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Potassium channels in barley: cloning, functional characterization and expression analyses in relation to leaf growth and development

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ABSTRACT

It is not known how the uptake and retention of the key osmolyte K^+ in cells are mediated in growing leaf tissue. In the present study on the growing leaf 3 of barley, we have cloned the full-length coding sequence of three genes which encode putative K^+ channels (*HvAKT1*, *HvAKT2*, *HvKCO1*/*HvTPK1*), and of one gene which encodes a putative K^+ transporter (*HvHAK4*). The functionality of the gene products of *HvAKT1* and *HvAKT2* was tested through expression in *Xenopus laevis* oocytes. Both are inward-rectifying K^+ channels which are inhibited by Cs^+. Function of HvAKT1 in oocytes requires co-expression of a calcineurin-interacting protein kinase (*At*CIPK23) and a calcineurin B-like protein (AtCBL9) from *Arabidopsis*, showing cross-species complementation of function. *In planta*, *HvAKT1* is expressed primarily in roots, but is also expressed in leaf tissue. *HvAKT2* is expressed particularly in leaf tissue, and *HvHAK4* is expressed particularly in growing leaf tissue. Within leaves, *HvAKT1* and *HvAKT2* are expressed predominantly in mesophyll. Expression of genes changes little in response to low external K^+ or salinity, despite major changes in K^+ concentrations and osmolality of cells. Possible contributions of *HvAKT1*, *HvAKT2*, *HvKCO1* and *HvHAK4* to regulation of K^+ relations of growing barley leaf cells are discussed.

Key-words: *Hordeum vulgare*; ion channel; leaf elongation; potassium nutrition; single-cell analyses; water relations; *Xenopus laevis* oocytes.

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INTRODUCTION

Cell expansion requires continuous uptake of water, which in turn is driven by osmosis (Fricke 2002). The types of prevailing solute used by cells to generate osmotic pressure can differ between leaf tissues and cell types (Leigh & Tomos 1993; Fricke, Leigh & Tomos 1996; Karley, Leigh & Sanders 2000a), but most cells use K^+ at significant (>50–100 mM) concentrations. In leaf epidermal cells of grasses, K^+ accounts for almost 50% of the cell osmotic pressure (Fricke *et al.* 1994). Most of the K^+ taken up by cells accumulates in the central vacuole. The extent of accumulation depends on the transport properties of both the tonoplast and plasma membrane.

Existing evidence suggests that osmotic pressure is kept constant during cell expansion (Pritchard, Fricke & Tomos 1996; Fricke, McDonald & Mattson-Djos 1997). A leaf cell which expands at a relative rate of 15% h^{-1} and maintains an osmotic pressure of 440 mosmol kg^{-1} has to accumulate solutes at a rate that is equivalent to 66 mosmol kg^{-1} h^{-1}. Up to 50% of this rate may be met through accumulation of K^+. One would expect transport systems which aid movement of K^+ across the plasma membrane to be particularly abundant in growing leaf cells. Surprisingly, there are no studies which have addressed this question in detail at the molecular level. Work on maize coleoptiles has pointed to involvement of Shaker-type K^+ channels and their importance in auxin- and gravitropically induced extension growth (Claussen *et al.* 1997; Philippar *et al.* 1999).

There are two principal routes through which K^+ can be taken up selectively into cells, channels and transporters. The best characterized K^+ channels in plants belong to the family of Shaker-type channels. Shaker K^+ channels were first discovered in the fruit fly *Drosophila* (for recent reviews, see Chérel 2004; Lebaudy, Véry & Sentenac 2007). There are nine different Shaker channels in *Arabidopsis*. All of them are voltage gated with open probabilities increasing either by hyperpolarization (inward rectifiers) or by depolarization (outward rectifiers). Although Shaker channels are expressed in a tissue-specific manner, some

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channels fulfil functions more cell or tissue specific than others. For example, SKOR, GORK and SPIK (AtAKT6) are, respectively, involved in xylem loading of K^+, in guard cell movement (stomatal closure) or pollen tube growth. Channels AtAKT1, AtAKT2, AtKAT1 and AtKAT2 are particularly expressed in roots (where AtAKT1 mediates K^+ uptake from the soil solution; Hirsch *et al.* 1998), in vascular bundles (where AtAKT2 facilitates K^+ loading of phloem; Lacombe *et al.* 2000; Pilot *et al.* 2001; Deeken *et al.* 2002) or in guard cells (AtKAT1, AtKAT2; Pilot *et al.* 2001). Some of these channels must also account for the bulk of K^+ inward current in other leaf tissues (mesophyll: AtAKT1, AtAKT2; Dennison *et al.* 2001). Plant K^+ transporters group into three possibly four major families (Ashley, Grant & Grabov 2006; Gierth & Mäser 2007; Grabov 2007). One family, the KT/KUP/HAK family (different research groups have given different acronyms), is thought to include members with functions in high affinity K^+ uptake (Rubio, Santa-María & Rodríguez-Navarro 2000), for example HvHAK1 in barley roots (Santa-Maria *et al.* 1997) and AtHAK5 in *Arabidopsis* roots, but functions may also be related to transport of auxin (Vicente-Agullo *et al.* 2004) or Na^+ (Takahashi *et al.* 2007).

In a related study on the K^+ relations of growing cells of the developing leaf 3 of barley, we have used the patch clamp technique to characterize K^+ currents in protoplasts from different leaf zones (Volkov *et al.* 2009). Comparison of net K^+ uptake rates with K^+ currents and of current characteristics pointed to the involvement of Shaker-type K^+ inward rectifiers in K^+ uptake under K^+-replete conditions, and the necessity of active transport systems under K^+ deficiency. There are no full-length sequences of Shaker-type inward-rectifying K^+ channels in barley. Therefore, the aim of the present study was first to obtain full-length sequences, by screening an in-house-created barley cDNA library, and then to analyse the function of the cloned channels using expression in *Xenopus laevis* oocytes (Miller & Zhou 2000). To test for involvement of other transport systems which might facilitate uptake and retention of K^+ in (growing) leaf cells, the cDNA library was screened for KCO1- (TPK1-, Czempinski *et al.* 2002; Véry & Sentenac 2002; Gobert *et al.* 2007) and HAK-related genes. Tissue localization of expression and changes in expression in response to K^+ treatments [K^+ limitation, low K^+:Na^+ ratios (salinity)] was analysed by *in situ* RT-PCR and semiquantitative or real-time RT-PCR, respectively. Potassium concentrations and osmolality were analysed at the cellular level, using established single-cell analysis techniques (Tomos *et al.* 1994; Fricke & Peters 2002). This made it possible to relate changes in gene expression to changes in the 'end product' of channel and transporter activity relevant to cell elongation.

MATERIALS AND METHODS

Plant growth

Barley [*Hordeum vulgare* L. cv. Golf (Svalöf Weibull AB, Svalöv, Sweden)] seeds were bubbled overnight in distilled water, germinated on 0.5 mM $CaSO_4$ for 6 d and then grown for a further 7–9 d on modified Hoagland nutrient solution (control plants; Fricke *et al.* 1997) before being analysed at an age of 14–16 d. At this developmental stage, leaf 3 was elongating at 2–3 mm h^{-1} and was 12–18 cm long, of which 7–8 cm was enclosed in and 5–11 cm emerged from the sheath of leaf 2. Plants were grown in a growth chamber (Microclima MC1000HE; CEC Technology, Glasgow, UK) at 21 °C day and 15 °C night temperatures, and photosynthetically active radiation of 350–400 μmol photons m^{-2} s^{-1}. Relative humidity was 70%, and the light/dark periods lasted 16/8 h, respectively.

Nutritional and salt treatments

The nutrient solution used for growth of 'control' plants contained 2 mM KNO_3, 0.5 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.5 mM $NH_4H_2PO_4$, 1 mM $(NH_4)_2HPO_4$, 0.5 mM NaCl, 12.5 μM H_3BO_3, 0.25 μM $CuSO_4$, 1 μM $MnSO_4$, 1 μM $ZnCl_2$, 0.38 μM Na_2MoO_4 and 54 μM FeNaEDTA.

The aim of nutritional treatments was to reduce external K^+ supply, to an extent that plants were limited in K^+ and forced to remobilize internal K^+ without showing signs of necrosis or severe reduction in growth. The plants were offered 50 μM KNO_3 (2.5%) rather than the usual 2 mM KNO_3 (100%). To compensate for associated reduction in NO_3^- supply and total cation charge in this 'low-K' nutrient solution, the concentration of $Ca(NO_3)_2$ was increased from 2 to 3 mM. In one K^+ treatment ('2 d low K'), the plants were bubbled overnight and germinated on 0.5 mM $CaSO_4$ as described earlier and then transferred for 5–6 d on control nutrient solution before being transferred 2 d prior to harvest on low-K solution. In the other K^+ treatment ('low-K' plants), the plants were bubbled overnight and germinated on 0.5 mM $CaSO_4$ as described above and then transferred directly on low-K solution and left on this solution for 7–9 d prior to harvest. The low-K solution was not replaced during culture, and it is possible that low-K plants depleted external K^+ within days, well before the harvest date.

The plants were also subjected to stress with 100 mM NaCl (reduced external K^+:Na^+ ratio), for a 3–5 d period (Fricke & Peters 2002) prior to harvest. Salt was applied 1–2 d before leaf 3 emerged from the sheath of leaf 2 and plants were typically 16–17 days old at the time of harvest.

The control plants and the treated plants were always grown in parallel. Averages from at least three independent experiments (= batches of plants) are shown.

Plant harvest

The plants were harvested at the same time of day (around 1000–1200 h, 4–6 h into the photoperiod) to avoid interference from possible diurnal changes in expression of candidate genes. Samples were taken from up to three developmental zones along the elongating leaf 3 of barley: the elongation zone (EZ), non-elongation zone (NEZ) and emerged blade (EmBL) (Fig. 1a). In addition, a sample was

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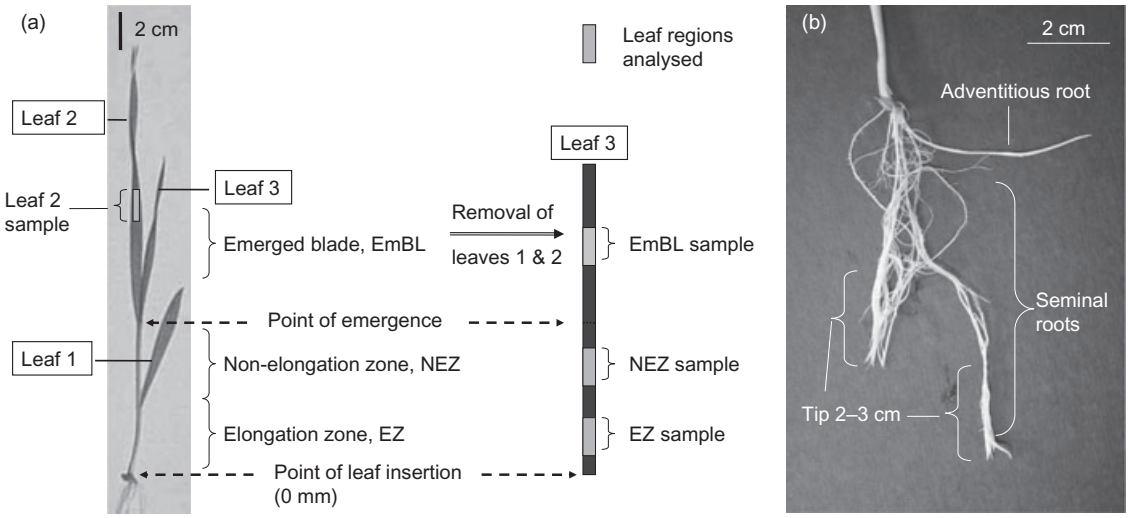

Figure 1. Scheme of a barley plant detailing regions which were analysed. (a) Leaf regions. The elongation zone (EZ), the non-elongation zone (NEZ) and the emerged portion of the blade (EmBL) of the developing leaf 3 were analysed, as was the blade of the mature leaf 2 (L2). The EZ of leaf 3 stretches from about 2 to 40 mm from the point of leaf insertion. Above the EZ is the NEZ, in which cells have ceased elongation, yet show some lateral expansion. Both EZ and NEZ are enclosed by sheaths of older leaves. Above the point of emergence of leaf 3 from the sheath of leaf 2 is the EmBL. It is important to remember that cells are displaced from one region into the next at considerable velocities. A cell which exits the EZ is displaced through the NEZ (which is 30–40 mm long) and into the open air in as little as 10–20 h. Messenger RNA that is transcribed in one leaf region (e.g. EZ) may still be present in the following leaf region(s) (e.g. NEZ) or facilitate, for example light-dependent (EmBL) translation of gene products (see also Richardson *et al.* 2007). (b) Root regions. At the time of harvest, the root system consists mainly of seminal roots; these emerge already during germination; adventitious roots appear when plants are about 13–15 days old. Either entire seminal roots, being enriched in mature tissue, or only the tip 2–3 cm, containing a higher proportion of growing and undifferentiated tissue, were harvested. For details, see Materials and methods, and Results sections.

taken from the mature blade of leaf 2 and from the root. Leaf samples consisted of 2-cm-long segments which were taken from the centre region of the respective zone (Fig. 1a). The root sample consisted of the tip 2–3 cm of seminal roots and included the root EZ (tip 0.5–1 cm) and adjacent root hair zone (Fig. 1b). This root sample presented a small portion (ca. 10–15%) of the root system of plants and lacked any of the older, more mature root tissue that is responsible for the bulk of K^+ uptake. Therefore, in one additional set of experiments for qPCR analyses of expression, entire seminal roots were harvested (Fig. 1b). Samples of a particular region were pooled from 5–10 plants for expression analyses. Single-cell solute concentrations and osmolality were analysed for individual plants (see below).

Isolation of candidate genes

Sequences of primers used in the present study are listed in Supporting Information Table S1.

Contig DNA sequences which showed high homology to *Arabidopsis* AtAKT1 and AtAKT2 were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) or from the Barleybase database (http://www.barleybase.org; Shen *et al.* 2005). As these sequences were principally incomplete 3′ end fragments, primers were developed to produce specific gene fragments. These were used to isolate full-length gene sequences from a whole-plant cDNA library of the barley cultivar Golf as described previously (Richardson *et al.* 2007). For isolation of HvHAK4, partial sequences reported by Rubio *et al.* (2000) were used; for isolation of HvKCO1, the full-length coding sequence reported by Sinnige *et al.* (2005) was used for design of primers.

Nucleotide sequence alignments and analysis were conducted as described by Richardson *et al.* (2007). For construction of phylogenetic trees, protein sequences of barley candidate genes were entered into BLAST search (http://www.ncbi.nlm.nih.gov/blast), and a selection of those sequences which showed highest homologies were aligned using Clustal X, incorporating default settings (Thompson *et al.* 1997). Sequences were aligned using maximum parsimony (MP) analyses with PAUP 4.0b10 (Swofford 2003). Amino acid positions were unweighted, and gaps were coded as missing data. Starting trees were obtained via stepwise addition. Bootstrap analyses included 100 replicates. Tree bisection- and reconnection-based heuristic searches were used in all analyses. Protein sequences used for construction of trees are listed in Supporting Information Table S2; alignments of selected sequences highlighting conserved regions are shown in Supporting Information Fig. S1a–d.

Expression analyses

Total RNA was extracted, and cDNA was synthesized as described previously (Richardson *et al.* 2007). For

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semiquantitative PCR, the reaction was assembled as follows in a total volume of 30 μL: 1× PCR buffer, 0.08 μM dNTP, 0.75 units of *taq* polymerase enzyme mix (all Qiagen, West Sussex, UK), 0.2 μM of both forward and reverse specific primers (Supporting Information Table S1) and 0.6 μL of cDNA template (40 ng of cDNA per reaction). PCR was conducted over a range of cycle number: 23, 25, 27, 29, 31, 33 or 35, so as to ensure that samples in the linear non-saturated PCR phase could be selected for each primer pair. Reaction conditions were 94 °C for 5 min, followed by the required number of cycles of 94 °C for 30 s, primer pair specific temperature for 30 s and 72 °C for 90 s. Then, 12 μL volumes were loaded onto 1% (w/v) agarose gels containing 0.1 μg mL^{-1} ethidium bromide, along with 0.5 μg of 1 kb ladder DNA (New England Biolabs, Herts, UK) to be used for PCR product quantification, and electrophoresed at 100 V for 25 min. Gel images were taken over UV light with a UVP BioDoc-IT imaging system (Ultra Violet Products Ltd, Cambridge, UK) using a standardized set of camera parameters for all gene-specific primers tested (thus allowing intercomparison of data findings) as described previously (Richardson *et al.* 2007). The intensity of the PCR products was quantified using the DocIT LS software package (Ultra Violet Products Ltd) against the five smallest 1 kB ladder bands (0.5 kB = 42 ng, 1.0 kB = 42 ng, 1.5 kB = 36 ng, 2.0 kB = 48 ng and 3.0 kB = 125 ng), and the quantification data exported to Microsoft Excel files.

Real-time PCR (qPCR) was carried out using a Stratagene Mx3000P rapid cycler and SYBR-Green as reagent (Takara Bio Inc., Otsu, Shiga, Japan) on 96-well plates, following the manufacturer's protocol. Each reaction contained 40 ng of cDNA. Four candidate genes were studied (*HvAKT1*, *HvAKT2*, *HvHAK4*, *HvKCO1*), and a further three genes were used as reference of expression [ubiquitin, ATPase, glycerinaldehyde-phosphate dehydrogenase (GAPDH)]. Three plant regions were analysed (root sample consisting of entire seminal root system, EZ of leaf 3 and mature blade of leaf 2), and three replicate reactions were run for each gene and plant region, resulting in a total of ($7 \times 3 \times 3 =$) 63 samples which were run in parallel. Expression was analysed for cDNA samples prepared from three independent batches of plants (= three experiments) as described previously (Wei *et al.* 2007). C_t values of replicate reactions were typically within 0.5 units (e.g. between C_t 27.00 and C_t 27.50). In each run, the average C_t for ubiquitin, ATPase and GAPDH was calculated for each plant region. The individual C_t values of candidate genes were related to these averages (according to plant region) using the ΔC_t ($C_{t[\text{candidate gene}]} - C_{t[\text{reference gene}]}$) method (Pfaffl 2001), resulting in three replicate ΔC_t values and fold changes in expression [$2^{-(\Delta Ct)}$] for each candidate gene, plant region and expression reference. The average of these fold changes was calculated and set to 1.0 in the root (*HvAKT1*) or leaf EZ (*HvAKT2*, *HvHAK4*, *HvKCO1*), and the values for the other plant regions related to this. As a result, one experiment yielded three patterns of expression of a particular candidate gene between plant regions, one based on ubiquitin, one based on ATPase and one based on GAPDH as reference gene. The nine patterns of expression which were obtained from three independent experiments were averaged and used for statistical analyses [*t*-test, analysis of variance (ANOVA)].

Efficiency of qPCR amplification was tested using series of dilutions of cDNA (Wei *et al.* 2007). Previous work (Richardson *et al.* 2007; Wei *et al.* 2007) and additional tests (not shown) showed that ubiquitin, ATPase and GAPDH are by far the most suitable reference genes for this particular study. Further genes which are generally used as reference genes of expression such as actin, tubulin, 18S or 26S-rRNA, elongation factor II, cyclosporine and heat shock protein HSP70 show very large differences in expression, either between root and leaf or between growing and non-growing tissue. Expression of ubiquitin, ATPase and GAPDH differs by only 3% between leaf EZ and mature blade. In the root, expression of reference genes is on average three times higher than in leaf tissue. Although this is not ideal for 'reference' genes, it is the best possible reference we could find in a study where we compared root with shoot, and growing with non-growing tissues. More important, three of the four candidate genes tested (*HvAKT1*, *HvAKT2*, *HvHAK4*) differ in expression between root and leaf far more than threefold (see Results). The fourth gene, *HvKCO1*, is expressed almost twice as high in leaf compared to root tissue; taking higher expression of reference genes in root tissue into consideration, this may result in 50% higher expression of *HvKCO1* in root compared to leaf tissue (this still leaves *HvKCO1* being expressed much more uniformly between plant regions than any other candidate gene tested).

In situ PCR

In situ PCR experiments were performed according to Popova, Dietz & Golldack (2003) with modifications as detailed in Wei *et al.* (2007). Gene-specific sense and antisense oligonucleotide primers amplified a 467–632 bp long fragment on the 3′-end of the different genes that included part of the 3′-non-coding region of the transcript. Negative control reactions were performed in parallel by omitting gene-specific oligonucleotide primers to test background fluorescence signals. Microscopic images were obtained with a cooled CCD camera coupled to an Axioskop fluorescence microscope using filter set 09 (Zeiss, Göttingen, Germany). Images show candidate gene transcripts with green fluorescence.

Functionality test of candidate genes in oocytes

cRNA was synthesized using an mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA), and injected into *X. laevis* stage V and VI oocytes, which were incubated in modified Barth's saline, as described previously (Wei *et al.* 2007). Two days following injection, the oocytes were tested for functionality of expressed candidate genes using

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two-electrode voltage clamp (Johansson *et al.* 2006). The basic bath medium (ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4 adjusted with NaOH. Various K^+ or Na^+ concentrations were achieved by substituting 96 mM NaCl and 2 mM KCl with the corresponding salt, and by adjusting osmotic pressure with sorbitol for 10 mM K^+ and 1 mM K^+ solutions. The pH was adjusted with KOH for bath solution containing 100 mM K-gluconate.

Solute and osmolality analyses

Solute concentrations and osmolality were determined in bulk leaf extracts obtained through a centrifugation technique and in sap extracted from individual epidermal cells of the abaxial epidermis (Fricke 2004, see also Tomos *et al.* 1994). Osmolality was determined by picolitre osmometry (Malone, Leigh & Tomos 1989; Fricke & Peters 2002). Solute concentrations in extracts of epidermal cells were determined by energy-dispersive X-ray (EDX) analysis of picolitre-sized droplets which were placed on a pioloform-coated double folding 100/200-mesh nickel electron microscope grid. Rubidium (Rb) was used as internal standard to account for varying X-ray yield because of topographical variation between pL droplets (Tomos *et al.* 1994). Bulk extracts were analysed using the same technique, as described by Fricke (2004), and results obtained for bulk extracts were within 5% of values obtained through inductively coupled plasma atomic emission spectrometry (checked for K^+; not shown). Grids were washed and freeze-dried in hexane and isopentane, respectively, and analysed with a scanning electron microscope (S-4100; Hitachi, Tokyo, Japan) equipped with an X-ray analyser (Link, Oxford, UK). Nickel, rather than copper, grids were used to avoid that the Cu signal of grids interfered with the Na signal of samples. Standard droplets containing Na^+ and K^+ in the expected range of concentrations were run in parallel on each grid.

Mesophyll osmolality and solute concentrations were calculated from values for epidermal cells and bulk leaf by assuming that the epidermis occupies about 26% of leaf symplast volume and that the mesophyll occupies the majority of the remaining volume (Fricke & Flowers 1998; Fricke 2004).

Statistical analyses

ANOVA (Excel), either one factor or two factors, was used to test for the existence of statistical significance of differences in variables between plant regions and treatments. For those cases where ANOVA showed statistically significant differences, Student's *t*-test (Excel) was used to test for significance in a variable between any pair of samples (e.g. expression of candidate gene between two leaf regions or between two treatments). Statistical data analyses are summarized in Supporting Information Tables S3–S6).

RESULTS

Isolation of gene clones

A cDNA library was established using combined leaf and root material of barley plants at a developmental stage where all remaining analyses were carried out. The library was screened for genes with high homology to known K^+ channels in *Arabidopsis* (AKT1/2 type) or putative channels in barley (HvKCO1). Three full-length clones encoding putative K^+ channels were isolated, and these were termed HvAKT1, HvAKT2 and HvKCO1. Prior to screening the library for HAK-related sequences, we used RT-PCR to check which of the known full-length (HvHAK1, HvHAK2) or partial (HvHAK4) barley HAK sequences reported by Rubio *et al.* (2000) were expressed in particular in the leaf EZ. Based on this, we selected a HAK4-related sequence and isolated a full-length clone which was termed HvHAK4.

The clone (open-reading frame) termed HvAKT1 (EMBL accession DQ465922) is 3120 bp long and encodes a protein of 898 amino acids (2697 bp), from bp 137 to bp 2833. The pore-forming domain (amino acids 262–285) includes the hallmark GYGD/E-motif typical of K^+ selective channels (see Supporting Information Fig. S1a). A Blast (NCBI–Blast2) search shows highest (95%) protein sequence identity with a full-length clone of an AKT1-like channel in wheat (*Triticum aestivum*; Uni-prot reference Q9M671), followed by 77% sequence identity with a K^+ uptake channel in maize (*Zea mays*; Q571X8) and 76% identity with a putative K^+ channel in rice (*Oryza sativa*; Q8VYX2). Sequence identity to *Arabidopsis* AtAKT1 (Q38998) is 62%; sequence identity to a partial sequence (593 amino acids) of a putative AKT1-like channel in barley (Q84ZX3) is 99%.

Clone HvAKT2 (EMBL accession DQ465923) is 2880 bp long and encodes a protein of 859 amino acids, from bp 129 to bp 2708. The pore-forming domain and GYGD motif are between amino acids 255 and 277 (Supporting Information Fig. S1b). Blast search shows highest protein sequence identity to full-length clones of two putative K^+ channels in rice (73% each; Q75HP9 and A2Y4X9), 72% identity to ZmK2 in maize and 54% sequence identity to *Arabidopsis* AtAKT2/3 (Q38898). Sequence identity to HvAKT1 and to *Arabidopsis* AtAKT1 is 39%. Phylogenetic tree analysis of AKT1- and AKT2-type channels, together with SPICK-type and KAT1/2-type channels, gives three major clusters (Fig. 2). One cluster contains HvAKT1 grouped together with AKT1-type channels of wheat, rice, maize and *Arabidopsis*, and with *Arabidopsis* KAT1 and KAT2, with closest phylogenetic relationship to AKT1-type channels of grass (and monocot) species, particularly wheat (see also Supporting Information Fig. S2a for extended phylogenetic analyses of HvAKT1 and AKT1-type channels); the second cluster contains HvAKT2 together with maize ZmK2 and two putative K^+ channels in rice; and the third cluster contains AKT2- and AKT1-type channels of, exclusively dicotyledonous species, including *Arabidopsis* AtAKT2/3 (but not AtAKT1). Among the full-length coding sequences available through Web search, HvAKT2 is related phylogenetically closest to ZmK2.

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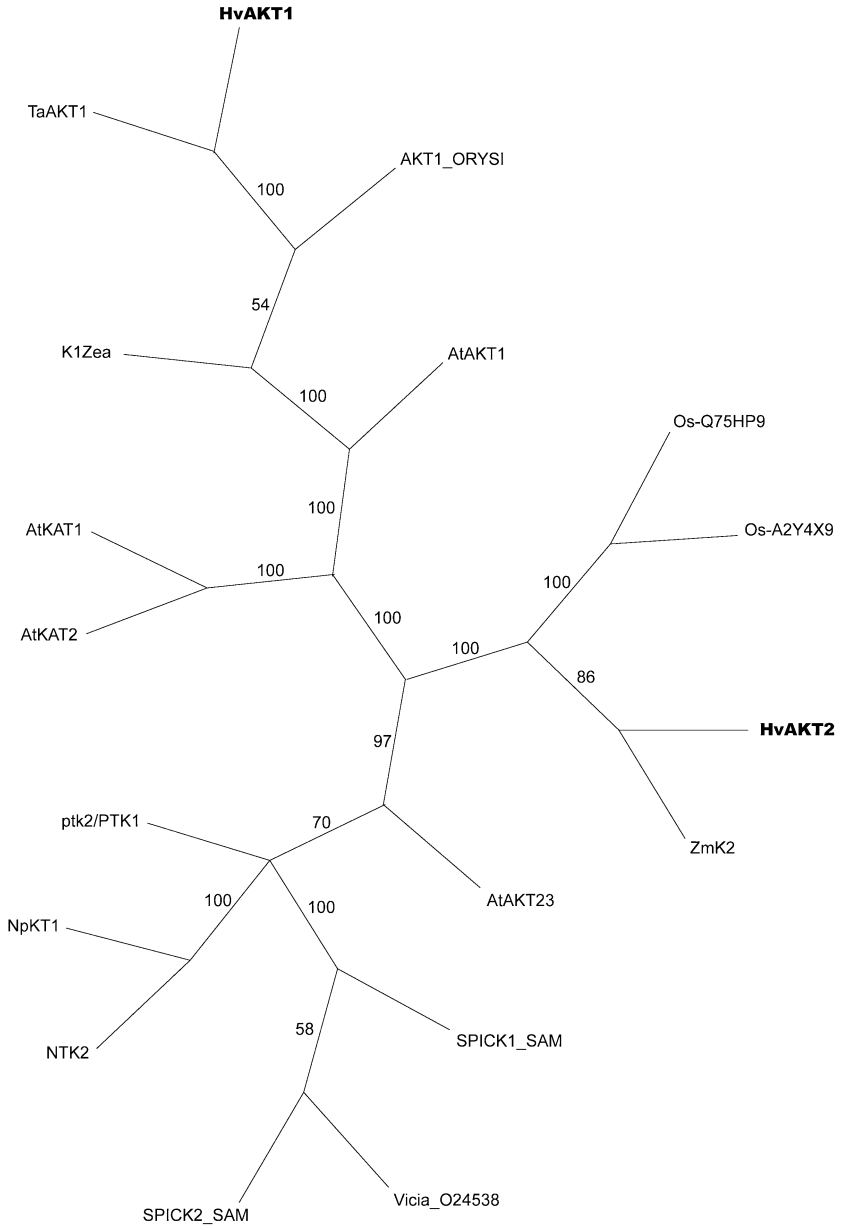

Figure 2. Phylogenetic tree of *HvAKT1* and *HvAKT2* (highlighted in bold) with related sequences in plants, particularly rice and *Arabidopsis*. Protein sequences of barley candidate genes were entered into BLAST search (http://www.ncbi.nlm.nih.gov/blast), and a selection of those sequences which showed highest homologies were aligned using ClustalX, incorporating default settings (Thompson *et al.* 1997). Sequences were aligned using maximum parsimony (MP) analyses with PAUP 4.0b10 (Swofford 2003). Amino acid positions were unweighted, and gaps were coded as missing data. Starting trees were obtained via stepwise addition. Bootstrap analyses included 100 replicates. Tree bisection- and reconnection-based heuristic searches were used in all analyses. Protein sequences used for alignment are given in Supporting Information Table S2; alignment of selected sequences with conserved regions highlighted is given in Supporting Information Fig. S1a,b.

Clone HvHAK4 (EMBL accession DQ465924) is 2808 bp long and encodes a protein of 785 amino acids, from bp 34 to bp 2391, with 12 transmembrane spans (Supporting Information Fig. S1c). Protein sequence identity to full-length sequences of HvHAK1 (AF025292; 773 amino acids long) and HvHAK2 (AF129479; 772 amino acids) is 40 and 72%, respectively. The partial HvHAK4 sequence (AF129484; 202 amino acids) that was used to isolated clone HvHAK4 shows 99% sequence identity. Phylogenetic tree analysis of HvHAK4 with full-length HAK-type sequences in *Arabidopsis* and rice, two species for which all HAK-type sequences are known, and partial sequences in barley results in three to four larger clusters (Supporting Information Fig. S2b). Each cluster contains sequences of grasses (monocotyledonous plants) and *Arabidopsis* (dicotyledonous plant), suggesting closer phylogenetic relationships

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between individual HAK-type transporters irrespective of plant group than between different HAK-type transporters of one particular plant group. HvHAK4 is more closely related to rice OsHAK2 than to OsHAK4, and, among *Arabidopsis* HAK-type sequences, closest related to AtKUP4 (Kim *et al.* 1998).

Clone HvKCO1/HvTPK1 (GenBank accession EU926490) is 1473 bp long and encodes a protein of 349 amino acids, from bp 143 to bp 1189; the pore domain including the GYGD motif is between amino acids 215 and 234 (Supporting Information Fig. S1d). The HvKCO1 clone isolated differs from that reported (but not cloned) by Sinnige *et al.* (2005; Q4G309) by only one amino acid (amino acid position 42); a full-length clone of *H. vulgare* var. distichum (two-rowed barley; Q216V9) shows 95% sequence identity to HvKCO1. Blast search shows putative outward-rectifying K^+ channels in several plant species (*Medicago truncatula*, *O. sativa*, *Eucalyptus camaldulensis*, *Solanum tuberosum*) with identical or almost identical protein length (348–349 amino acids) and 54–78% sequence identity. *Arabidopsis* AtKCO1/AtTPK1 (363 amino acids long) shows 52% sequence identity to HvKCO1. Phylogenetic tree analysis groups HvKCO1 in a cluster with KCO1-like sequences of other grasses; KCO-like sequences of dicotyledonous plants form either a separate cluster or cluster together with a sequence of the monocot, rice (Supporting Information Fig. S2c).

Functional characterization of HvAKT1 and HvAKT2

HvAKT1 and *HvAKT2* cRNA were injected into *X. laevis* oocytes. Channel activity was measured using voltage clamping. Expression of HvAKT2 results in the appearance of weakly inward-rectifying currents that display both time-dependent and instantaneous activation upon a hyperpolarizing voltage step. Currents are highly selective for K^+ over Na^+ (Fig. 3a,b) and blocked by 5 mM Cs^+, (Fig. 4a; see also Supporting Information Fig. S3 for typical recordings of individual protoplasts). Increasing the H^+ concentration in the bath medium from pH 7.5 to pH 5.5 leads to an increase in total HvAKT2 current at voltages between –20 and –160 mV; this is mainly caused by an increase in the instantaneous component (Fig. 4b). Paired *t*-test confirms a significantly higher instantaneous current at pH 5.5 at voltages between –20 and –140 mV ($P < 0.05$), while there is no significant difference in time-dependent current. Increasing the Ca^{2+} concentration in bath medium from 1 to 10 mM leads to a decrease in both components of HvAKT2 currents at voltages between –80 and –140 mV (Fig. 4c).

Expression of HvAKT1 cRNA in oocytes does not result in gene-specific K^+ currents (Fig. 5a), even when the potentially regulatory beta-unit of HvAKT1 is co-expressed (not shown). However, co-injection with cRNA for *Arabidopsis* calcineurin B-like protein (*AtCBL1* or *AtCBL9*, the latter resulting in the larger currents which are shown) and CBL-interacting protein kinase (*AtCIPK23*), previously shown to be required for functional heterologous expression of *AtAKT1* (Li *et al.* 2006; Xu *et al.* 2006), results in the appearance of a measurable time-dependent inward current (Fig. 5b), albeit smaller than the current evoked by similar amounts of injected cRNA for AtAKT1 (with *AtCBL1* and *AtCIPK23*; Fig. 5c). Figure 5d shows the average I–V curves for both channels. Exchanging K^+ in the external medium for Rb^+ or Na^+ considerably decreases the current indicating selectivity for K^+ over both ions. As in the case of other Shaker-type channels, currents are strongly inhibited by the addition of Cs^+ (Fig. 5e).

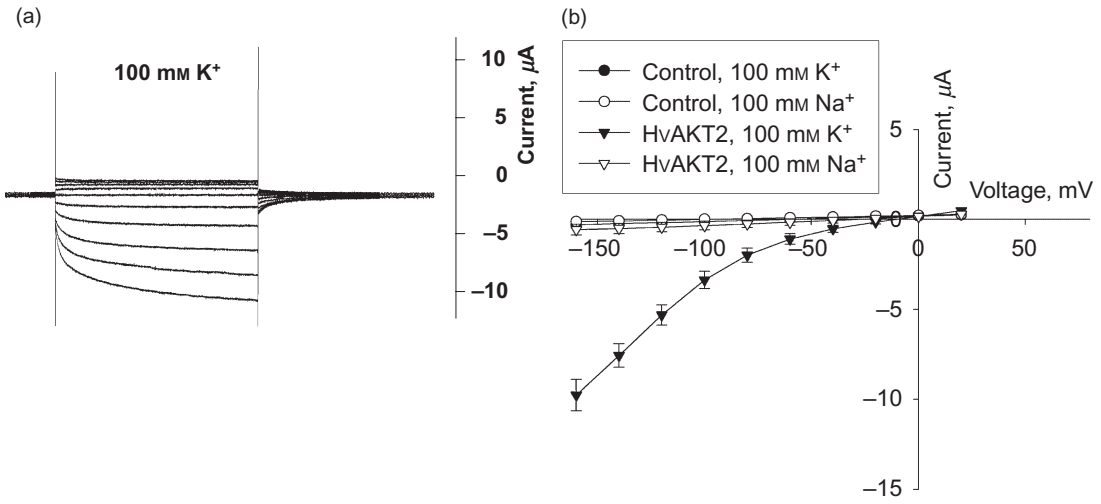

Figure 3. HvAKT2-mediated current in *Xenopus laevis* oocytes. (a) Typical current recording in an oocyte injected with HvAKT2 cRNA. (b) Current–voltage relationships determined for *HvAKT2*-injected oocytes (triangles, average current ± SE, $n = 6$ oocytes from 3 toads) and water-injected oocytes (circles, average current ± SE, $n = 8$ oocytes from 3 toads). Holding potential was –60 mV; voltage was clamped from –160 to +20 mV in 20 mV steps. Total current was measured 3 s after the start of the voltage pulse. The bath medium contained 100 mM K^+ (added as K-gluconate) (solid symbols) or 100 mM Na^+ (added as Na-gluconate) (open symbols). For other components in the bath, see Materials and methods.

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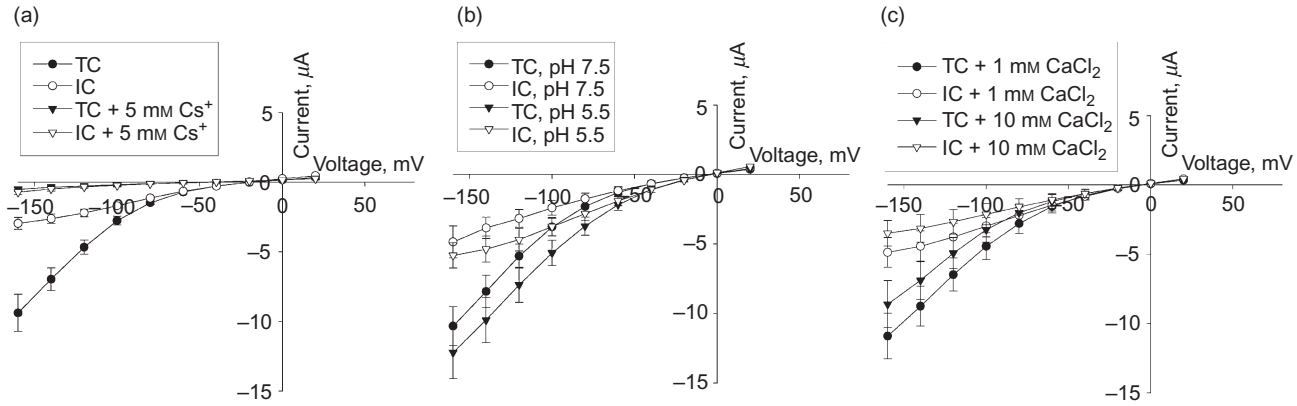

Figure 4. Effects of Cs^+, pH and Ca^{2+} on HvAKT2 currents in *Xenopus laevis* oocytes. Average total current (TC, closed symbols) and instantaneous current (IC, open symbols, $n = 4$–6 oocytes from 2 toads), measured at 3 s after the start of the voltage pulse. Holding potential was –60 mV; voltage was clamped from –160 to +20 mV in 20 mV steps. Bath medium always contained 100 mM K^+, added as K-gluconate. (a) Currents before (circles) and after (triangles) addition of 5 mM CsCl (means ± SE, $n = 4$ oocytes from 2 toads). (b) Currents at pH 7.5 (circles) or pH 5.5 (triangles; means ± SE, $n = 6$ oocytes from 2 toads). (c) Currents in the presence of 1 mM (circles) or 10 mM (triangles) Ca^{2+} (means ± SE, $n = 5$ oocytes from 2 toads).

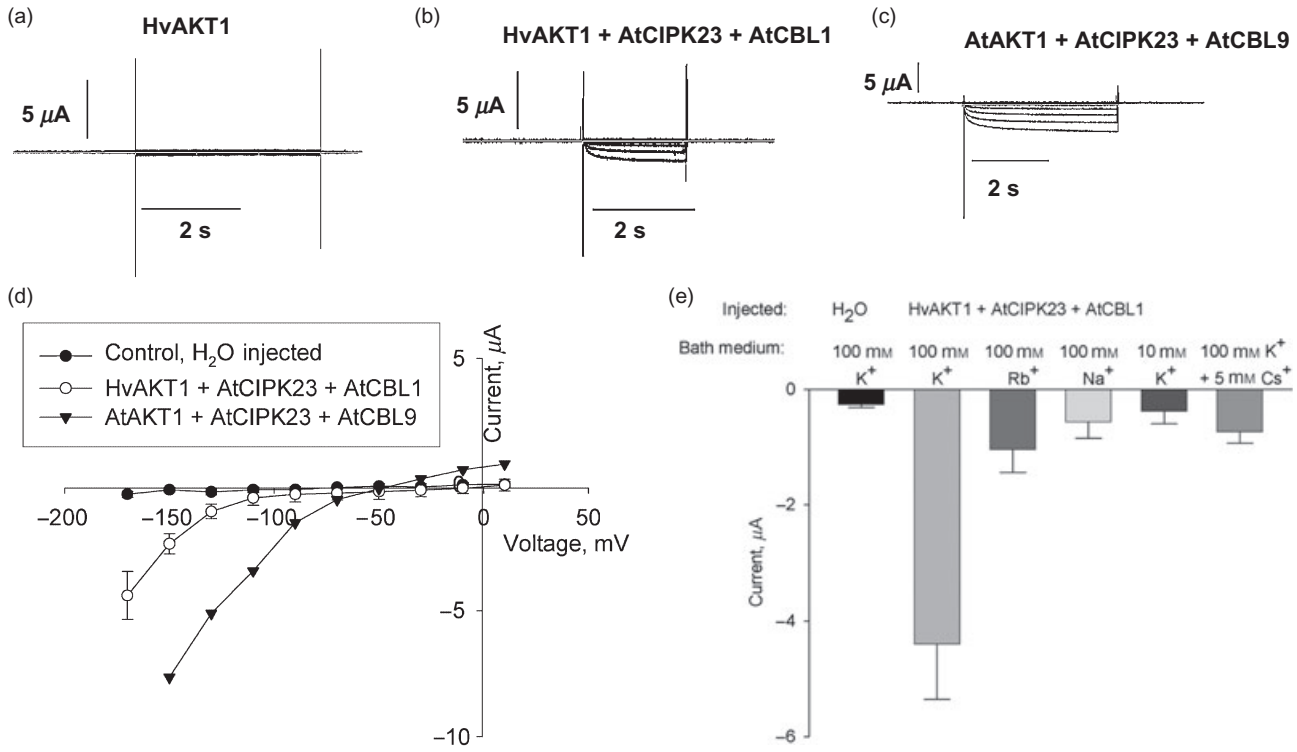

Figure 5. Heterologous expression of barley *HvAKT1* in *Xenopus laevis* oocytes. The oocytes were injected with cRNA of HvAKT1 and/or with cRNA of other genes as indicated, and currents were recorded 2 d later. Voltage was clamped from –180 to +20 mV in 20 mV steps; holding potential was –30 mV. (a) Expression of HvAKT1 on its own does not result in detectable currents. (b) Co-expression of *HvAKT1* with *At*CIPK23 and *At*CBL1 results in the appearance of inward-rectifying currents. (c) Co-expression of AtAKT1 with *At*CIPK23 and *At*CBL9 results in large inward currents. (d) Averaged I–V relations of water-injected oocytes (closed circles, means ± SD, $n = 4$ oocytes, standard deviations are smaller than symbols), and oocytes injected with *HvAKT1*, *AtCIPK23* and *AtCBL1* (open circles, means ± SD, $n = 8$ oocytes), or *AtAKT1*, *AtCIPK23* and *AtCBL 9* (triangles, mean of two oocytes). (e) Mean (±SD) current at –170 mV in water-injected oocytes with 100 mM K^+ in the bath, and in oocytes injected with *HvAKT1*, *At*CIPK23 and *At*CBL1 with 100 mM K^+ ($n = 8$ oocytes), 100 mM Rb^+ ($n = 6$ oocytes), 100 mM Na^+ ($n = 5$ oocytes), 10 mM K^+ ($n = 3$ oocytes) or 100 mM K^+ and 5 mM Cs^+ ($n = 4$ oocytes) in the bath.

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Expression of channel genes in leaf tissues and root

The same pattern of expression of candidate genes between plant regions is observed using semiquantitative and qPCR (Fig. 6a,b; see also Supporting Information Fig. S4). *HvAKT1* is expressed predominantly in roots, and shows much weaker expression in leaves (3–4% of expression in roots) with no significant differences between leaf zones (Fig. 6; for statistical analyses of data, see Supporting Information Tables S3 & S4). By contrast, *HvAKT2* is expressed about 20 times higher in leaf compared to root tissue (Fig. 6b). Of the genes tested, *HvHAK4* is expressed most specifically in the leaf EZ. Expression is 30 times higher than in other leaf regions and about 10 times higher than in root samples. In contrast, *HvKCO1*/*HvTPK1* is expressed uniformly between root and shoot (Fig. 6b).

In an additional series of (three) experiments, we used only ubiquitin as reference gene of expression and included the EmBL portion of leaf 3 in qPCR analyses. In addition, only the tip 2–3 cm of seminal roots (being enriched in growing tissue) rather than the entire seminal roots (consisting mainly of fully differentiated tissue) were analysed as root sample. The basic pattern of expression of candidate genes between plant regions does not differ from that when three reference genes are used. Expression in the EmBL portion is similar to that in the mature blade of leaf 2 (not shown). The difference in expression of HvHAK4 between root (tip 2–3 cm) and shoot sample (leaf EZ) is fivefold and not as large (10-fold) as when expression is compared between entire seminal roots and the leaf EZ (not shown); this points to higher expression of HvHAK4 in growing compared to mature root tissue.

We tested the effects of K^+ deficiency and of salt treatment on the expression of candidate genes using semiquantitative RT-PCR (see Supporting Information Fig. S4; for statistical analyses of data, see Supporting Information Table S4). The pattern of expression of candidate genes between plant regions is not affected by treatments, and the level of expression remains largely unchanged. ANOVA analysis shows a significant treatment effect for *HvAKT1*. Expression of *HvAKT1* increases in response to salt and low K in the EZ, and in all leaf tissues tested in response to low K.

Cell- and tissue-specific expression of *HvAKT1* and *HvAKT2* within leaves was analysed by *in situ* RT-PCR. The EZ, and the midportion and tip region of the emerged part of leaf 3 were studied. *HvAKT2* is expressed more strongly than *HvAKT1*, particularly in mesophyll cells bordering to the epidermis. Both genes are expressed predominantly in mesophyll cells (Fig. 7a–m). At the margins of the tip of leaves, weak expression can be detected in a few cells of the epidermis, whereas stomata and vascular bundles

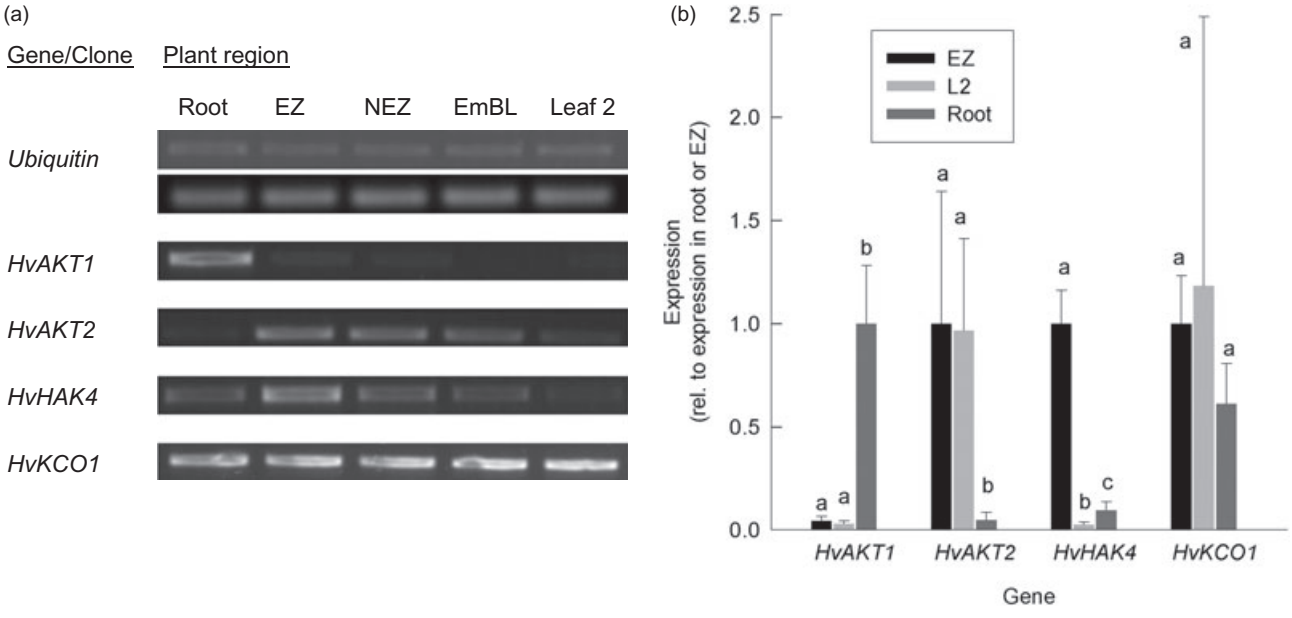

Figure 6. Expression of candidate genes in root and shoot tissue of barley plants. (a) Gel images of semiquantitative RT-PCR. Each gene was tested at three or four different cycle numbers. Gel images for that cycle number that did not suffer from saturation and for which the band of a particular gene was visible for as many plant regions as possible are shown. Cycle number was 27 and 29 (ubiquitin), 31 (*HvAKT1* and *HvAKT2*), 33 (*HvHAK4*) and 29 (*HvKCO1*), respectively. Ubiquitin was used as reference gene of expression, and images for two cycle numbers are shown. (b) Real-time qPCR analyses of expression. Expression of candidate genes was first related to expression of reference genes ubiquitin, ATPase and GAPDH. The resulting value was then compared between plant regions by setting the expression ratio of 'candidate-gene' : 'reference gene' to one in that plant region where the gene is expressed highest [elongation zone (EZ): HvHAK4, HvAKT2, HvKCO1; root: HvAKT1]. Shown are averages with standard deviations of three independent experiments. Abbreviations: NEZ, non-elongation zone; EmBL, emerged blade portion (all leaf 3); L2, mature blade of leaf 2. Statistical analyses were carried out by single-factor analysis of variance (ANOVA), followed by *t*-test to test for difference in expression of a particular candidate gene between any pair of plant regions (see Supporting Information Table S3); statistical significance is indicated by different letters.

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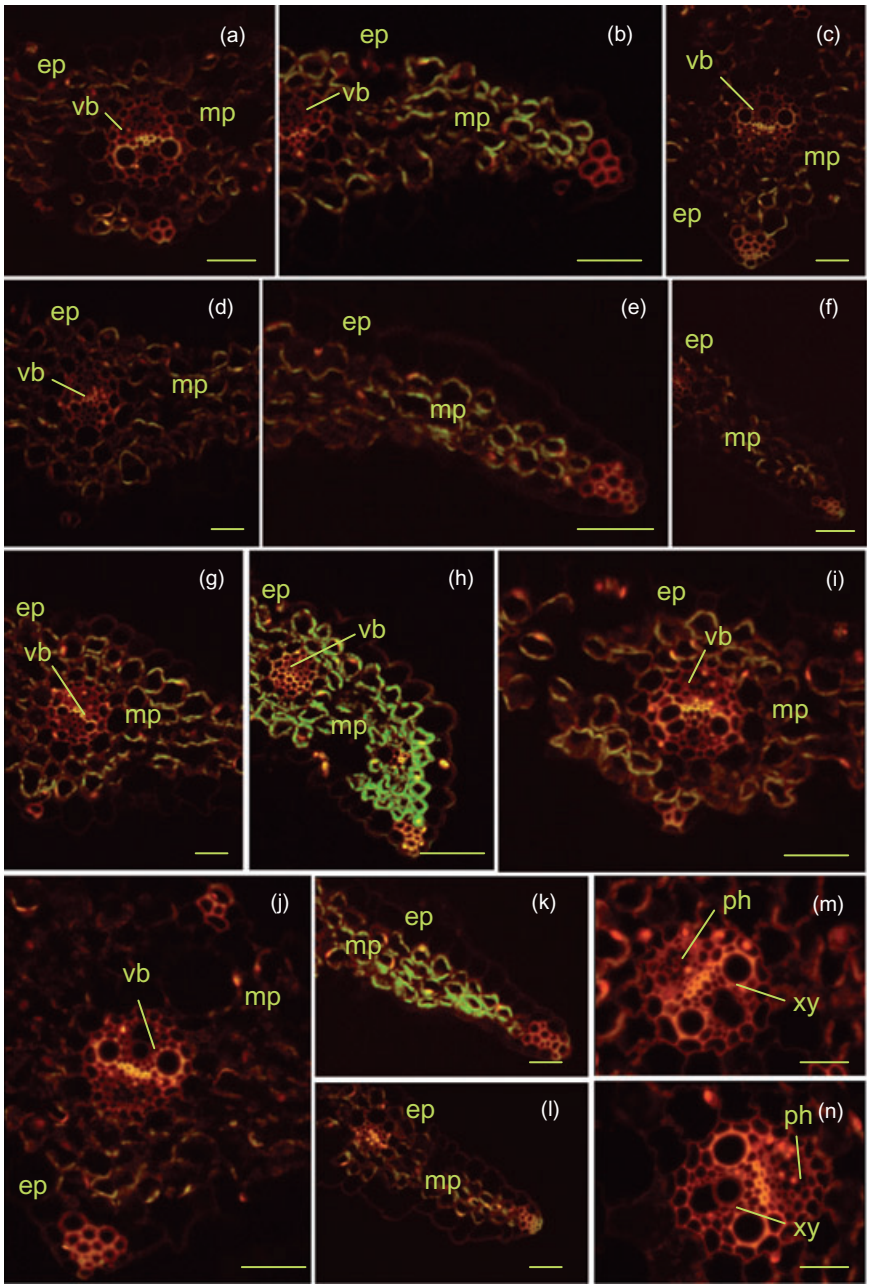

Figure 7. *In situ* PCR localizations of expression of candidate genes in cross-sections of barley leaves. Microscopic images were obtained with a cooled CCD camera coupled to an Axioskop fluorescence microscope using filter set 09 (Zeiss). Expression of candidate genes is shown by green fluorescence; orange–yellowish red colour [as in (a), vascular bundle, or (b), leaf margin] is caused by autofluorescence of chloroplasts (chlorophyll) and cell walls, in particular of walls of vascular elements and walls of collenchyma in leaf margins and beneath leaf ridges. (a–f) *HvAKT1*. (a) Tip region. (b) Lateral area of tip region. (c) Emerged blade (EmBL). (d) Elongation zone (EZ). (e) Lateral area of EmBL. (f) Lateral area of the EZ. (g–m) *HvAKT2*. (g) Tip region. (h) Lateral area of tip region. (i) EmBL. (j) EZ. (k) Lateral area of EmBL. (l) Lateral area of the EZ. (m) Vascular bundle, EmBL. (n) Vascular bundle, background control. ep, epidermis; vb, vascular bundle; mp, mesophyll; ph, phloem; xy, xylem. (m, n) The bar equals 100 μm. Other images: the bar equals 200 μm.

show no expression; the same applies to HvHAK4 (not shown; HvKCO1 was not tested).

Nutritional and stress treatments

Reduction in external K^+ to 2.5% the level in control nutrient solution has no effect on elongation velocity of leaf 3, despite a decrease in osmolality of mesophyll and epidermis from 400–450 (control) to 310–360 mosmol kg^{-1} (Fig. 8a), and regardless whether plants are exposed to low K^+ for only 2 d prior to sampling or for the entire period (7–9 d) while being on nutrient solution. In control plants, the K^+ concentration in epidermis and mesophyll of the leaf EZ is about 150 mM; it is slightly lower (mesophyll) or almost

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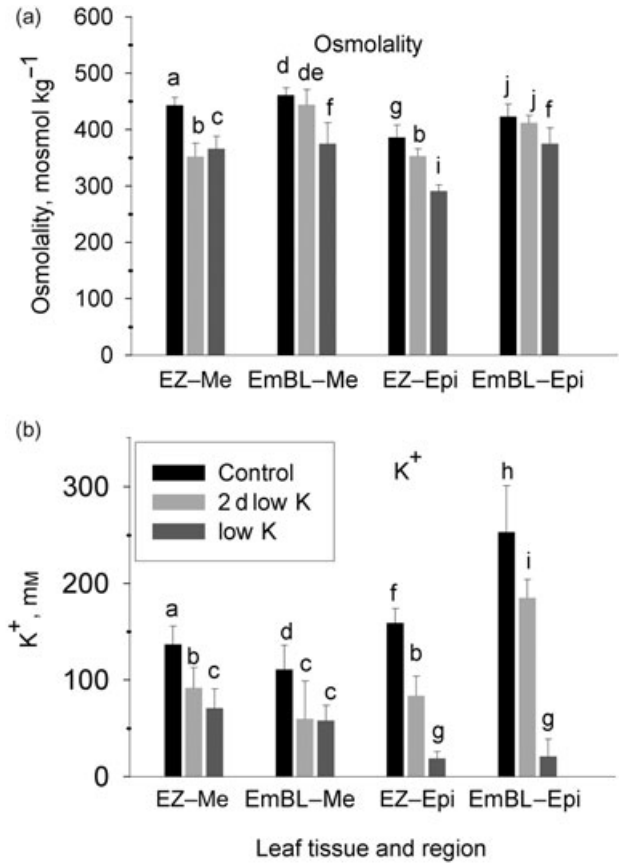

Figure 8. Osmolality and K^+ concentrations in epidermal (Epi) and mesophyll (Me) cells of leaf 3 of barley grown under control conditions or at two different regimes of low K^+. The elongation zone (EZ) and emerged blade (EmBL) portion were analysed. Control plants were grown on half-strength modified Hoagland solution containing, in terms of K^+ and Ca^{2+}, 2 mM K^+ and 2 mM Ca^{2+}. The 2 d low-K plants grew initially on the same nutrient solution as the control plants, except for the last 2 d prior to sampling when they received reduced K^+ (50 μM), and, in compensation, increased Ca^{2+} (3 mM) at unchanged Na^+ (0.5 mM). Low-K plants received reduced K^+ (and increased Ca^{2+}) for the entire period (7–9 d) on nutrient solution. Averages (±SD) of 10 (control), eight (2 d low K) and seven (low K) plant analyses are shown. Statistical differences in K^+ concentrations and osmolality between treatments, leaf regions and tissues (mesophyll, epidermis) were analysed by two-factor analysis of variance (ANOVA), followed by *t*-test for any pair of data (see Supporting Information Tables S5 & S6). Statistical significance is shown by different letters.

twice as high (epidermis) in the EmBL (Fig. 8b). Reduction in external K^+ for 2 d prior to harvest (2 d low K) lowers K^+ significantly, in the mesophyll by 50 mM and in the epidermis by 80 mM in both the EZ and EmBL (statistical analyses of K^+ and osmolality data are given in Supporting Information Tables S5 & S6, respectively). Prolonged limitation in K^+ (low-K plants) does not further decrease mesophyll K^+, whereas epidermal K^+ decreases to 20 mM. The latter suggests that plants are increasingly starved by K^+. Changes in the concentrations of Ca^{2+}, a solute which barley leaf epidermal cells use to osmotically replace K^+, are small (10 mM) compared to changes in K^+ (not shown).

DISCUSSION

Characteristics of HvAKT1 and HvAKT2

When *HvAKT1* is expressed in *X. laevis* oocytes, K^+-dependent inward currents are observed only after co-expression with a calcineurin B-like protein (AtCBL1) and a calcineurin-interacting protein kinase (AtCIPK23) from *Arabidopsis thaliana*. The experiment shows that *HvAKT1* encodes an inward-rectifying K^+ channel and is activated by the CBL/CIPK signalling pathway, as its *Arabidopsis* homolog. The barley CBL and CIPK homologs that activate HvAKT1 *in planta* remain to be identified, but the results suggest that barley plants have a Ca^{2+}-dependent signalling network in regulation of cellular K^+ similar to that described for *A. thaliana* (Li *et al.* 2006; Xu *et al.* 2006). The diversity of CBL proteins and CIPKs is comparable in the fully sequenced genomes of the dicot *A. thaliana* and the monocot rice (Kolukisaoglu *et al.* 2004). Disruption of this regulatory network during isolation of mesophyll protoplasts from the EmBL could be one explanation for low occurrence of K^+-dependent inward currents in these protoplasts (see accompanying paper, Volkov *et al.* 2009).

Co-expression with calcineurin-interacting protein kinase and B-like protein is not an absolute requirement for functional expression of AKT1-type channels in *X. laevis* oocytes. Hartje *et al.* (2000) measured currents through the AKT1 homolog of tomato (*Lycopersicon esculentum*, LKT1) in oocytes without co-expression of other genes. The authors also functionally expressed an AKT1 homolog of the phylogenetically closely related potato (*S. tuberosum*, SKT1) following removal of 3′ overhangs in the linearized template DNA.

HvAKT2 expressed in *X. laevis* oocytes shows gating characteristics as its *Arabidopsis* homolog AtAKT2 (Dennison *et al.* 2001; Deeken *et al.* 2002), notably weak inward rectification and a voltage-independent component. Changes in external pH and Ca^{2+} concentration affect channel activity of HvAKT2 expressed in *X. laevis* oocytes (see Fig. 4). A decrease in external pH increases total inward current because of an increase in instantaneous component of current. Stimulation of current is observed over a voltage range (–20 to –140 mV) that covers the normal resting membrane potential of cells (–88 to –25 mV measured *in planta*, at bathing medium/apoplastic K^+ of 0.1–100 mM; see accompanying paper, Volkov *et al.* 2009), and could contribute to K^+ uptake into growing barley leaf cells provided the cell wall space is more acidic than in non-growing tissues. In contrast to AtAKT2 (Marten *et al.* 1999; Latz *et al.* 2007) and the maize homolog ZmK2 (Philippar *et al.* 1999), HvAKT2 currents are increased, not reduced, by lowering the pH in the bath medium. Geiger *et al.* (2002) studying AtAKT3 concluded that outer pore residues His228 and Ser271 are involved in sensing of proton concentration. The open-reading frame of AtAKT3

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is identical to that of AtAKT2, except that the first 15 amino acids are missing at the 5′-end of AtAKT3. His228 and Ser271 are absent from ZmK2 and HvAKT2, and are replaced through an aspartic acid and glutamine residue, respectively (see Supporting Information Fig. S1b). Coincidentally, at positions 228 and 271 of AtAKT2 (not AtAKT3) is also a His and Ser residue, respectively, which is highly conserved among AKT2-type channels including ZmK2 and HvAKT2 (Supporting Information Fig. S1b). We conclude that residues His228 and Ser271 identified as essential for pH sensing in *AtAKT3* are not essential for pH sensing in HvAKT2.

Increase in bathing medium Ca^{2+} from 1 to 10 mM decreases HvAKT2 inward currents by 20–30%. This is in the same range as reported for *Arabidopsis* AtAKT2/AtAKT3 (Marten *et al.* 1999). Plants grown on low K^+ received 3 mM rather than 2 mM external $Ca(NO_3)_2$. If anything, Ca^{2+} in epidermal and mesophyll cells increases, but does not decrease (not shown). Therefore, it is unlikely that apoplastic Ca^{2+} decreases during K^+ deficiency, and to an extent that causes an increase in K^+ currents through Ca^{2+} sensitivity of HvAKT2 to aid K^+ acquisition.

Role of cloned genes in barley leaf growth and K^+ nutrition

It was not within the scope of this study to test through transgenic approaches for changes in growth and K^+ accumulation as a result of altered expression of candidate genes. Rather, by analysing K^+ concentrations at tissue resolution, we were able for the first time to relate tissue-specific levels of expression of candidate genes to the 'end product' of channel/transporter activity, which is of biophysical relevance to growth (in wild-type plants).

Expression of *HvAKT1* is about 20–30 times higher in roots compared to leaves. This is in line with the supposed role of AKT1 in root uptake of K^+, particularly in the presence of inhibitory concentrations of ammonium in the growth medium (as in the present study; Hirsch *et al.* 1998; Spalding *et al.* 1999; Santa-María, Danna & Czibener 2000; Su *et al.* 2002). In the leaf EZ, expression of HvAKT1 is about 10% that of HvAKT2. Dennison *et al.* (2001), using a mutant approach, concluded that AtAKT1 and AtAKT2 contribute 50% each to the K^+ permeability of mesophyll cells in *Arabidopsis*. In rice, *OsAKT1* expression in leaves was observed in xylem parenchyma, phloem and the mesophyll (Golldack *et al.* 2003). The present observations that *HvAKT1* is expressed in the mesophyll of the EZ and that K^+ inward currents observed in protoplasts isolated from the leaf EZ can be mediated by AKT1-type channels (Volkov *et al.* 2009, accompanying paper) point to a role for HvAKT1 in the K^+ nutrition of growing barley leaf cells. *HvAKT1* is also expressed in the mesophyll of the EmBL portion, and expression doubles in response to low K^+, yet K^+ inward currents are not detected in mesophyll protoplasts isolated from this region (only untreated control plants analysed; Volkov *et al.* 2009). One likely explanation is that HvAKT1 activity in the EmBL is regulated at the post-transcriptional level in such a way that activity is lost during isolation of protoplasts, at least under experimental conditions which we used (see Karley, Leigh & Sanders 2000b, who measured time-dependent K^+ inward currents in mature barley leaf cells).

K^+-dependent currents in protoplasts of the leaf EZ are strongly inward rectifying (Volkov *et al.* 2009), whereas currents in oocytes expressing HvAKT2 are weakly inward rectifying. These data point to a minor role for HvAKT2 in K^+ uptake into growing barley leaf cells. However, Latz *et al.* (2007) recently reported for *Arabidopsis AtAKT2* that transient expression in a plant-based system gives current characteristics more reminiscent of AKT1-type channels. Possibly, this involves a CBL/CIPK signalling pathway as identified in the present study for the successful expression of HvAKT1 in *X. laevis* oocytes, and could imply that part of the AKT1-type currents observed in barley protoplasts of the leaf EZ reflect activity of HvAKT2.

The homologs of *HvAKT2* in *Arabidopsis* and maize, *AtAKT2*/*AtAKT3* (Marten *et al.* 1999; Deeken *et al.* 2002) and *ZmK2* (Bauer *et al.* 2000) have been implicated in K^+ loading of phloem in source leaf tissue. The present data do not support such a primary role for HvAKT2 in barley, judging from expression pattern in the developing leaf 3. *HvAKT2* is expressed in the mesophyll.

HvHAK4 is expressed about 30 times higher in the EZ compared to other leaf regions, and is mainly expressed in mesophyll. We do not know whether HvHAK4 transports K^+. Plasma membrane potential of mesophyll and epidermal cells in the leaf EZ is around –88 mV (Volkov *et al.* 2009). Assuming that HvHAK4 is located at the plasma membrane and that cytosolic K^+ is in the range 60–90 mM (Walker, Leigh & Miller 1996; Carden *et al.* 2003; Cuin *et al.* 2003), cells can take up K^+ from an apoplastic K^+ environment as low as 3–5 mM using channels. HvHAK4 may aid K^+ uptake under these apoplastic conditions. For HvHAK2, which is a close relative of HvHAK4, a role in low-affinity K^+ transport has been proposed (Senn *et al.* 2001).

Arabidopsis TRH1 (AtKUP4), a close homolog of HvHAK4, has been implicated in K^+ transport in growing roots, but has also been ascribed a function related to auxin transport (Rigas *et al.* 2001; Vicente-Agullo *et al.* 2004). Similarly, mutation in another member of the KT/KUP/HAK family, SHY3 (AtKT2/AtKUP2; Quintero & Blatt 1997; Fu & Luan 1998; Kim *et al.* 1998), mediates K^+-dependent cell expansion in growing leaf tissues in *Arabidopsis* in a manner that may be related more to a regulatory function of the protein than to impairment of K^+ transport (Elumalai, Nagpal & Reed 2002). A similar function of HvHAK4 could explain its preferential expression in growing tissue. In addition, there is evidence that some KT-HAK-KUP transporters significantly contribute to Na^+ influx (Takahashi *et al.* 2007), which may be critical to sustain cation uptake and growth in leaves of plants exposed to low K^+.

The functionality of the tandem-pore K^+ channel HvKCO1/HvTPK1 was not tested in the present study. However, Maathuis and colleagues (Maathuis, personal

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communication) using the barley clone isolated here were able to rescue the wild-type phenotype in *Arabidopsis* AtTPK1 knock-outs through expression of HvTPK1. This suggests that HvTPK1 is a vacuolar K^+ channel that fulfils a role similar to its *Arabidopsis* homolog *AtTPK1*, and is involved in the maintenance of intracellular K^+ homeostasis (Czempinski *et al.* 2002; Gobert *et al.* 2007). Comparatively uniform expression of *HvKCO1*/*TPK1* between root and leaf tissues supports such a 'housekeeping' role of HvKCO1 in cellular K^+ relations.

K^+ concentrations and gene expression in plants grown at low K^+

In plants which grow on low K^+ throughout, K^+ concentrations do not differ between EZ and EmBL. The most likely explanation is that net import of K^+ into cells which have completed elongation, as observed in K^+ replete plants, is discontinued in plants which are increasingly limited (or starved) by K^+. Preferential (net) accumulation of solutes in the EZ has been observed for barley plants exposed to salinity (Fricke *et al.* 2006). We do not know what defines this sink strength, but it can be either or both, an increased capacity of growing mesophyll and epidermal leaf cells to: (1) take up and retain K^+ ('cell property'); or (2) to unload K^+ from xylem stream and phloem sap as it passes through the EZ towards the leaf tip ('tissue property'). *In situ* PCR data suggest that neither HvAKT1 nor HvAKT2 is involved in the latter mechanism. Instead, expression of these channels in mesophyll, but not epidermis, may contribute to the maintenance of higher K^+ concentrations in mesophyll during K^+ limitation, as observed during salinity (Fricke *et al.* 1996; Cuin *et al.* 2003).

Salinity and K^+ treatments cause few changes in expression of candidate genes. This points to regulation at the level of protein and is supported through micro-array data at Genevestigator (http://www.genevestigator.ethz.ch; see Supporting Information Fig. S5). Lack of responsiveness to K^+ and Na^+ at the transcriptional level was also found for *AtAKT1* and *AtAKT2* in *Arabidopsis* (Maathuis *et al.* 2003). The only K^+ transporter gene showing a consistent induction by low K^+ in *Arabidopsis* is *AtHAK5* (Armengaud, Breitling & Amtmann 2004; Gierth, Mäser & Schroeder 2005).

CONCLUSIONS

HvAKT1 and HvAKT2 encode inward-rectifying Shaker-type K^+ channels which are expressed in leaf and root. Functional expression of these channels in *X. laevis* oocytes suggests that barley plants have a Ca^{2+}-dependent CBL/CIPK signalling network involved in the regulation of cellular K^+ similar to that described for *A. thaliana*. HvAKT1 and HvAKT2 may contribute to K^+ uptake into mesophyll cells of growing leaf tissue and maintenance of higher K^+ in mesophyll compared to epidermis during K^+ limitation. HvHAK4 may fulfil a similar role. Expression analyses of candidate genes suggest that transport activity is regulated at the protein level in response to low K^+ or salinity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) Alignment of sequences of members of the Shaker family of potassium channels with high homology to *HvAKT1*, the gene cloned in the present study. Alignment was carried out with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/), and regions of homology were highlighted using BoxShade (http://www.ch.embnet.org/software/BOX_form.html). Based on information provided for *Arabidopsis* AtAKT1 (Sentenac *et al.* 1992, *Science* 256: 663–665), the position of the six transmembrane spans S1–S6 is marked in green; the pore region containing the conserved GYGD motifs is highlighted in purple (PORE). Sequences are from monocotyledonous plants (*Hordeum vulgare*, HvAKT1; *Zea mays*, ZmK1; *Oryza sativa*, OsAKT1; *Triticum aestivum*, TaAKT1) or from the dicotyledonous plants *Arabidopsis thaliana* (AtAKT1), *Solanum tuberosum* (SKT1), *Lycopersicon esculentum* (LKT1) and *Daucus carota* (DKT1). Sequences are given in Supporting Information Table S2. (b) Alignment of sequences of members of the Shaker family of potassium channels with high homology to *HvAKT2*, the gene cloned in the present study. Alignment was carried out with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/), and regions of homology were highlighted using BoxShade (http://www.ch.embnet.org/software/BOX_form.html). Based on information provided for *Arabidopsis* AtAKT2 (Cao *et al.* 1995, *Plant Physiology* 109: 1093–1106), the position of the six transmembrane spans S1–S6 is marked in green; the pore region containing the conserved GYGD motifs is highlighted in purple (H5). The two residues identified in AtAKT2 as being essential for pH sensing (Geiger *et al.* 2002, *The Plant Cell* 14: 1859–1868), His228 and Ser271 are highlighted in red. These residues are replaced through aspartic acid (D) and glutamine (Q) in ZmK2 and HvAKT2. Note that AtAKT2, which has a 15-amino acid longer 5′-end, has at equivalent amino acid positions (228 and 271), also a His and Ser residue which are highly conserved among AKT2-type channels and highlighted in red. Sequences are from monocotyledonous plants (*H. vulgare*, HvAKT2; *Z. mays*, ZmK2; *O. sativa*, Os-Q75HP9) or from the dicotyledonous plants *A. thaliana* (AtAKT2, AtAKT3), *Nicotiana tabacum* (NTK2), *Populus tremula* (PTK2) and *Samanea saman* (Spick2). Sequences are given in

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Supporting Information Table S2. (c) Alignment of sequences of members of the HAK/KUP/POT family of potential potassium transporters with *HvHAK4*, the gene cloned in the present study. Alignment was carried out with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/), and regions of homology were highlighted using BoxShade (http://www.ch.embnet.org/software/BOX_form.html). Based on information provided for *Arabidopsis* AtKUP1 (Kim *et al.* 1998, *The Plant Cell* 10: 51–62), the position of the 12 transmembrane spans is marked in green. Sequences are from monocotyledonous plants (*H. vulgare*, HvHAK4, HvHAK2, HvHAK1; *O. sativa*, OsHAK2, OsHAK3, OsHAK4, OsHAK1) or from the dicotyledonous plant *A. thaliana* [AtKUP1/AtKT1 and AtKUP2/AtKT2; see Kim *et al.* 1998 and Quintero & Blatt 1997 (*FEBS Letters* 415: 206–211) for original names given, respectively, AtKUP3, AtKUP4 (Kim *et al.* 1998) and AtHAK5]. Sequences are given in Supporting Information Table S2. (d) Alignment of sequences of members of the KCO/TPK family of potassium channels with high homology to *HvKCO1*, the gene cloned in the present study. Alignment was carried out with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/), and regions of homology were highlighted using BoxShade (http://www.ch.embnet.org/software/BOX_form.html). Based on information provided for *Arabidopsis* AtKCO1 (Czempinski *et al.* 1997, *EMBO Journal* 16: 6896), the position of the four transmembrane spans M1–M4 is marked in green; the two pore regions (P1, P2) are highlighted in purple, and the two EF hand motifs (EF1, EF2) and the region rich in K and R (KR) are highlighted in turquoise. Sequences are from monocotyledonous plants (*H. vulgare*, HvKCO1 and Q2I6V9_HORVD; *O. sativa*, A2YHFO_ORYSI or from the dicotyledonous plants *A. thaliana* (AtKCO1), *S. tuberosum* (StKCO1), *S. saman* (SPOCK1) and *Eucalyptus camaldulensis* (Q9LLM5_EUCCA). Sequences are given in Supporting Information Table S2.

Figure S2. Phylogenetic tree of (a) *HvAKT1* and *HvAKT2*, (b) *HvHAK4* and (c) *HvKCO1*/*HvTPK1* with related channels in plants, particularly rice and *Arabidopsis*. Protein sequences of barley candidate genes were entered into BLAST search (http://www.ncbi.nlm.nih.gov/blast), and a selection of those sequences which showed highest homologies were aligned using ClustalX, incorporating default settings (Thompson *et al.* 1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. (*Nucleic Acids Research* 25: 4876–4882.). Sequences were aligned using maximum parsimony (MP) analyses with PAUP 4.0b10 [Swofford 2003, PAUP* 4.0. Phylogenetic Analysis Using Parsimony (and other methods), version 4.0b10.]. Amino acid positions were unweighted, and gaps were coded as missing data. Starting trees were obtained via stepwise 18 addition. Bootstrap analyses included 100 replicates. Tree bisection- and reconnection-based heuristic searches were used in all analyses. Annotations of genes together with protein sequences used for alignment are given in Supporting Information Table S2. In (b), one non-plant sequence is included (*HAK1* of the yeast *Debaryomyces*); *Arabidopsis AtKUP1* and *AtKUP2* have been referred to originally also as *AtKT1* and *AtKT2*, respectively (Quintero & Blatt 1997, *FEBS Letters* 415: 206–211).

Figure S3. Heterologous expression of barley *HvAKT2* in *Xenopus laevis* oocytes. Oocytes were injected with cRNA of HvAKT2 and analysed 2 d later for HvAKT2-specific currents. Typical recordings are shown of (a) inhibition of currents by 5 mM Cs^+ in the bathing medium, and (b) Na^+-independent inward-rectifying currents; these were virtually absent because of K^+ selectivity of currents.

Figure S4. Expression of (a) *HvAKT1*, (b) *HvAKT2* and (c) *HvHAK4* in root and shoot tissue of barley plants grown under control conditions or exposed to K^+ limitation for 2 d (2 d low K) or 7–9 d (low K) and salinity (3–5 d NaCl). Expression was analysed by semiquantitative RT-PCR. Ubiquitin was used as reference gene of expression. Data are averages with standard deviations of three independent experiments, using three batches of plants. Cycle number was 29 (*HvAKT1*), 31 (*HvHAK4*) and 33 (*HvAKT2*). Abbreviations used for leaf regions: EZ, elongation zone; NEZ, non-elongation zone; EmBL, emerged blade portion (all leaf 3); L2, mature blade of leaf 2. Results are expressed in arbitrary units. To test for treatment effects on expression, statistical analysis 21 was carried out by two-factor analysis of variance (ANOVA), followed by *t*-test to test for difference in expression of a particular candidate gene in a particular plant region between any pair of treatments (see Supporting Information Table S4); statistical significance of expression within a plant region (treatment effect) is indicated by different letters.

Figure S5. Genevestigator (http://www.genevestigator.ethz.ch/) was used to screen the sequences on the barley Affymetrix chip which are closest related to the candidate genes cloned in the 23 present study for expression in relation to tissue, organ and treatment. (a) Comparison of the expression level of HAK4, AKT2, KAT1, KAT2, KCO1, KAB1 (the potassium channel subunit) and the reference gene (ubiquitin, Ubi) used in the present study in 706 micro-arrays available in the GENEVESTIGATOR database. The meta-profile analysis with the different tools of the GENEVESTIGATOR Web browser gives the expression profile of candidate genes in function of (b) developmental stage, (c) anatomy and organ specificity and (d) different stimuli.

Table S1. Primers used for isolation of barley (*Hordeum vulgare* L.) candidate genes and expression analyses.

Table S2. Sequences used for construction of phylogenetic trees.

Table S3. Statistical significance of differences in expression (qPCR analyses) of candidate genes between plant regions of barley (*Hordeum vulgare* L.) plants.

Table S4. Statistical significance of differences in expression (semiquantitative PCR analyses) of candidate genes between leaf regions and between treatments of barley (*Hordeum vulgare* L.) plants.

Table S5. Statistical significance of differences in K^+ concentrations between leaf tissues (epidermis, mesophyll, bulk leaf), leaf regions [elongation zone (EZ), emerged blade

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(EmBL)] and treatments (control, 2 d low K, low K) of barley (*Hordeum vulgare* L.) plants.

Table S6. Statistical significance of differences in osmolality between leaf tissues (epidermis, mesophyll, bulk leaf), leaf regions [elongation zone (EZ), emerged blade (EmBL)] and treatments (control, 2 d low K, low K) of barley (*Hordeum vulgare* L.) plants.

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