.
(b) Northern blot analysis of *AtCHX17* expression in the mutant (*chx17-1*) and the wild-type (Wt) roots. Plants were grown in hydroponic conditions for 6 weeks in standard medium then transferred to K^+-free nutrient solution for 6 days. Total RNA (20 μg) extracted from roots of control or treated plants, was blotted and hybridized with a ^{32}P-labeled *AtCHX17*-specific probe. Ethidium bromide-stained rRNAs is shown as a loading control.

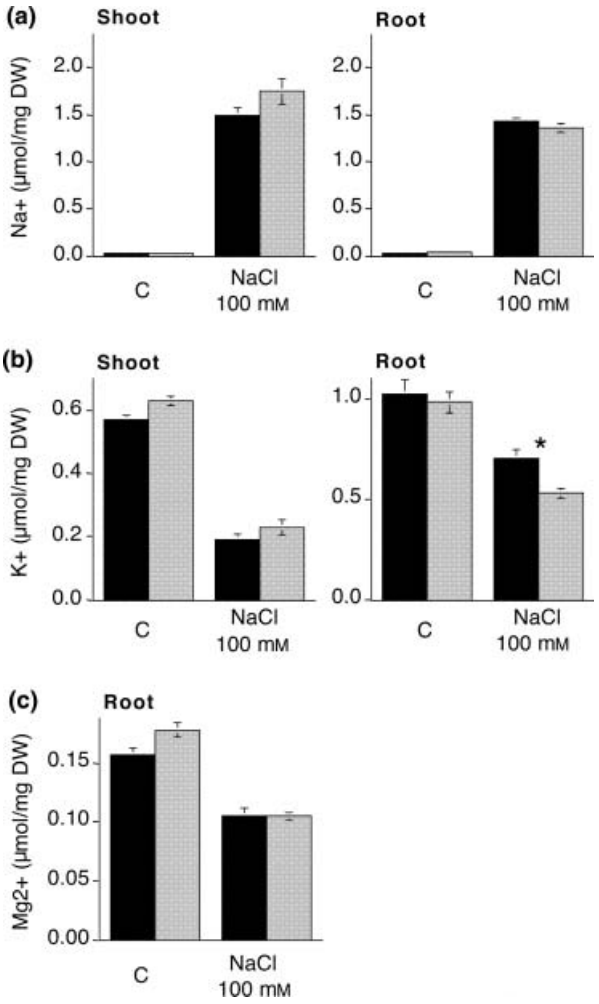

Figure 7. Phenotypic analysis of *chx17-1* in response to salt stress.
Six-week-old wild-type (black bars) or *chx17-1* (gray bars) plants grown in hydroponic conditions in basal nutrient solution were transferred in nutrient solution containing 100 mM NaCl. Six days after the transfer, shoots and roots were collected for Na^+ (a), K^+ (b) or Mg^{2+} (c) content determination by flame photometry. Data are mean ± SE ($n = 10$). Statistically significant differences between the wild-type and the knockout mutant are indicated by asterisks as determined by *T*-test ($P < 0.01$). Data are representative of three independent experiments.

confirm that the line was homozygous. To amplify the 3′ junction of the *CHX17*::T-DNA insertion, the P2 primer also had to be combined with the LBb1 primer and not with a right border- located primer, to lead a 640 bp *CHX17*:: T-DNA chimeric DNA fragment. The T-DNA insertion therefore probably carries two left arms at its extremities. Inverted tandem T-DNA insertions are commonly observed and offer a straightforward interpretation of this result. The P1/LBb1 and P2/LBb1 fragments were sequenced to determine the *AtCHX17*/T-DNA junction sequences (Figure 6a). To identify the number of T-DNA insertion events, Southern blot analysis was performed with an LB-located probe indicating that *chx17-1* carries a single T-DNA insertion locus (data not shown). Finally the expression of *AtCHX17* was analyzed by northern experiments in roots of *chx17-1* and wild-type (Col-0) plants subjected to K^+ starvation (Figure 6b). The *AtCHX17* transcripts were detected in the wild-type roots in response to K^+ starvation but were completely absent in the mutant.

Phenotypic characterization of chx17 knockout mutants

Morphological phenotypes of *chx17-1* were analyzed under various growth conditions. When grown *in vitro* or in hydroponic conditions in basal nutrient medium or under salt stress or K^+ starvation, no differences in germination time, primary root length, root branching and shoot growth were observed between the wild-type (Col-0) and *chx17-1* (data not shown). Phenotypic characteristics of *chx17-1* were further investigated by determining Na^+ and K^+ content in response to salt stress and K^+ starvation (Figures 7 and 8). Wild-type and mutant plants were grown in hydroponic conditions in the same tanks in basal nutrient solution. After 6 weeks of culture, plants were transferred in nutrient solution containing 100 mM NaCl or in K^+-depleted nutrient solution. Six days after the transfer, the second fully expanded rosette leaves and the mature zone of the roots (without the root tip) were collected and washed for 5 min in

0.2 mM $CaSO_4$ before ion content determination. In control conditions Na^+ and K^+ contents were equivalent in both genotypes (Figure 7 and 8). In response to both treatments, the differences between the mutant and the wild type were systematically observed in three independent series of experiments. As expected, the NaCl treatment led to an increase in Na^+ content (Figure 7a) and to a decrease in K^+ content in shoots and roots of both genotypes, indicating that the plants were K^+-deprived (Figure 7b). In shoots of salt-treated plants, the Na^+ or K^+ content was similar in both genotypes (Figure 7a,b). In roots, the Na^+ content was also equivalent in both genotypes (Figure 7a), but the K^+ content was reduced by 24.3% in *chx17-1* compared with the wild type (Figure 7b). In wild-type roots, 6 days of NaCl treatment led to a loss of 0.32 μmol mg^{-1} dry weight (DW) of K^+. The reduction of K^+ content was increased by 40% in the mutant (0.45 μmol mg^{-1} DW). Interestingly, the root Mg^{2+} content was found to be similar in both genotypes (Figure 7c), indicating that alteration in ion content was specific for K^+ in the mutant. As expected, the K^+ starvation experiment led to a decrease in K^+ content in shoots and roots (Figure 8a). This decrease was similar in shoots of both genotypes. However, in roots the K^+ content was reduced by 22.8% in the mutant compared with the wild type (Figure 8a). Again, this difference was specific to K^+, the root Mg^{2+} content being similar in both genotypes in response to the treatment (Figure 8b). Under conditions of reduced K^+ availability (salt stress or K^+ starvation), the K^+ content is decreased in *chx17-1* roots compared with the wild type, suggesting that *AtCHX17* function is related to K^+ homeostasis.

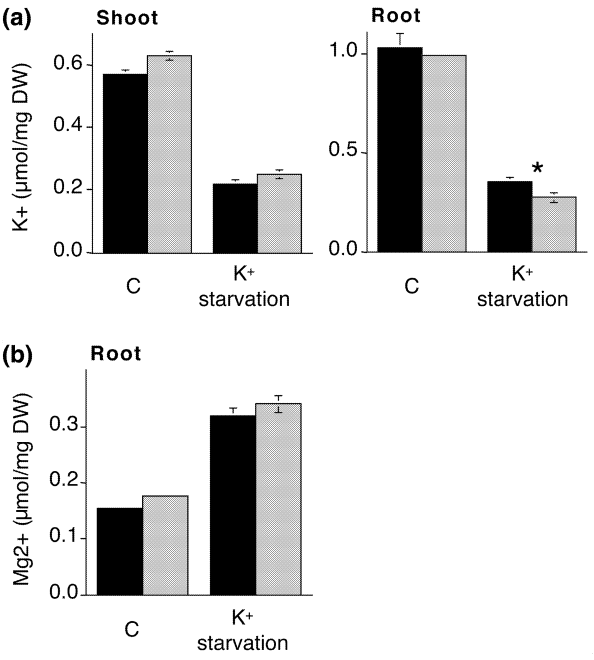

Figure 8. Phenotypic analysis of *chx17-1* in response to K^+ starvation.
Six-week-old wild-type (black bars) or *chx17-1* (gray bars) plants grown in hydroponic conditions in basal nutrient solution were transferred in K^+-free nutrient solution. Six days after the transfer, shoots and roots were collected for K^+ (a) or Mg^{2+} (b) content determination by flame photometry. Data are mean ± SE ($n = 10$). Statistically significant differences between the wild-type and the knockout mutant are indicated by asterisks as determined by *T*-test ($P < 0.01$). Data are representative of three independent experiments.

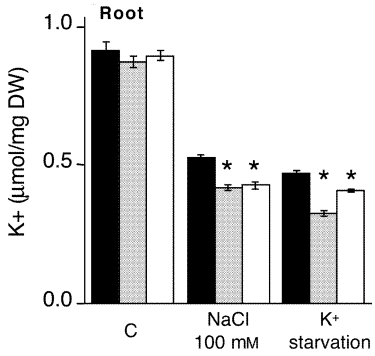

Figure 9. Phenotypic analysis of *chx17-1bc* and *chx17-2* in response to salt stress or K^+ starvation.
Six-week-old wild-type (black bars), *chx17-1bc* (gray bars) or *chx17-2* (white bars) plants grown in hydroponic conditions in basal nutrient solution were transferred in nutrient solution containing 100 mM NaCl or in K^+-free nutrient solution as indicated. Six days after the transfer, the roots were collected for K^+ content determination by flame photometry. Data are mean ± SE ($n = 10$). Statistically significant differences between the wild-type and the knockout mutants are indicated by asterisks as determined by *T*-test ($P < 0.01$).

To rule out the possibility that the observed phenotype was caused by an unknown point mutation unlinked to *chx17-1*, an homozygous line (named *chx17-1bc*) resulting from the back-cross of *chx17-1* with Col-0 was selected and analyzed. Furthermore, to confirm that the observed phenotype was the result of loss of function of *AtCHX17* and was not specific to the *chx17-1* allele, we have analyzed a second mutant with a T-DNA inserted within *AtCHX17* (sequenced-indexed line Salk-060180, SIGnal database). In this mutant (*chx17-2* allele) the T-DNA was inserted within the third exon. A homozygous line was selected, selfed and used for phenotypic analysis. Phenotypic characteristics of *chx17-1bc* and *chx17-2* were investigated by determining the root K^+ content in response to salt stress and K^+ starvation (Figure 9). As in the mutant *chx17-1*, the root K^+ content was reduced in both mutants compared with the wild type in response to both treatments (Figure 9). Two independent mutants within the *AtCHX17* gene led to an equivalent phenotype regarding root K^+ content. Therefore, we conclude that AtCHX17 function relates to K^+ acquisition and homeostasis in *Arabidopsis*.

Discussion

AtCHX17 is homolog of Na^+,K^+/H^+ antiporters

Plant members of the CPA2 (or CHX) family were proposed to encode cation/proton antiporters (Mäser *et al.*, 2001). So

far, no plant members of this family have been characterized. It was predicted that cation/H^+ antiporters extrude cations from the cytosol to the outside or into intracellular compartments such as the vacuole, because of protons pumps at the plasma membrane and endomembranes of plant cells (Sze *et al.*, 1999). AtCHX17 shows substantial similarities with Na^+,K^+/H^+ antiporters from other organisms, particularly with KHA1 from *S. cerevisiae*. KHA1 was proposed to function as a K^+/H^+ antiporter localized at the plasma membrane involved in the regulation of intracellular pH, K^+ efflux and cell volume control (Ramirez *et al.*, 1998). These homologies suggest that AtCHX17 function as a Na^+,K^+/H^+ antiporter.

AtCHX17 *regulation upon salt stress and potassium starvation suggest a role in* K^+ acquisition and homeostasis

AtCHX17 expression was strongly induced in roots in response to salt stress. Plant responses under salinity are not only direct results of Na^+ toxicity but may be also the consequence of a reduced K^+ availability due to the competition between Na^+ and K^+. Indeed, in response to K^+ starvation or NaCl treatment, similar reduction of K^+ content was observed (Figure 2d). Consistent with this hypothesis of K^+ limitation having a major role in salt-induced *AtCHX17* expression, is the strong induction of *AtCHX17* in roots of plants under K^+ starvation, in the absence of sodium. Therefore, Na^+ is not required for the expression of the gene. Very recently, using transcriptome analysis, conflicting data were reported regarding *AtCHX17* induction by salt stress or K^+ starvation. Consistent with our data, *AtCHX17* was found to be overexpressed in roots of *Arabidopsis* plant treated with NaCl 100 mM (Kreps *et al.*, 2002). In a second report, *AtCHX17* upregulation was not observed in response to salt stress or K^+ starvation (Maathuis *et al.*, 2003). These differences are likely to be explained by the growth stage of the plants. In our experiments, at the beginning of each treatment rosette growth was not completed (stage 3.50–3.90 according to Boyes *et al.*, 2001) and we clearly observed an inhibition of rosette growth in response to salt stress or K^+ starvation. In the work of Maathuis *et al.* (2003) plants have almost completed their growth (stage 5.10 according to Boyes *et al.*, 2001) and whole plant K^+ demand might be low at the time of the treatment. Consistent with this interpretation, the root K^+ content was not affected in response to K^+ starvation in this experiment. All together our expression data point out that the function of AtCHX17 might be related to K^+ transport rather than to Na^+ transport as predicted by sequence analysis.

Upregulation of K^+ uptake in plants under K^+-limitation is an adaptative response to adjust the uptake capacity to the whole plant demand. In split-root experiments, withdrawal of external K^+ from one part of the root system leads to an increase of K^+ influx into the K^+-deprived roots as well as into the K^+-supplied roots suggesting that the control is exerted by nutritional status of the whole plant through inter-organ signaling pathway (Drew and Saker, 1984). Increase in high affinity K^+ uptake in response to K^+ starvation in K^+-deprived roots has been shown to involve an increase in the number of carriers and protein synthesis (Drew *et al.*, 1984; Fernando *et al.*, 1990). To our knowledge, upregulation of plant transporters by both salt stress and K^+ starvation was only reported for two members of the HAK K^+-transporters family in common ice plant (Su *et al.*, 2002). These transporters were proposed to be major contributors to K^+ homeostasis under high salinity conditions both by facilitating uptake and by transport through the vasculature. Similarly, *AtCHX17* is induced upon salt stress and K^+ starvation suggesting that AtCHX17 might also contribute to K^+ acquisition and homeostasis under high salinity in *Arabidopsis*.

In roots, *AtCHX17* expression is strongly induced by ABA treatment. The transport and accumulation of K^+ was reported to be at least in part ABA-regulated. ABA regulation of K^+ transport has been extensively evidenced in guard cells (review in Schroeder *et al.*, 2001). In higher plant roots, there is evidence of modulation of K^+ channel activity in stelar cells by ABA (Roberts, 1998; Roberts and Snowman, 2000). Additionally, treatment with ABA has been shown to downregulate the expression of the pericycle-localized root K^+ channel SKOR (Gaymard *et al.*, 1998). However, ABA regulation of other root K^+ transporters was never reported. Whether the induction of *AtCHX17* by K^+ starvation might be ABA-mediated remains to be elucidated.

Together, these results show that various environmental stresses, including high salt, K^+ deficiency, ABA and acidic medium, upregulate *AtCHX17* transcript suggesting that this gene has a role in adaptative response to those stresses. Whether *AtCHX17* acts directly or indirectly to modulate K^+ acquisition and homeostasis remains to be clarified.

Role of AtCHX17 in K^+ *homeostasis*

The localization of *AtCHX17* expression in root cells is consistent with a role in K^+ acquisition. The tissue localization of *AtCHX17* expression was determined using a GUS reporter gene strategy and confirmed by *in situ* RT-PCR experiments. *AtCHX17* was expressed in root cell types specialized in ions uptake. *AtCHX17* expression is strong along the mature root in epidermal and cortical cells and absent at the root tip. In roots, *AtCHX17* is expressed in zones (epidermis and cortex) where ions can reach cell surface by apoplastic diffusion. In the mature root, the Casparian strip present at the endodermis blocks such diffusion into the stele (Pitman, 1982). Therefore, root epidermal and cortical cells mediate the net uptake of ions into the root symplasm, whereas the stelar cells of the root mediate the net loss of ions from the root symplasm into the xylem vessels (Clarkson, 1988).

The phenotype of *chx17* knockout mutants supports a role in K^+ acquisition and homeostasis. The functional characterization of *AtCHX17* was investigated in two T-DNA insertional mutants (*chx17-1* and *chx17-2*) of *Arabidopsis*. The mutant plants were compared with the wild type, in response to salt stress and potassium starvation. In response to both treatments, the root K^+ content was found to be lower in the mutants compared with the wild type whereas no differences were detected for other cations such as Na^+ and Mg^{2+}. These data give further support to the hypothesis of a role of AtCHX17 in increasing net K^+ uptake. It is unclear whether this is due to an increase in K^+ uptake or a reduction in K^+ loss especially in the roots.

In the absence of functional characterization of AtCHX17 protein, direct evidence is lacking regarding the mechanism of AtCHX17 function. However, our data provide circumstantial evidences for stimulating working hypothesis to further investigate the role of this gene. Potassium could enter root cells via K^+ channels or K^+,H^+ symporters (review in Véry and Sentenac, 2003) and is then accumulated into vacuoles by a K^+_{in}/H^+_{out} exchange. Long-distance transport could involve export of K^+ from xylem parenchyma cells to the apoplast by a K^+_{out}/H^+_{in} exchange at the plasma membrane.

Assuming a working model of AtCHX17 as a K^+/H^+ exchanger, different hypothesis might be proposed. Plant cells contain two major pools of K^+, one in the vacuole and one in the cytosol. In conditions of K^+ sufficiency, it was reported that, in epidermal and cortical cells the net uptake of K^+ into vacuoles is mediated by K^+_{in}/H^+_{out} antiporters (walker 96), and AtCHX17 may be part of this mechanism. However, in K^+-deficient cells the net K^+ release out of the vacuole is proposed to be achieved by H^+,K^+ symporters (Walker *et al.*, 1996). The hypothesis of AtCHX17 being a K^+/H^+ antiporter localized at the tonoplast activated under K^+ starvation is somehow not consistent with this model. In plants there is some biochemical evidence suggesting the existence of K^+/H^+ antiporter at the plasma membrane (Cooper *et al.*, 1991; Hassidim *et al.*, 1990). These antiporters are proposed to fulfill functions in the control of cellular pH and K^+ content such as those described for fungi (review in Rodrìguez-Navarro, 2000). Such antiporters would exclude K^+ from the cytosol to the apoplasm. However, AtCHX17 being involved in such a role, is not consistent with the mutant phenotype. According to such a hypothesis, a loss of function mutation is not expected to reduce tissue K^+ content. Recently, a tomato K^+/H^+ antiporter, LeNHX2, was reported to be located in the intracellular compartment (Venema *et al.*, 2003). This exchanger is proposed to maintain higher K^+ concentration in intracellular compartments under salt-stress conditions and might be involved in intracellular K^+ homeostasis. The contribution of AtCHX17 in such a function in *Arabidopsis* may be an alternate hypothesis but remains to be elucidated.

In experiments presented in this paper, we have monitored the accumulation of Na^+ and K^+, that is the end result of processes involving multiple transporters. We cannot rule out that compensation by other transporters involved in Na^+ or K^+ homeostasis, including other CHX members, and/or deregulation mechanisms, occurred in the mutant leading to the phenotype observed. This concerns specially other members of the CHX family closely related to AtCHX17 (AtCHX15, AtCHX16, AtCHX18, AtCHX19 and AtCHX20 shares more than 50% identities). Excepted *AtCHX18*, all of them were found to be expressed using RT-PCR experiments (F. Cellier, unpublished data). We also consider a model where activation of *AtCHX17* modulates activities of other K^+ transporters, such as high-affinity K^+,H^+ symporters, that bring in K^+ into plants under K^+-deficient conditions (Maathuis and Sanders, 1994; Rodrìguez-Navarro, 2000; Walker *et al.*, 1996).

This study shows that *AtCHX17* has a role in K^+ homeostasis and that it may assist plants to withstand various environmental stresses. Clearly, further investigations are required to determine the membrane location of AtCHX17 protein and to test whether it affects K^+ content in roots mainly via a K^+/H^+ exchange, a K^+,H^+ symport or a combination of several mechanisms.

Experimental procedures

Plant material and culture conditions

Arabidopsis thaliana ecotypes Wassilewskija (Ws) and Columbia (Col-0) were used in this study. The Salk-002039 mutant line is of the ecotype Columbia and was provided by ABRC *Arabidopsis* stock center as segregating T3 lines. Plants were grown in hydroponic conditions, *in vitro* or in the greenhouse. For hydroponic culture, seeds were sown on sand laid on a mesh floating on tap water in a 10 l tank. After 2 weeks, the culture system was transferred on aerated basal nutrient solution containing 1 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4$, 50 μM Na-Fe-EDTA, 50 μM H_3BO_3, 0.05 μM CoCl, 0.05 μM $CuSO_4$, 15 μM $ZnSO_4$, 50 μM $MnSO_4$, 3 μM MoNa, pH adjusted to 6 with KOH if needed. The growth-chamber environmental parameters were as follows: light/dark cycle 8/16 h, light intensity 300 μmol sec^{-1} m^{-2} PAR, temperature 22°C/20°C, 70% hygrometry. The nutrient solution was renewed twice a week during the first part of the culture, and daily the last week before the experiment and during the experiment.

For K^+ starvation experiment, roots were rinsed in 0.2 mM $CaSO_4$ before being transferred to a K^+-free medium. In K^+-free medium, KH_2PO_4 and KNO_3 were replaced by 1 mM NaH_2PO_4 and 0.5 mM $Ca(NO_3)_2$ and the pH was adjusted to 6 with MES-Tris (pH 6). For salt treatment, the basal nutrient solution was supplemented with 100 mM NaCl. For medium maintained at pH 5, 6 or 7, the basal nutrient solution was buffered with 4.4 mM MES (pH 5 or 6 with Tris) or 4.4 mM BES (pH 7 with Tris). For ABA treatment, the basal nutrient solution was supplemented with 50 μM ABA from a 100 mM stock solution. ABA (± *cis*,*trans*-abscisic acid; Sigma A4906 (Sigma, St Quentin Fallavier, France)) stock solution was prepared in 1 N NaOH.

For all experiments, plants of the same age cultivated in various conditions were harvested at the same time in the middle of the light period. At the beginning of each treatment, plants were at a

vegetative stage corresponding to rosette growth (between stage 3.50 and 3.90 according to Boyes *et al.*, 2001) and rosette growth was not completed. When necessary, treatments were initiated for various times before the harvest. During all the experiments, the pH of the nutrient solution was monitored daily. During salt stress, K^+ starvation or ABA treatments, the pH of the nutrient solution (initially at 6) was always comprised between 6.3 and 6.6 the day of the harvest.

For *in vitro* culture, seeds were surface-sterilized by soaking in a solution containing 1.5% (w/v) Bayrochlor (Indusco France, Gargenville, France) in 50% ethanol for 30 min under strong agitation, rinsed three times in 100% ethanol and dried overnight under a sterile air flow. Seeds were sown on the same basal nutrient solution containing 0.7% agar and supplemented with 1% sucrose. After 10 days of culture, seedlings were transferred to the appropriate agar-solidified medium depending on the treatment as described above. For K^+ starvation experiment, agar was replaced by agarose. The *in vitro* growth conditions were as follows: light/dark cycle 16 h/8 h, light intensity 150 μmol sec^{-1} m^{-2} PAR, temperature 21°C/18°C, 65% hygrometry.

In the greenhouse, plants were grown on compost (Neuhaus Humin Substrat N2; Klasman-Deilmann GmbH, Geeste, Germany) and subirrigated with tap water. Greenhouse culture conditions were as follows: light/dark cycle 16 h/8 h, sunlight intensity limited to 300 μmol sec^{-1} m^{-2} PAR, temperature 25°C/24°C.

Northern analysis

Total RNA was extracted as described (Ausubel *et al.*, 1991). Total RNA samples (20 μg) were resolved by electrophoresis on Mops-formaldehyde agarose gel (Lehrach *et al.*, 1977) and blotted to a Hybond-N nylon membrane (Amersham, Orsay, France). Northern blot hybridizations, to randomly primed radiolabeled probes (Prime-a-Gene Labeling System; Promega, Charbonnieres, Les Bains, France), were performed at 42°C in 50% formamide, 5x SSPE (0.72 M NaCl, 0.05 M NaH_2PO_4 and 5 mM EDTA, pH 7.4), 1% sarkosyl, 10% dextran sulfate and 100 μg/ml salmon sperm DNA. Membranes were washed at room temperature in 2x SSC and 0.1% SDS for 20 min, at 42°C in the same buffer for 20 min, and then twice at 42°C in 0.1x SSC and 0.1% SDS for 20 min. For each experiment, RNA extraction and Northern blot were triplicated.

Amplification of gene-specific AtCHX17 cDNA fragment and AtCHX17 cDNA cloning

Total RNA was isolated from *A. thaliana* ecotype Ws. First-strand cDNA was synthesized using the M-MLV reverse transcriptase (Promega) from total RNA (5 μg), and used as template for PCR amplification. PCR amplification was performed using Extra Poll DNA polymerase (Eurobio, Courtaboeuf, France) and a pair of *AtCHX17*-specific primers 18F (5′-GTGCGAATGGCTGAACATCC-3′), and 18R (5′-ATCCCTGACGCTACAGAACC-3′). The PCR reaction mixture contained 5 μl of 10X Extra Poll buffer, 2.5 μl of 50 mM $MgCl_2$, 1 μl of 10 mM dNTP mix, 1 μl of each primers (10 μM stock) and 1 unit Extra Poll. The PCR program cycle was as follows: 30 sec at 94°C, 45 sec at 65°C–1°C/cycle, 2 min at 72°C, for 9 cycles then 30 sec at 94°C, 45 sec at 55°C, 2 min at 72°C for 29 cycles. The PCR product was gel purified and used as a probe.

The complete *AtCHX17* coding region was amplified by PCR as described above using the pfu DNA polymerase (Promega) and specific primers that annealed to sequences that included the start and stop codons (underlined): (5′-TTTAAAGCATGGGAACAAACGG-TACAACATGTCCAG-3′) and (5′-CCAAATAGAAAATTCACTAAGGA-CTC-3′), respectively. CDNAs obtained from four independent PCR amplifications were cloned in pCR-Blunt Vector (Invitrogen, Cergy Pontoise, France) and sequenced.

Transgenic plants

A 2 kb long fragment corresponding to the AtCHX17 promoter region was amplified from genomic DNA (ecotype Columbia) by PCR with the pfu DNA polymerase (Promega) using the primers 5′-TTTTCCATGGTTTAAAGATCTGACAAATGATGAATATG-3′ and 5′-TTTTGGATCCTCTACCTGAGTTTGTTTTAACC-3′ introducing a unique *Nco*I site at the ATG initiation codon of the *AtCHX17* coding sequence and a *Bam*HI site at the 5′ end. The PCR product was digested by *Nco*I and *Bam*HI and the resulting fragment was cloned in pBi320.X (R. Derose, RHOBIO, Evry, France; PBi320.X bears a unique *Nco*I site at the initiation codon of a promoterless GUS coding sequence located upstream the nopaline synthase terminator) leading to a traductional fusion between the *AtCHX17* promoter region and the GUS coding sequence. The *AtCHX17* promoter sequence of this construct was verified by sequencing and the corresponding complete expression cassette was cloned in pMOG 402 binary vector (Dr H. Hoekema). The resulting plasmid was transferred in the GV3101 *Agrobacterium tumefaciens* strain (Koncz and Shell, 1986). The transformation of *Arabidopsis* (ecotype Columbia) was performed using the floral dip procedure (Clough and Bent, 1998). Transgenic plants were screened *in vitro* on a Murashige and Skoog standard medium (Sigma M5519) supplemented with 50 mg l^{-1} kanamycin. Ten lines of the T1 progeny, mono-locus for the transgene, were selected from 50 kanamycin-resistant T0 plants. Among those lines, five independent homozygous lines of the T2 progeny were selected for further analysis.

GUS assays

GUS histochemical staining was performed as described (Lagarde *et al.*, 1996). Cross-sections of GUS-stained material were prepared with a microtome (LKB, Bromma, Sweden) from tissues embedded in hydroxyethyl methacrylate (Technovit 7100; Heraus-Kulzer GmbH, Wehrein, Germany) and counter stained in purple with periodic acid Schiff reagents. For quantitative assays, GUS activity was determined by measuring 4-methylumbelliferone (MU) produced from the glucuronide precursor 4-methylumbelliferyl-β-D-glucuronide as described (Jefferson, 1987). GUS activity was calculated in units of pmol MU mg^{-1} protein min^{-1}.

Liquid-phase in situ *RT-PCR of* Arabidopsis *root*

The present protocol is modified from Koltai and Bird (2000). *Arabidopsis* roots were cut into 1 cm small pieces and immediately fixed in PAA [2% (v/v) paraformaldehyde, 63% (v/v) ethanol, 5% (v/v) acetic acid] overnight at 4°C. Fixed root pieces were washed three times for 10 min each in DEPC-treated water, embedded into molten 9% (w/v) low-melting point agarose in PBS and cooled to room temperature. Embedded root pieces were cut using a vibratome microtome (Bio-Rad, Marnes la Coquette, France). Sections (50 μm) were transferred into a tube containing DEPC-treated water, heated and washed at 65°C three times with DEPC-treated water to remove agarose. For reverse transcription, sections were incubated in 40 μl RT-mix [1x RT buffer (Promega), 1 mM each of dTTP, dCTP, dGTP, dATP and 0.1 μM 18R primer]. The tubes containing the sections in RT-mix were heated to 65°C for 5 min and then returned to ice. M-MLV reverse transcriptase

(Promega) was added to each tube to a final concentration of 0.5 units μl^{-1} and incubated at 42°C for 1 h. The tubes were washed once with DEPC-treated water. Forty microliters of PCR-mix [1x PCR buffer (Eurobio), 1.5 mM $MgCl_2$, 200 μM each dTTP, dCTP, dGTP, dATP, 10 mM each primer 18F and 18R, 10 μM digoxigenin-11-dUTP (La Roche, Meylan, France) and 0.125 units μl^{-1} EurobioTaqII (Eurobio)] were added to each tube, followed by 72°C for 5 min and 25 cycles (92°C for 30 sec; 55°C for 30 sec; 72°C for 30 sec). Following RT-PCR, the tubes were washed three times for 10 min in 1x PBS (5 mM Na_2HPO_4, 130 mM NaCl, pH 7.5). Sections were then dehydrated in graded ethanol series (25, 50, 70, 95, 100%) for 15 min each, with three changes in 100% ethanol. After dehydration, the sections were transferred into graded LR White (Sigma) series (3:1, 1:1, 1:3 mixtures of ethanol/LR White) for 30 min each and into 100% LR White for overnight at 4°C. Next day, the sections were left a day long with several changes in 100% LR White. Polymerization of LR White was effected with long-wave length (365 nm) UV light for overnight at 4°C. Thin sections (3 μm) made with a Histomicrotome (Leica, Wetzlar, Germany) were collected onto glass slides coated with 3-aminopropyltriethoxysilane (Sigma). After blocking with 2% (w/v) BSA in 1x PBS for 1 h, thin sections were incubated with alkaline phosphatase-conjugated anti-digoxigenin-Fab fragment (La Roche) diluted 1:250 in blocking solution for 2 h. The slides were rinse three times with 10x washing buffer (100 mM Tris–HCl, 150 mM NaCl, pH 9.5) for 15 min each. Detection of alkaline phosphatase was carried out for 1 h using NBT/BCIP ready-to-use stock solution (La Roche) diluted in 1x washing buffer.

Genetic analysis of chx17-1 and chx17-2

Plants homozygous or heterozygous for the *chx17* locus were selected by PCR with different sets of primers. The primers P1, P2, P3 and P4 are located in *AtCHX17*, the primer LBb1 is located in the T-DNA LB. The sets of primers (P1, LBb1) and (P4, Lbb1) amplify a 770 bp and a 400 bp CHX17::T-DNA chimeric DNA fragment in *chx17-1* and *chx17-2*, respectively. The set of primers (P1, P2) and (P3, P4) amplify a 1.1 kb and a 948 bp region of genomic DNA and was used to identify the non-tagged *chx17* alleles. Homozygous lines were selected and selfed for further studies.

Determination of ion contents

Plant material was dried for at least 24 h at 80°C. The DW was measured. Plant material was incubated in 0.1 N HCl at 55°C for 30 min for ions extraction. Cations Na^+, K^+ or Mg^{2+} concentrations in the extracts were determined by flame photometry (SpectrAA 220FS; varian Australia Pty Ltd, St Helens, Australia).

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