The Potassium Transporters HAK2 and HAK3 Localize to Endomembranes in Physcomitrella patens. HAK2 is Required in Some Stress Conditions

Rosario Haro*, Ana Fraile-Escanciano, Pablo González-Melendi and Alonso Rodríguez-Navarro

Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid, Campus de Montegancedo, Carretera M-40, km 38, 28223 Pozuelo de Alarcón, Madrid, Spain

*Corresponding author; E-mail, rosario.haro@upm.es; Fax, +34-91-715-7721.

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The function of HAK transporters in high-affinity K+ uptake in plants is well established; this study aims to demonstrate that some transporters of the same family play important roles in endomembranes. The PpHAK2–PpHAK4 genes of Physcomitrella patens encode three transporters of high sequence similarity. Quantitative PCR showed that PpHAK2 and PpHAK3 transcripts are expressed at approximately the same level as the PpACT5 gene, while the expression of PpHAK4 seems to be restricted to specific conditions that have not been determined. KHA1 is an endomembrane K+ / H+ antiporter of Saccharomyces cerevisiae, and the expression of the PpHAK2 cDNA, but not that of PpHAK3, suppressed the defect of a kha1 mutant. Transient expression of the PpHAK2–green fluorescent protein (GFP) and PpHAK3–GFP fusion proteins in P. patens protoplasts localized to the endoplasmic reticulum and Golgi complex, respectively. To determine the function of PpHAK2 and PpHAK3 in plants, we constructed ΔPphak2 and ΔPphak2 ΔPphak3 plants. ΔPphak2 plants were normal under all of the conditions tested except under K+ starvation or at acidic pH in the presence of acetic acid, whereupon they die. The defect observed under K+ starvation was suppressed by the presence of Na+. We propose that PpHAK2 may encode either a K+–H+ symporter or a K+ / H+ antiporter that mediates the transfer of H+ from the endoplasmic reticulum lumen to the cytosol. PpHAK2 may be a model of the second function of HAK transporters in plant cells. The disruption of the PpHAK3 gene in ΔPphak2 plants showed no effect.

Keywords: Endomembranes ● HAK transporters ● Potassium fluxes.

Abbreviations: ACT, actin; ER, endoplasmic reticulum; EST, expressed sequence tag; GFP, green fluorescent protein; qRT-PCR, quantitative real-time PCR; UTR, untranslated region; YFP, yellow fluorescent protein.

Introduction

Potassium is the most abundant cation in all types of living cells; among the many cellular roles which K+ plays, osmotic and pH adjustments might have been the most primordial. To control the osmotic potential, K+ and anions move in parallel across the plasma membrane, either inward or outward, to increase or decrease cellular osmolality, respectively, while preserving charge balance. For pH adjustments, K+ and H+ are exchanged, which brings about net H+ movement, either uptake or loss, to decrease or increase the cellular pH, respectively. These processes are especially important in bacteria, fungi and plants because their cells are exposed and have to adapt to environments in which osmolality and pH are highly variable.

Genetic studies of K+ transport in non-animal cells were performed in bacteria and fungi by selecting and studying defective mutants (Ramos et al. 1985, Dosch et al. 1991). However, after the advent of the whole-genome sequencing era, many genes encoding putative K+ transporters have been identified by the similarities between their translated sequences and those of previously studied K+ transporters. Thereafter, the real functions of the identified genes may be determined by reverse genetics. This process can be followed with bacteria, fungi and plants; however, plants present specific complications, which occasionally make it difficult to identify the functions (Alonso and Ecker 2006).

One important family of K+ transporters in fungi and plants show sequence homology to the Escherichia coli Kup transporter (Rodriguez-Navarro 2000). Escherichia coli Kup is a transporter with a modest affinity and rate that is important only at low pH, where its maximum rate exceeds that of the Trk system (Epstein 2003). In contrast to the E. coli Kup transporter, its sequence homologs in fungi, named HAK, show a very high affinity for K+ and an astonishing capacity to deplete K+ from the external medium and concentrate it within cells (Bañuelos et al. 1995, Haro et al. 1999). The K+–H+ symport mechanism described for the high-affinity K+ uptake of Neurospora crassa (Rodriguez-Navarro et al. 1986) probably applies to all HAK fungal transporters and explains the striking concentrative capacity of these transporters (Rodriguez-Navarro 2000). Remarkably, the main characteristic of the E. coli Kup transporter may also be its K+–H+ symport mechanism (Zakharyan and Trchounian 2001).

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Similar to fungi, plant roots are also able to deplete K\(^+\) down to extremely low concentrations, and it has been demonstrated or substantially supported that HAK transporters mediate this function in barley (Santa-Maria et al. 1997), Arabidopsis (Rubio et al. 2000, Gierth et al. 2005, Rubio et al. 2008), rice (Bañuelos et al. 2002), and several Arabidopsis transporters (Whiteman et al. 2008) localize to the tonoplast. HAK transporters are distributed in six phylogenetic groups (Garcia deblás et al. 2007). Although the K\(^+\)–H\(^+\) symport mechanism has not been demonstrated in plant HAK transporters, it fits perfectly into the mechanistic model proposed for the high-affinity K\(^+\) uptake of Arabidopsis (Maathuis and Sanders 1997), which is mediated by AtHAK5 (Rubio et al. 2000, Gierth et al. 2005, Rubio et al. 2008). Recently, it has been proposed that some HAK transporters mediate high-affinity Na\(^+\) uptake (Benito et al. 2012).

These observations demonstrate that the function of some plant HAK transporters is to mediate high-affinity K\(^+\) or Na\(^+\) uptake in plant root cells and perhaps in the cells of other tissues. However, other functions are possible because there are HAK transporters, some named KUP, that localize to the membranes of organelles. For example, the AtKUP12 protein has been identified in the chloroplast proteome (Kleffmann et al. 2004, Peltier et al. 2004), and a rice transporter (Bañuelos et al. 2002) and several Arabidopsis transporters (Whiteman et al. 2008) localize to the tonoplast. HAK transporters are distributed in six phylogenetic groups (Gómez-Porras et al. 2012); those involved in high-affinity K\(^+\) uptake belong to Groups I and IV, while nothing is known about the function of the HAK transporters that cluster in the other phylogenetic groups. This situation prompted a general study of HAK transporters.

The genome of P. patens contains 18 HAK genes, and three of the encoded transporters, PpHAK2–PpHAK4, form Group V (Gómez-Porras et al. 2012). Because the PpHAK2–green fluorescent protein (GFP) fusion protein localizes to endomembranes (Garcia deblás et al. 2007), the Group V HAK transporters offered an attractive possibility to study the HAK transporters of organelles. Here we report that the PpHAK2 transporter localizes to the endoplasmic reticulum (ER) and PpHAK3 localizes to the Golgi complex. PpHAK2 plays an essential role when P. patens plants are stressed by the absence of K\(^+\) or by organic acids.

**Results**

**The transcript expression of the PpHAK2–PpHAK4 genes, and subcellular location of PpHAK2p and PpHAK3p**

The existence of expressed sequence tags (ESTs) corresponding to the PpHAK2–PpHAK4 genes demonstrates that these genes are expressed (Garcia deblás et al. 2007). To gain insight into the function of these genes in relation to K\(^+\) nutrition, we determined their expression at three K\(^+\) concentrations, 100, 4 and 0 mM K\(^+\) (K\(^+\) starvation), by quantitative real-time PCR (qRT-PCR) (Fig. 1), normalizing the results with reference to the expression of the PpACT5 gene. As a reference, we include the expression of the PpHAK1 gene that encodes a high-affinity K\(^+\) transporter (Garcia deblás et al. 2007). PpHAK2 showed expression levels similar to those of PpACT5, except at 100 mM K\(^+\), where it was slightly inhibited. PpHAK3 also showed an expression level similar to that of PpACT5 at 4 mM K\(^+\), but it was inhibited by approximately 10-fold at both high and low K\(^+\) (100 and 0 mM). In contrast to PpHAK2 and PpHAK3, the expression of PpHAK4 was not detected under our experimental conditions. We confirmed that two PpHAK4 ESTs existed in the NCBI database (http://www.ncbi.nlm.nih.gov/, accessed on October 10, 2012). However, these ESTs were sequenced from a cDNA library including hormone-treated plants (Nishiyama et al. 2003), which suggested that the expression of PpHAK4 might be hormone dependent. We did not explore this possibility and continued our study with the other two genes.

A previous transient expression of the PpHAK2–GFP fusion protein in P. patens proplasts suggested that PpHAK2 is not a plasma membrane transporter (Garcia deblás et al. 2007). Although the position of the GFP signal in the reported images was compatible with an ER location, the images did not have sufficient detail to demonstrate the exact location of the protein. In those experiments the PpHAK2-GFP fusion gene was under the control of the Cauliflower mosaic virus (CaMV) 35S gene promoter, which shows a weak expression
in *P. patens* (Horstmann et al. 2004). Therefore, we repeated the experiments using a new construct in which the expression of the fusion gene was under the control of the 5′ untranslated region (5′ UTR) of the *PpACT5* gene (Weiss et al. 2006), which we found to be expressed similarly to *PpHAK2* and *PpHAK3* (Fig. 1).

When expressed from the new construct, the *PpHAK2–GFP* protein formed a network through the cytoplasm and particularly around the nucleus, which was consistent with localization in the ER (Fig. 2; Sparkers et al. 2009). To confirm this localization, we co-expressed *PpHAK2–GFP* with an ER fluorescent marker. The ER marker was created by combining the signal peptide of AtWAK2 at the N-terminus of the yellow fluorescent protein (YFP) and the ER retention signal His-Asp-Glu-Leu at the YFP C-terminus (Nelson et al. 2007). The results showed that the green fluorescence from the *PpHAK2–GFP* protein co-localized with the yellow fluorescence from the ER marker (Fig. 2A–C). This result strongly suggested that the *PpHAK2* protein localized to the ER membrane.

Next, we performed similar expression experiments with the *PpHAK3–GFP* fusion gene, also using the 5′ UTR of the *PpACT5* gene to control the expression. In this case, the GFP signal suggested that the protein was targeted to the Golgi membranes. To confirm that localization, we co-expressed *PpHAK3–GFP* with the G–YFP Golgi marker, which was based on the cytoplasmic tail and transmembrane domain (first 49 residues) of GmMan1 soybean α-1,2-mannosidase I (Saint-Jore-Dupas et al. 2006, Nelson et al. 2007) (Fig. 2D–F). It is worth noting that the amino acid sequences of *PpHAK2* and *PpHAK3* are 82% identical (Supplementary Fig. S1), and most of the differences accumulate in the variable region of HAK transporters, which is comprised of residues 660–750 (Rodríguez-Navarro 2000).

**Heterologous expressions of *PpHAK2* and *PpHAK3***

Routinely, plasma membrane HAK transporters have been functionally tested in yeast *trk1 trk2* mutants, but it would be physiologically inexplicable that an endomembrane transporter suppressed the defect of these mutants. In fact, the *PpHAK2* cDNA does not suppress the defective K⁺ uptake of the yeast *trk1 trk2* mutant (Garciadeblas et al. 2007), and we obtained the same negative result for *PpHAK3*.

Therefore, we expressed the *PpHAK2* and *PpHAK3* cDNAs in *kha1* mutants, following the approach used for the functional study of endomembrane CHX K⁺ transporters (Chanroj et al. 2011). The rationale of this approach is that KHA1 takes part in...
the regulation of intracellular cation content and pH (Maresova and Sychrova 2005, Flis et al. 2005). Single kha1 mutants do not show a clearly defective phenotype, but kha1 mutants in the ena1-4 nha1 background (strain LMB01) do not grow at low K⁺, pH 7.5. Addition of the trk1, trk2 and tok1 mutations (strain LMM04) makes the strain more sensitive at low K⁺, due to the trk1 trk2 mutations, and especially defective at low K⁺ and high or low pH. Therefore, we tested the physiological expression of PpHAK2 and PpHAK3 cDNAs in these two types of mutants. In the kha1 ena1-4 nha1 mutant, PpHAK2 showed no effects (Supplementary Fig. S2), but it clearly improved the growth of the kha1 ena1-4 nha1 trk1 trk2 tok1 mutant (strain LMM04) at pH 4.5. At this pH and 100 mM K⁺, the mutant strain transformed with the empty plasmid and with PpHAK2 grew similarly well. In contrast, at 5 and 10 mM K⁺, the strains expressing PpHAK2 grew significantly better than the control. In contrast, at pH 6.0, 5 mM K⁺, strain LMM04 showed a slightly decreased growth rate that was not improved by PpHAK2 (Fig. 3). PpHAK3 did not suppress the defect of either LMB01 or LMM04. Frequently, plant HAK transporters are not functional in yeast and mutations have to be introduced to observe their function (Rubio et al. 2000, Garcia-debla´s et al. 2007). To date, we have not determined whether there are mutations that activate PpHAK3 in yeast cells.

**ΔPphak2 plants are strongly affected by K⁺ starvation; ΔPphak3 does not show a distinctive phenotype**

To elucidate the in planta roles of PpHAK2 and PpHAK3, we decided to construct ΔPphak2, ΔPphak3 and ΔPphak2 ΔPphak3 plants, using the disruption fragments shown in Supplementary Fig. S3. We obtained many ΔPphak2 lines and isolated five for further study, but the disruption of the PpHAK3 gene proved to be extremely difficult both in wild-type and in ΔPphak2 lines. In our hands, this occurs with some P. patens genes and is not necessarily associated with an indispensable function of the gene. After many attempts, we isolated two ΔPphak2 ΔPphak3 lines but no ΔPphak3 line. These seven lines were named PpHAK2-(1–5) and PpHAK3-DPpHAK3-(1–2). In all of these lines, the disrupted gene did not exist in the genome and gene transcripts were not detected.

In normal medium or modified media, involving increased NaCl concentrations or altered pH values (ranging from 4 to 7), ΔPphak2 plants grew normally, without any observable differences from wild-type plants. In contrast, under K⁺ starvation in liquid medium, ΔPphak2 plants showed clear signs of stress, with abundant production of a brown pigment, and cellular and developmental changes (Fig. 4). This response was independent of whether the liquid medium was inoculated with protonemata or gametophores, and was never observed in wild-type plants.

When wild-type protonemata were transferred to K⁺-free medium, the plants grew normally for 4–6 d, producing gametophores of 4–6 leaves. The growth then slowed down, but the plants maintained their green color and normal appearance, exhibiting many gametophores with 10–12 leaves after 10–15 d (Fig. 4A–C). During this period, the mass of the plants duplicated, and, consequently, the K⁺ content of the
plants decreased by a half per unit mass (Fig. 5). The Chl content was normal during the first 8 d and then started decreasing rapidly (Fig. 6). In contrast to this response, soon after the transfer of ΔPphak2 protonemata to K⁺-free medium, the medium started becoming brown (Fig. 4A). After 4–6 d, gametophores were absent and, 2 or 3 d later, there were many chloronemal filaments showing brown tip cells and brown caulonemata with buds that eventually became brown cell clusters unable to produce leaves. Interestingly, some gametophores with 2–4 leaves on brown stems were observed (Fig. 4D). The brown pigment of the liquid cultures was associated with solid material that was solubilized only by treatment with alkali, which suggested that it was a cell wall-bound phenolic compound (Semerdjieva et al. 2003). Compared with wild-type plants, the decrease of the K⁺ content per unit mass in ΔPphak2 plants was slower during K⁺ starvation (Fig. 5), which was compatible with a slower growth and a slower dilution of the plant K⁺ content. The decrease of the Chl content of ΔPphak2 plants was continuous, starting almost immediately after transfer to K⁺-free medium, in a manner that was also very different from wild-type plants (Fig. 6).

As for ΔPphak2 plants, we applied a battery of growth tests to ΔPphak2 ΔPphak3 plants, including high K⁺ or Na⁺ concentrations, high and low pH values, high Ca²⁺ concentrations and the combination of some of them. In all of these tests, ΔPphak2 ΔPphak3 plants behaved exactly as wild-type and ΔPphak2 plants. Then we tested K⁺ starvation in ΔPphak2 ΔPphak3 and ΔPphak2 plants in parallel, finding exactly the same
response for the two lines. In summary, the ΔPphak3 mutation was silent, at least in ΔPphak2 plants.

**Further characterization of ΔPphak2 plants**

To continue the characterization of ΔPphak2 plants, we first investigated whether brown cells were energetically competent by measuring K⁺ uptake after 20 d of K⁺ starvation in wild-type and ΔPphak2 plants. In these plants, K⁺ uptake is very fast, which requires rapid ATP generation (Rodríguez-Navarro 2000) and a functional plasma membrane. The test revealed that there were no significant differences between wild-type and mutant plants, which indicated that most brown cells were energetically competent and maintained a functional plasma membrane after 10 d of K⁺ starvation (Supplementary Fig. S4).

At the ultrastructural level, K⁺ starvation produced important changes in ΔPphak2 plants. After 10 d of K⁺ starvation, the cells from wild-type plants maintained an unaltered overall structure (inset in Fig. 7C); short strands of ER also had a normal appearance (Fig. 7C). In contrast, Pphak2 mutant plants showed an altered structure soon after K⁺ removal. After 6 d of K⁺ starvation, ΔPphak2 plants contained two types of cells with different subcellular arrangements. One of these types showed a dense cytoplasm with abundant ribosomes, mitochondria and lenticular-shaped chloroplasts (inset in Fig. 7A); long cisternae of ER were clearly recognized (Fig. 7A). The other type displayed a collapsed cytoplasm, clustering the organelles together (inset in Fig. 7B). The chloroplasts lost their typical structure, presenting an irregular shape and curved thylakoids (Fig. 7B); long strands of ER cisternae surrounding organelles and connecting cells through plasmodesmata were also observed (Fig. 7B). The most striking morphology was observed in the cells of ΔPphak2 plants after 10 d of K⁺ starvation, with shrunken protoplasm, severe collapse of the organelles (inset in Fig. 7D), long strands of ER between the organelles (Fig. 7D) and round-shaped chloroplasts (Fig. 7D).

To determine the causes of the sensitivity of ΔPphak2 plants to K⁺ starvation, it was necessary to consider that K⁺ starvation in our K⁺-free medium produced more effects than only a decrease in the plant K⁺ content. First, the process could involve Cl⁻ starvation because the Cl⁻ concentration was greatly reduced in the K⁺-free medium (to 4 μM, added as MnCl₂), and, secondly, the decrease of the cytosolic K⁺ concentration runs in parallel with the decrease of the cytosolic pH (D. J. Walker et al. 1996, Walker et al. 1998). To investigate which of these decreases was responsible for the detrimental effect observed under K⁺ starvation, we performed growth experiments in K⁺-free medium supplemented with NaCl (4 mM), NH₄Cl (4 mM), Na₂SO₄ (2 mM), K₂SO₄ (2 mM) and MgCl₂ (10 and 20 mM). The results of these experiments (Table 1; Supplementary Fig. S5) demonstrated that the Cl⁻ concentration in the growth medium was not related to the defects of the ΔPphak2 plants and that Na⁺ corrected the problem as effectively as K⁺. During K⁺ starvation in the presence of 4 mM Na⁺, the K⁺ content decreased similarly in wild-type and ΔPphak2 plants (Fig. 5B); in both cases, the sum of the plant Na⁺ and K⁺ contents matched the K⁺ content of plants growing in 4 mM K⁺, which suggested that the cytosolic pH did not decrease in the presence of Na⁺. Under these conditions, ΔPphak2 plants were completely normal and indistinguishable from wild-type plants (Supplementary Fig. S5). The partial correction of the defect by Mg²⁺ when the concentration was sufficiently increased, >20 mM, might be a Na⁺-mimetic effect due to the large Mg²⁺ concentration. Taken together, these results suggested that a decrease in the cytosolic pH caused the defect in the ΔPphak2 plants (Supplementary Fig. S5).

This conclusion prompted us to test the effect of acetic acid in ΔPphak2 plants. Weak acids, such as acetic, propionic or benzoic, acidify the cytoplasm of microorganisms because the non-dissociated molecules that are abundant at low external pH values cross the membrane and dissociate at the neutral cytosolic pH. This effect has been extensively studied in yeast cells (Macpherson et al. 2005, Mira et al. 2010) and has also been described in plant cells (Brunner et al. 1984, Guern et al. 1986, Dunlop et al. 1988). In P. patens, acetic acid was in fact toxic; 5 mM acetic acid inhibited the growth in 4 mM K⁺, pH 5.6 liquid KFM. After 2 or 3 d, the culture was clearly blanching, and, after 1 week, the cultures had lost most of their green color and were nearly transparent. These symptoms were the same for wild-type and ΔPphak2 plants; in both cases, no brown pigment was produced.

In addition to this general effect, low acetic acid concentrations inhibited the growth of ΔPphak2 plants in a K⁺
**Fig. 7** Ultrastructure of protonema cells from wild-type and ΔPphak2 knockout lines. Electron microscope images at low (insets) and high (main panels) magnifications. Two types of cell morphologies were observed at 6 d under K⁺ starvation in ΔPphak2 plants (A, B); (A) a long cisterna of ER (arrows) can be seen next to the cell nucleus (n) in a ribosome-dense cytoplasm (ct) also containing mitochondria (m) and chloroplasts (cl) filled with starch (s) (inset); (B) a general collapsed structure with irregularly shaped chloroplasts (cl), which display unparalleled distribution of thylakoids. Abundant ER cisternae (arrows) are seen between the organelles and connecting neighbor cells through plasmodesmata (pl). After 10 d under K⁺ starvation, wild-type plants (C) showed cells with the typically turgid morphology, pushing the nucleus (n) and cytoplasmic organelles towards the cell wall, as compared with cells from ΔPphak2 plants after 6 d under K⁺ starvation, (inset) and the ER strands (arrows) looked shorter (main panel). After 10 d under K⁺ starvation, ΔPphak2 plants (D) showed more severe morphological changes; long ER strands (arrows) were clearly recognized in between round-shaped chloroplasts (cl). Bars in insets = 1 μm; bars in main images = 0.5 μm.
concentration-dependent manner, producing a toxic response that was indistinguishable from that of K⁺ starvation. In liquid KFM containing 1 mM acetic acid and 0.4 mM K⁺ at pH 5.6, the response of Δpphak2 plants was identical to that shown in Fig. 4 for K⁺ starvation, while wild-type plants grew normally (Supplementary Fig. S5). During the browning process, Δpphak2 plants maintained the normal K⁺ content that they had before adding the acetic acid (in three experiments, 2,868 ± 318 and 2,905 ± 403 nmol mg⁻¹ DW before and after 20 d of treatment, respectively). In the same medium with 4 mM K⁺, Δpphak2 plants showed no signs of toxicity. This suppression of the acetic acid toxicity by the increase in the K⁺ concentration in the external medium might be explained because this increase enhances K⁺ uptake and, subsequently, the K⁺/H⁺ exchange, which alleviates the decrease of cellular pH.

### Discussion

The possibility that some HAK-KUP transporters function in internal membranes was proposed several years ago (Senn et al. 2001). This proposal was later supported by the apparent localization of the rice HAK10 (Bañuelos et al. 2002) and several Arabidopsis KUPs (Whiteman et al. 2008) to the tonoplast, and AtKUP12 to the thylakoid membrane (Peltier et al. 2004). One of these Arabidopsis transporters, TRH1, shows a double localization to the plasma membrane and tonoplast (Rigas et al. 2013). The results of the current study lend further support to that notion because we obtained physical evidence showing that the PpHAK2 localizes to the ER membrane and PpHAK3p to the Golgi complex. It is worth noting that the expression of the two HAK-GFP fusion genes was controlled by the PpACTS promoter, which shows a transcript expression very similar to those of the PpHAK2 and PpHAK3 genes (Fig. 1).

Considering the results obtained with the yeast kha1 mutant (Fig. 3), the function that seems most likely for PpHAK2 is the exchange of K⁺ and H⁺ in internal membranes. A similar conclusion has been reported previously for some Arabidopsis CHX transporters (Maresova and Sychrova 2006, Chanroj et al. 2011). Although this function is not consistent with the K⁺–H⁺ symport function that is normally assigned to plasma membrane HAK transporters (Rodríguez-Navarro 2000), the mechanistic model is currently of secondary importance because it is not clearly established even for the CHX transporters (Mottaleb et al. 2013).

The only defect of Δpphak2 plants was their evident incapacity to support a K⁺-free medium, in which they suffered a large number of cellular and developmental defects (Figs. 4, 7). However, although this response is clear, the physiological basis of the response may be complex. In the first place, K⁺ starvation implies a decrease of both the cytosolic K⁺ concentration and pH (Ramos et al. 1990, N.A. Walker et al. 1996, Walker et al. 1998). Although either of these two decreases might cause the defect of the mutant plants, the suppression of the K⁺ starvation defect by Na⁺ indicates that only cytoplasmic acidification was the cause of the defect. The rationale of this conclusion is that in the presence of Na⁺, K⁺ starvation results in the substitution of Na⁺ for K⁺, which prevents a decrease in the cytoplasmic pH although the decrease of the K⁺ content is very important. Furthermore, the intolerance of Δpphak2 plants to acetic acid at low pH also points to the intolerance of Δpphak2 plants to a decrease of the cytoplasmic pH, because this treatment did not decrease the K⁺ content. As described above, weak acids, such as acetic, propionic or benzoic, acidify the cytoplasm of cells. Remarkably, acetic acid concentrations that were toxic in wild-type plants produced the same toxic effect in Δpphak2 plants. In contrast, mild acetic acid treatments that were not toxic to wild-type plants reproduced the effects of K⁺ starvation in Δpphak2 plants. Taken together, these results suggest that Δpphak2 plants do not tolerate decreases of the cytosolic pH that are innocuous for wild-type plants.

Assuming this defect, the localization of PpHAK2 to the ER membrane indicates that the function of PpHAK2 is necessary to maintain the function of the ER when the cytosolic pH decreases. Environmental conditions that impair the ER function activate a signaling pathway termed the unfolded protein response. If this response is unable to restore the ER function, cell death is activated (Liu and Howell 2010). After 6 d of K⁺ starvation, Δpphak2 plants changed their overall cell ultrastructure.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Differential appearance</th>
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<tr>
<td>Standard</td>
<td>No difference, good growth (as in the wild-type in Fig. 4)</td>
</tr>
<tr>
<td>High NaCl, pH variation from 4.0 to 7.0</td>
<td>No difference, good growth (as in the wild-type in Fig. 4)</td>
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<tr>
<td>K⁺-free medium (i.e. K⁺ and Cl⁻ starvation)</td>
<td>Stress and cell death (shown in Fig. 4)</td>
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<tr>
<td>K⁺-free medium plus 4 mM NaCl</td>
<td>No difference, good growth (as in the wild-type in Fig. 4)</td>
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<tr>
<td>K⁺-free medium plus 2 mM Na₂SO₄</td>
<td>Stress and cell death (as in Fig. 4)</td>
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<tr>
<td>K⁺-free medium plus 4 mM NH₄Cl</td>
<td>Stress and cell death (as in Fig. 4)</td>
</tr>
<tr>
<td>K⁺-free medium plus 10 mM MgCl₂</td>
<td>Stress and cell death (as in Fig. 4)</td>
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<tr>
<td>K⁺-free medium plus 20 mM MgCl₂</td>
<td>Stress and cell death (as in Fig. 4)</td>
</tr>
<tr>
<td>0.4 mM K⁺ medium pH 5.6, 1 mM acetic acid</td>
<td>Stress and cell death (as in Fig. 4)</td>
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and particularly the shape and internal organization of the chloroplasts (Fig. 7B, D) in a manner not seen in wild-type plants even after much longer K⁺ starvation periods (Fig. 7C). In addition to these changes, the frequent observation of continuity in individual ER cisternae in thin sections can be related to their abundance and thickness. The long ER strands observed in ΔPphak2 plants were not observed in wild-type plants, in which only short ER portions were identified. These results suggest that K⁺-starved ΔPphak2 plants enter into a process of cell disorganization and cell death. In this process, in addition to other changes, the ER is overdeveloped. Interestingly, ΔPphak2 plant cells maintained many physiological capacities for quite a long time because they almost maintained intact their capacity to transport K⁺ after 10 d of K⁺ starvation (Supplementary Fig. S4).

Taken together, these observations are consistent with the hypothesis that the luminal pH of the ER in ΔPphak2 plants is affected when the cytosolic pH is acidic. It is likely highly that the ER pH is established similarly to that of organelles of the secretory pathway, i.e., a steady state that results from H⁺ pumping and leakage, K⁺/H⁺ exchange, and K⁺ and Cl⁻ conductances (Demarex 2002, Paroutis et al. 2004, Casey et al. 2010). In this model, PpHAK2 might mediate the return of H⁺ to the cytosol. Apparently, no other transporter can mediate this function when the cytosolic pH is acidic, but other pathways exist when the cytosolic pH is normal. The possibility that this return occurs as either K⁺/H⁺ antiport or K⁺−H⁺ symport is discussed in Mottaleb et al. (2013).

Although this reasoning might explain the basic response, it does not imply that the defect of ΔPphak2 plants requires an extensive decrease of the cytosolic pH. In fact, the decrease of the chl content started very soon after the transfer of the mutant plants to the K⁺-free medium (Fig. 6). As a matter of fact, P. patens plants do not lose K⁺ in a K⁺-free medium, and plant growth is the only cause of the decreased K⁺ content. Because growth is slow and the buffering capacity of the cellular components is high, it is unlikely that the transfer of ΔPphak2 plants to a K⁺-free medium produced an important decrease in the cellular pH in the short term. Similarly, the detrimental effect of acetic acid disappeared when the K⁺ concentration of the medium was increased from 0.4 to 4 mM, although this increase cannot substantially affect the cellular pH or the K⁺ content. These two observations indicate that the pH change that triggers the browning process might be very small. Perhaps a pH signal is incorrectly processed if PpHAK2 is not functional.

The absence of a distinctive phenotype of ΔPphak2 ΔPphak3 vs. ΔPphak2 plants does not allow the function of the PpHAK3 transporter to be discussed. PpHAK3 localizes to the Golgi complex and, in principle, the above-mentioned function of returning H⁺ from the Golgi lumen to the cytosol might apply to PpHAK3. As for PpHAK2 in normal growth conditions, other transporters substitute the function of PpHAK3 in all of the conditions that we tested; therefore, the mutation seems harmless. One of these transporters might be PpCHX1, which co-localizes with PpHAK3 (compare fig. 2B with fig. 2A in Mottaleb et al. 2013); among NHX transporters, there are also substitute candidates. In Arabidopsis, NHX5 and NHX6 are associated with the Golgi and trans-Golgi network (Bassil et al. 2011), and two NHX transporters in P. patens show high sequence homology with AtNHX5 and AtNHX6 (Chanroj et al. 2012).

It might be possible that CHX, HAK, and NHX transporters work in endomembranes performing functions that, although not identical, can individually be substituted for the function of other (or several) transporters when they are missing in a mutant. This can be seen for the ER transporter AtCHX23: this transporter seems to be involved in the reception or transduction of ionic signals that are critical to direct pollen growth towards the ovule. However, the mutation is physiologically deleterious only in chx21 plants, and the double mutant pollen is normal seen in the early phases of tube growth and guidance (Lu et al. 2011). The impaired fertility of chx21 chx23 Arabidopsis plants has also been explained by the failure of K⁺ homeostasis in the female gametophyte (Evans et al. 2012), but the notion that AtCHX23 is able to substitute for the function of AtCHX23 in chx23 plants is not affected by this alternative explanation.

The most relevant conclusion of this study is the notion that HAK transporters are expressed in endomembranes and that this family of transporters performs functions that are more extensive than K⁺ uptake through the plasma membrane. These findings open up new lines of research. For example, the shy3-1 (Elumalai et al. 2002) and trh1 (Rigas et al. 2001, Rigas et al. 2013) Arabidopsis mutants, in which two members of the HAK family of transporters are involved, show developmental defects that are not easily explained by a defective K⁺ uptake. These mutants should be re-examined addressing the location of the corresponding HAK wild-type proteins and considering the possibility that their functions might be necessary for the correct functioning of organelles.

**Materials and Methods**

**Plants and growth conditions**

The moss *P. patens* was routinely grown in BCDAT medium (Ashton et al. 1979), supplemented with 7 g l⁻¹ agar when required, as described elsewhere (Garciadeblas et al. 2007). Physiological tests were performed in liquid medium with continuous white light (Haro et al. 2010). Plants were normally grown in glass bottles with air bubbling in a phytochamber at 25°C. The bottles were inoculated with moss suspensions that were fragmented using a Polirion PT2100 homogenizer (Kinematica AG). KFM is a K⁺- and Na⁺-free medium, pH 5.8 (Garciadeblas et al. 2007) that was used for K⁺ starvation and supplemented with K⁺ or Na⁺ for growing plants at controlled cation concentrations. Control plants were grown in KFM with 4 mM KCl. K⁺-starved plants were obtained by transferring moss plants grown for 1 week in KFM medium supplemented
with 0.4 mM KCl to K⁺-free medium. The inocula with which we started all experiments were almost exclusively protonemata.

### Yeast strains and plasmids, media and growth conditions

The yeast strains LMB01 (Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mally1 ena1::HIS3::ena4A nha1::LEU2 kha1::kanMX) and LMM04 (Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mally1 ena1::HIS3::ena4A nha1::LEU2 trk1::LEU2 trk2::HIS3 kha1::kanMX::tok1::A) (Maresova and Sychrova 2005) were used for functional tests. The full-length PpHAK2 and PpHAK3 cDNAs were cloned into vector pYPGE15 (Brunelli and Pall 1993) have been previously described (Garcia-Deblás et al. 2007). Yeast transformants were routinely grown in SD medium (Sherman 1991) supplemented with 50 mM K⁺. For growth experiments, the yeast strains were inoculated on arginine phosphate (AP) medium (Rodríguez-Navarro and Ramos 1984) supplemented with the indicated K⁺ concentrations and with 10 mM MES-Ca²⁺ for pH 6.0, 10 mM tartaric acid-Ca²⁺ for pH 4.5 or 10 mM HEPES-Ca²⁺ for pH 7.5. The growth of yeast cells was monitored by recording the absorbance at 540 nm in a Microbiology Reader Bioscreen C workstation (Growth Curves Oy), which enables many samples to be run at the same time.

### Recombinant DNA techniques

The E. coli strain DH5α was routinely used for plasmid DNA propagation. The manipulation of nucleic acids was performed by standard protocols or, when appropriate, according to the manufacturer’s instructions. Total Physcomitrella RNA was prepared using the RNeasy Plant Kit and DNeasy Plant Kit (Qiagen USA, http://www.qiagen.com). The PCR amplification of cDNAs was performed with a double-stranded cDNA Synthesis System Kit (GE Healthcare, http://www.gehealthcare.com). PCR was performed in a Perkin-Elmer thermocycler, using the Expand-High-Fidelity PCR System (Roche Molecular Biochemicals, http://www.roche-applied-science.com). The resulting PCR fragments were first cloned into the PCR2.1-Topo vector and verified by sequencing. For yeast expression, the cDNA fragments were cloned into vector pYPGE15 (Brunelli and Pall 1993) and transformed into the yeast cells.

### Real-time PCR assays

Quantitative real-time PCR (qRT-PCR) of derived cDNA was performed using the TaqMan® probe with a FAM™ dye label (Roche Applied Science) for absolute quantification, as previously described (Fraile-Escanciano et al. 2010). Primers and probes for each gene assay were designed using the Universal Probe Library Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html). The PCR primers were designed to amplify the following fragments numbered as cDNA: PpHAK2 (60 nucleotide amplicon, from nucleotide 1,494 to 1,553, probe #75), PpHAK3 (72 nucleotide amplicon, from nucleotide 1,972 to 2,043, probe #71), PpHAK4 (75 nucleotide amplicon, from nucleotide 1,060 to 1,135, probe #10) and PpAct5 (87 nucleotide amplicon, from nucleotide 1,248 to 1,334, probe #55). The reported results are the transcript level ratios between the studied HAK gene and the ACT5 gene from the same cDNA preparation. All samples were analyzed in two PCR runs in which all samples were performed in triplicate.

### Localization of PpHAK2–GFP and PpHAK3–GFP

Localization of the PpHAK2–GFP and PpHAK3–GFP fusion proteins in P. patens was performed by transient expression in protoplasts. The PpHAK2–GFP and PpHAK3–GFP genes were cloned under the control of the PpACT5 promoter containing a large intron in the 5’ UTR. The expression plasmid, pRHACT5, was constructed from the PCR2.1-Topo vector. For this purpose, the 5’ UTR-PpAct5 fragment was amplified by PCR using the primers published by Weiss et al. (2006): number 13 as the forward primer and number 14 as the reverse primer; the latter was modified to contain a BamHI cloning site at the 3’ end of the primer. Then, a BamHI– HindIII fragment from the pMF6 vector containing the GFP nos-3’ terminator (Rubio-Somoza et al. 2006) was cloned downstream of the PpACT5 promoter. Finally, the PpHAK2 and PpHAK3 cDNA were cloned in-frame into the BamHI site at the 5’ end of the GFP gene. Experiments of co-localizations were performed with organelle markers used in others plants (Nelson et al. 2007; http://www.bio.utk.edu/cellbiol/markers/). The ER fluorescent marker combines the signal peptide of an ER protein of Arabidopsis at the N-terminus of the YFP and the ER retention signal His-Asp-Glu-Leu at its C-terminus. The Golgi marker was based on the fusion of YFP with the cytoplasmic tail and transmembrane domain (first 49 amino acids) of GmMan1, soybean α-1,2-mannosidase I (Saint-Jore-Dupas et al. 2006).

The resulting construct was used for transient expression in P. patens protoplasts. After transformation, the protoplasts were kept in the dark for 24 h in BCDAT medium supplemented with 6% mannitol and 5% glucose, followed by
cultivation in the same medium for 3–4 d under normal growth conditions.

Protoplasts were co-transformed with either PpHAK2–GFP and the ER marker tagged with YFP or PpHAK3–GFP and a Golgi marker tagged with YFP. Z-series of protoplasts were obtained on a Leica TCS SP8 confocal microscope (Leica Microsystems). The GFP signal and Chl autofluorescence were collected simultaneously under the laser excitation lines of 488 and 633 nm, respectively. To rule out any cross-talk between GFP and YFP, the YFP signal was collected sequentially (sequential scan) under the laser excitation line of 514 nm. Images were processed using the LAS AF Lite 3.1.0 (Leica Microsystems).

Generation of \( \Delta PpHAK2 \) and \( \Delta PpHAK3 \) knockout lines

The pTN82 vector (T. Nishiyama, http://moss.nibb.ac.jp/) was used to construct the PpHAK2:Neo knockout fragment (Supplementary Fig. S3). In this fragment, the neomycin resistance cassette, which contained the \( nptII \) gene under the control of the promoter and terminator of the 35S gene, was flanked by two fragments of 924 and 854 bp of the \( ntpII \) resistance cassette, which contained the \( \text{neomycin} \) used to construct the PpHAK2:Neo knockout fragment.

Generation of \( \Delta PpHAK2 \) and \( \Delta PpHAK3 \) knockout lines

The pTN82 vector (T. Nishiyama, http://moss.nibb.ac.jp/) was used to construct the PpHAK2:Neo knockout fragment (Supplementary Fig. S3). In this fragment, the neomycin resistance cassette, which contained the \( nptII \) gene under the control of the promoter and terminator of the 35S gene, was flanked by two fragments of 924 and 854 bp of the PpHAK2 gene. These fragments were amplified by PCR using primers that included the restriction enzymes sequences to direct the cloning into the corresponding sites of the pTN82 vector polylinker. The \( 5' \) fragment extended from nucleotide 9 to 928 and was inserted between the \( SalI \) and \( HindIII \) sites; the \( 3' \) fragment extended from nucleotide 3,281 to 4,139 and was inserted into the \( BamHI \) site. The \( \Delta PpHAK2 \) knockout lines were generated by transforming \( P. \ patens \) protoplasts with 25 μg of the linear fragments obtained by digesting the pTN82:PpHAK2 plasmid with the \( SalI \) and \( NotI \) restriction enzymes. Stable antibiotic-resistant clones were selected after two rounds of incubation in BCDAT medium supplemented with 30 μg ml\(^{-1} \) of neomycin.

The pTN86 vector (T. Nishiyama, http://moss.nibb.ac.jp/) was used to construct the PpHAK3:Hyg knockout fragment (Supplementary Fig. S3). In this fragment, the hygromycin-B resistance cassette, which contained the \( aph4 \) gene under the control of the promoter and terminator of the 35S gene, was flanked by two fragments of the non-coding \( 5' \) and \( 3' \) regions of the PpHAK3 gene. These fragments were amplified by PCR using primers that included the restriction enzyme sequences to direct the cloning into the corresponding sites of the vector polylinker. The \( 5' \) fragment extended from nucleotide positions 3 to 877 of the ATG and was inserted between the \( SalI \) sites; the \( 3' \) fragment extended from positions 4,481 to 5,296 and was inserted between the restriction sites \( BamHI \) and \( SacI \). The \( \Delta PpHAK3 \) knockout lines were generated by transforming \( P. \ patens \) protoplasts with 25 μg of the linear fragment obtained by digesting the pTN86:PpHAK3 plasmid with the \( Apol \) and \( SacI \) restriction enzymes. Stable antibiotic-resistant clones were selected after two rounds of incubation in BCDAT medium supplemented with 30 μg ml\(^{-1} \) of hygromycin-B.

The screening of putative disrupted clones was performed by PCR on genomic DNA purified from transformant plants. Four independent PCRs were performed to confirm that both the \( 5' \) and \( 3' \) insertion sites were sequentially correct. For both sites, one primer corresponded to a chromosomal fragment outside the knockout construction and was used in both PCRs; of the other two primers, one was specific for the marker and the other for the wild-type gene (Supplementary Fig. S3; Supplementary Table S1). Clones in which the sequences of the knockout insertions were correct and the fragments of the wild-type gene could not be amplified were selected.

Cation uptake experiments and cation contents

Uptake experiments were performed in \( K^+ \)-starved cultures of \( P. \ patens \) suspended in KFM. After the addition of the tested cation, the depletion from the external medium was followed by atomic emission spectrophotometry (Bañuelos et al. 2002). To determine the \( K^+ \) content, samples of \( \text{Physcomitrella} \) were transferred to a filter, washed, weighed, and extracted with 0.1 M HCl (Garcia-Deblás et al. 2007).

All experiments were repeated at least three times. The variability of the experimental results using different plant or yeast batches was low. Although not recorded, the standard deviations of the kinetic parameters were <20% of the mean.

Determination of total Chl

Chl was determined by absorption spectrophotometry in aqueous acetone extracts of Chl (Arnon 1949, Mackinney 1941). Samples of 5–25 mg of fresh tissues were extracted with 1.5 ml of 80% acetone in an Eppendorf tube for 1 h on ice in the dark. The concentration of Chl \( a \) and \( b \) in the extracts were determined at 663 and 645 nm, respectively, using the specific coefficients given previously (Mackinney 1941; total Chl in mg ml\(^{-1} \) = 20.2 \( A_{665} \) + 8.02 \( A_{645} \)).

Electron microscopy

Wild-type and \( \Delta PpHAK2 \) plants were fixed in a mixture of 4% formaldehyde and 5% glutaraldehyde (GS882-50ML, Sigma) in PBS (phosphate-buffered saline: 137 mM NaCl; 0.27 mM KCl; 1 mM phosphate buffer, pH 7.4, Sigma P4417). A fresh formaldehyde solution was prepared from paraformaldehyde powder (Sigma P6148-500G). Small pieces of the specimens were sliced and fixed under vacuum until the samples sank in the fixative solution, then were left overnight at 4°C in the same solution. Next, the fixed material was washed in PBS and dehydrated in a graded ethanol series: 30, 50, 70, 90 and 100%. The dehydrated material was progressively infiltrated in the resin LR White Resin Medium (Agar Scientific), containing 2% benzoyl peroxide (BS907, Sigma) as catalyst, in a series of mixtures (3:1, 1:1, and 1:3) of 100% ethanol:LR white for 1 h each, at 4°C. Finally the specimens were maintained in pure resin overnight at the same temperature. Polymerization in gelatin capsules was performed at 60°C for 24 h. Sections of 70–80 nm were cut in a...
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Disclosures

The authors have no conflicts of interest to declare.

References


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