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RESEARCH PAPER

Competition between uptake of ammonium and potassium in barley and *Arabidopsis* roots: molecular mechanisms and physiological consequences

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Abstract

Plants can use ammonium (NH_4^+) as the sole nitrogen source, but at high NH_4^+ concentrations in the root medium, particularly in combination with a low availability of K^+, plants suffer from NH_4^+ toxicity. To understand the role of K^+ transporters and non-selective cation channels in K^+/NH_4^+ interactions better, growth, NH_4^+ and K^+ accumulation and the specific fluxes of NH_4^+, K^+, and H^+ were examined in roots of barley (*Hordeum vulgare* L.) and Arabidopsis seedlings. Net fluxes of K^+ and NH_4^+ were negatively correlated, as were their tissue concentrations, suggesting that there is direct competition during uptake. Pharmacological treatments with the K^+ transport inhibitors tetraethyl ammonium (TEA^+) and gadolinium (Gd^{3+}) reduced NH_4^+ influx, and the addition of TEA^+ alleviated the NH_4^+-induced depression of root growth in germinating *Arabidopsis* plants. Screening of a barley root cDNA library in a yeast mutant lacking all NH_4^+ and K^+ uptake proteins through the deletion of *MEP1–3* and *TRK1* and *TRK2* resulted in the cloning of the barley K^+ transporter HvHKT2;1. Further analysis in yeast suggested that HvHKT2;1, AtAKT1, and AtHAK5 transported NH_4^+, and that K^+ supplied at increasing concentrations competed with this NH_4^+ transport. On the other hand, uptake of K^+ by AtHAK5, and to a lesser extent via HvHKT2;1 and AtAKT1, was inhibited by increasing concentrations of NH_4^+. Together, the results of this study show that plant K^+ transporters and channels are able to transport NH_4^+. Unregulated NH_4^+ uptake via these transporters may contribute to NH_4^+ toxicity at low K^+ levels, and may explain the alleviation of NH_4^+ toxicity by K^+.

Key words: Ammonium toxicity, *Arabidopsis*, barley, competition, gadolinium, potassium nutrition, tetraethyl ammonium.

Introduction

Ammonium (NH_4^+) is a central nitrogen compound in all organisms. In the autotrophic anabolism of plants, NH_4^+ is generated through the reduction of nitrate, or is directly taken up from the soil. In addition, NH_4^+ is generated via deamination of organic compounds during protein turnover, photorespiration, and lignin biosynthesis (Joy, 1988).

It is well known that high concentrations of NH_4^+ can be toxic to plants leading to severe growth depression (Gerendás *et al.*, 1997). Various hypotheses have been put forward aimed at identifying the cause of NH_4^+ toxicity. The majority of these hypotheses deal with the physiological changes associated with NH_4^+ assimilation and ion imbalances resulting from decreased uptake of essential cations such as K^+, Mg^{2+}, and Ca^{2+} (Barker *et al.*, 1967; Gerendás *et al.*, 1997; Roosta and Schjoerring, 2007). Rapid assimilation of NH_4^+ in roots is associated with a large requirement

Abbreviations: FW, fresh weight; ICP-OES, Inductively Coupled Plasma-Optical Emission Spectrometry; MIFE, Microelectrode Ion Flux Estimation; NSCC, non-selective cation channel; ORF, open reading frame; TEA, tetraethyl ammonium; UTR, untranslated region; YNB, yeast nitrogen base.

for carbohydrates and may, consequently, result in root carbon depletion (Kronzucker *et al.*, 1998; Schjoerring *et al.*, 2002). NH_4^+ assimilation also leads to a net release of H^+ and a subsequent reduction in the concentration of dicarboxylic acids by decarboxylation, a cellular response to maintain cytoplasmic pH (Raven and Smith, 1976). Decarboxylation of dicarboxylic acids puts a further strain on the carbon budget of the root, and results in an increased uptake of anions for charge balance. In addition, NH_4^+ toxicity has been associated with hormonal imbalances (Barker, 1999; Walch-Liu *et al.*, 2000) and a decrease in photosynthesis (Putrich and Barker, 1967; Gerendás *et al.*, 1997).

Recently, an alternative hypothesis was put forward suggesting that growth depression of barley plants at high NH_4^+ was due to a futile cycling of NH_4^+ across the plasma membrane, where active extrusion of excess NH_4^+ by an as yet unknown transporter led to extensive energy consumption (Britto *et al.*, 2001). The high respiration rate of roots in conjunction with the energy demand for active extrusion of NH_4^+ was suggested to be the fundamental cause of the damaging effects of high external NH_4^+. Thus, toxicity was explained by an excessive cycling at the plasma membrane as opposed to consequences of assimilation and accumulation in the cells. Several bacteria also show elevated respiration at high NH_4^+ and low K^+ levels, caused by the energetically unfavourable cycling of NH_4^+ at the plasma membrane (Buurman *et al.*, 1989, 1991). It was suggested that cycling of NH_4^+ across bacterial plasma membranes is caused by the disruption of the regulation of NH_4^+ uptake and non-specific influx through the kdp K^+ transporter (Buurman *et al.*, 1991).

It has long been known that toxicity of NH_4^+ is particularly pronounced during K^+ deficiency (Wall, 1939). Similarly, addition of K^+ can alleviate NH_4^+ toxicity (Barker *et al.*, 1967; Cao *et al.*, 1993). Toxicity of NH_4^+ and alleviation by K^+ has also been shown in yeast, where high concentrations of NH_4^+ are toxic only when K^+ levels are low (Hess *et al.*, 2006).

NH_4^+ and K^+ are highly similar regarding charge, size, and hydration energy, characteristics that are important for membrane transport (Wang *et al.*, 1996; White, 1996). NH_4^+ has been shown to influence the uptake and accumulation of K^+ (see, for example, Rufty *et al.*, 1982; Allen and Raven, 1987; van Beusichem *et al.*, 1988; Finnemann and Schjoerring, 1999; Angeles Martinéz-Cordero *et al.*, 2005; Szczerba *et al.*, 2008*b*, and references therein), and K^+ influences the uptake and accumulation of NH_4^+ (Scherer *et al.*, 1984; Nielsen and Schjoerring, 1998; Szczerba *et al.*, 2008*a*). Reduced NH_4^+ uptake and accumulation through the provision of K^+ may prevent competition with NH_4^+ in subsequent metabolic processes that require K^+. It is therefore likely that the toxicity of NH_4^+ at high external concentrations is closely linked to its interference with K^+ uptake and cellular homeostasis.

The genome of *Arabidopsis thaliana* contains around 20 genes encoding K^+-selective transporters and 57 genes encoding cation channels (Mäser *et al.*, 2001; Véry and Sentenac, 2002). The presence of the non-selective cation channel (NSCC) blocker lanthanum (La^{3+}) reduced the uptake of NH_4^+ in leaves and roots (Nielsen and Schjoerring, 1998; Szczerba *et al.*, 2008*a*) and several experiments have indicated that NSCCs can facilitate the transfer of NH_4^+ across membranes (Gassman and Schroeder, 1994; White, 1996; Davenport and Tester, 2000; Demidchik and Tester, 2002; Balleza *et al.*, 2005). In addition, experiments using different heterologous expression systems have suggested that the *Arabidopsis* K^+ channel AtKAT1 (Schachtman *et al.*, 1992; Bertl *et al.*, 1995; Cao *et al.*, 1995; Uozumi *et al.*, 1995; Moroni *et al.*, 1998), K^+ transporters AtKCO1 (Czempinski *et al.*, 1997) and HvHKT2;1 (Santa-María *et al.*, 2000) are also candidates for mediating NH_4^+ transport.

The aim of this work was to investigate the effect of different concentrations of NH_4^+ and K^+ on the growth of barley (*Hordeum vulgare* L.) and *Arabidopsis* seedlings, and to demonstrate the interdependence of K^+ and NH_4^+ fluxes during uptake. Pharmacological treatments with TEA^+ and Gd^{3+} supported the indication from earlier studies that K^+-selective channels as well as NSCCs transport NH_4^+ in the roots. Strikingly, treatment with TEA^+ alleviated NH_4^+-induced growth depression in germinating *Arabidopsis* seedlings, indicating a role for K^+-selective channels in mediating NH_4^+ uptake. A yeast complementation screen using a cDNA library from barley roots indicated that HvHKT2;1 transports NH_4^+, and that growth on NH_4^+ was inhibited by increasing K^+ concentrations. Similar results were seen for AtAKT1 and AtHAK5. K^+ transport through HvHKT2;1, AtAKT1, and AtHAK5 was inhibited by NH_4^+. Together these results demonstrate interference between K^+ and NH_4^+ at uptake via HvHKT2;1, AtAKT1 and AtHAK5.

Materials and methods

Plant cultivation

Arabidopsis seeds (Col-0, Lehle seeds) were surface-sterilized by washing for 1 min with 50% ethanol, followed by incubation in 5% NaOCl and 0.02% SDS for 10 min. Thereafter, seeds were rinsed five times with sterile, double-deionized water. They were submerged in ±0.05% agarose and stratified for 2 d at 4 °C, either in the agarose-solution or on media in square 50 ml Petri dishes with the same composition as described by Cao *et al.* (1993). The medium was supplied with 0.4% agarose and 1% sucrose, and different combinations of 0.2 mM KCl, 6 mM NH_4Cl, 1 mM $GdCl_3$ or 10 mM TEACl. On the top of each plate two rows of seeds were sown, with 30 seeds in each row. Plates were organized horizontally, allowing seedlings to grow along the surface of the medium, in a controlled environment growth-chamber with a 85–110 μmol m^{-2} s^{-1} photon flux density for 8 h d^{-1}, 75–80% air humidity, and 20 °C air temperature. Plants were cultured for 10 d prior to analysis.

Barley seeds (*Hordeum vulgare* L., cv. Antonia) were sown on vermiculite in a greenhouse (250–300 μmol m^{-2} s^{-1} photon flux density, 75–80% humidity, at 20 °C and 18 °C during 16/8 h light/dark, respectively). Seedlings were watered with (K^+-free) double-deionized water. When the shoot was about 8 cm, four seedlings were transferred to 4.0 l buckets containing hydroponic medium without K^+ or NH_4^+. The medium consisted of

0.3 mM $MgSO_4$-$7H_2O$, 0.1 mM NaCl, 0.2 mM NaH_2PO_4-H_2O, 0.2 mM Na_2SO_4-$10H_2O$, 0.15 mM $Mg(NO_3)_2$-$6H_2O$, 0.6 mM $Ca(NO_3)_2$-$4H_2O$, 1.5 mM Fe(III)-EDTA-Na, 150 μM $MnSO_4$-H_2O, 105 μM $ZnSO_4$-$7H_2O$, 120 μM $CuSO_4$-$5H_2O$, 300 μM H_3BO_3, and 120 μM Na_2MoO_4-$2H_2O$. The pH was kept at 6.0 with 1.2 mM MES/NaOH. The solution was aerated with filtered air supplied through a syringe needle. After 3 d of growth in hydroponic medium, various concentrations of NH_4^+ and K^+ were added using $(NH_4)_2SO_4$ and/or K_2SO_4. The nutrient solution was changed daily. Five days after the initiation of the K^+/NH_4^+ treatments, plants were harvested in a mixed sequence to reduce artefacts caused by circadian rhythms. For NH_4^+ and K^+ determination, roots were washed in 2.5 g l^{-1} $CaSO_4$, and three times in double-deionized water, then dried on paper tissue. Plants were fractionated into (i) oldest leaf, (ii) young leaves, (iii) stems, and (iv) roots. Each fraction was weighed, frozen in liquid nitrogen, and stored at –80 °C prior to extraction.

Root length measurements

Arabidopsis roots were magnified and measured using a scanner or a microscope (Leica Microsystems M500 or MZ FL III) connected to a camera (Leica Microsystems DC 300 F).

Measurement of soluble NH_4^+ content

Samples were homogenized with a steel pin, after which 200 mg was taken out and crushed with 100 μl acid-washed sand and 2 ml 10 mM ice-cold formic acid, in an agate mortar on ice. This suspension was centrifuged at 10 000 *g* and 4 °C. The supernatant was centrifuged again and the supernatant of this last centrifugation-step was diluted ten times with 10 mM formic acid. The NH_4^+ concentration was determined by measuring the fluorescence in 3 mM *o*-phthalaldehyde, 10 mM 2-mercaptoethanol, and 100 mM phosphate buffer adjusted to pH 6.8 as previously described (Husted *et al.*, 2000).

Potassium determination with ICP-OES

Samples from the NH_4^+-extraction (described in the previous paragraph) were diluted ten times with 7% HNO_3. The K^+ concentration was determined by Induced Coupled Plasma-Optical Emission Spectroscopy (ICP-OES; Perkin-Elmer Optima 3000XL) at axial mode using a wavelength of 766.474 nm.

Flux measurements

Net fluxes of NH_4^+, K^+, and H^+ at the surface of *Arabidopsis* roots were measured non-invasively using the MIFE® technique (UTas Innovation, Hobart, Australia) essentially as described by Shabala *et al.* (1997, 2001) and Shabala (2000). Briefly, microelectrodes were pulled and salinized with tributylchlorosilane. After backfilling with an appropriate electrolyte, electrode tips were filled with commercially available ionophore cocktails (09882 for NH_4^+; 60031 for K^+; 95297 for H^+; all from Fluka) and mounted on a 3D-micromanipulator (MMT-5; Narishige), with their tips close together, 20 μm above the root surface. During measurements, a computer-controlled stepper motor moved the electrodes between two positions (20 μm and 40 μm, respectively) from the root surface in a 10 s square-wave manner. The CHART software (Shabala *et al.*, 1997; Newman, 2001) recorded the potential difference between two positions and converted them into electrochemical potential differences using the calibrated Nernst slope of the electrodes. H^+ fluxes were then calculated using the MIFE-FLUX software for cylindrical diffusion geometry (Newman, 2001). NH_4^+ and K^+ fluxes were calculated using the algorithm described in Knowles and Shabala (2004) taking into account the mutual interference between the two ions resulting from non-ideal selectivity of both the K^+ and NH_4^+ ionophore cocktails.

Arabidopsis Col-0 plants were pre-grown on plates for 10 d as described. Eight to 10-mm-long apical root segments were excised and mounted horizontally in a Perspex holder using agar (see Babourina *et al.*, 2000, for details). The holder was immediately placed in a 4 ml measuring chamber filled with 0.1 mM $CaCl_2$, pH 5.7 (unbuffered), mounted on a computer-driven 3D-micromanipulator (MMT-5; Narishige) and left to equilibrate for 60 min before the addition of treatment. Steady-state ion fluxes were recorded over a period of 5 min, 15 min after the application of the appropriate treatment. Unless stated differently, 1 mM NH_4^+, 0.2 mM K^+, 10 mM TEA, and 0.1 mM Gd^{3+} were used in the various treatments.

Yeast strains and expression of plant transporters

Three deletion mutants in the yeast *Saccharomyces cerevisiae* were generated in this study: a triple NH_4^+ transporter deletion mutant Δ*mep1-3*, a K^+ transporter double deletion mutant Δ*trk1,2* and a 5-fold deletion mutant Δ*mep1-3* Δ*trk1,2*. All mutants were made in the BY background from the Euroscarf collection. To generate Δ*mep1-3*, *HIS3* was first generated by PCR using primers: fw 5′-CACATGTGCTAACCAAACATCAGTGGGTAGTAATCA-TTCGGCGTCAGCGGGTGTTGG-3′ and rev 5′-CGTTGGCA-TGCGATGAGGTCAGTTTCCTGCGCCATTTCAGTCTCGG-TCTATTCTTTTGATTTAT-3′ to form a deletion cassette for *MEP2*. The PCR product was transformed into Δ*mep1* (*Y04751, Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YGR121c::kanMX4*), thus yielding a double mutant Δ*mep1,2*. The double mutant was crossed with Δ*mep3* (*Y15553, Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YPR138c::kanMX4*) to yield Δ*mep1-3* (*Matα leu2Δ0 lys2Δ0 ura3Δ0 YGR121c::kanMX4 YNL142w::HIS3 YPR138c::kanMX4*). Yeast strain Δ*trk1,2* (*Mata his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YKR050w::kanMX4*, *YJL129c::kanMX4*) was generated by crossing Δ*trk1* (*Y15121 Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YKR050w::kanMX4*) and Δ*trk2* (*Y01296 Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YJL129c::kanMX4*). Likewise, the 5-fold deletion mutant strain Δ*mep1-3 trk1,2* (*Mata leu2Δ0 ura3Δ0 YGR121c::kanMX4 YNL142w::HIS3 YPR138c::kanMX4, YKR050w::kanMX4, YJL129c::kanMX4*) was generated by crossing the two aforementioned yeast strains *Δmep1-3* and *Δtrk1,2*. All crossings (mating and sporulation) were done as described by Kassir and Simchen (1991). The deletion of genes was successfully verified by PCR, using a reverse primer for the flanking kanamycin gene (rev 5′-CTAGTAATCTTCAGGGGCC-3′) in combination with forward primers for the genes: *MEP1* 5′-ATGGCA-ACATCAGCTCG-3′, *MEP3* 5′-ATGGCTACATCTGCTAG-AAGA-3′, *TRK1* 5′-TTTCACGAAAAGAGGGACAATGTAC-3′, and *TRK2* 5′-AGGATTCGTTGTGCTTGTTGAATCG-3′. The genotypes of the resulting yeast strains were characterized by cultivation on different amino acids and NH_4^+ and K^+ conditions and their mating type was determined by mating with haploid strains with supplementing marker-genes and known mating type. To enable selection using two markers only, and to grow cells on media without supplementation by amino acids, yeast strains were transformed with single copy vectors from the pRS series containing appropriate marker genes.

Yeast strains were transformed with pYES2 containing cDNAs encoding *Arabidopsis* hak5L_{776}H (Rubio *et al.*, 2000), *AtAKT1* in plasmid pFL61 (Sentenac *et al.*, 1992) or a barley root cDNA library (Pedas *et al.*, 2008). Transformation was done by electroporation, as described at http://www.agr.kuleuven.ac.be/dp/logt/protocol/yeastelectroporation.htm. After transformation, yeast cells were pre-grown on synthetic YNB medium without NH_4^+, supplemented with 100 mM K_2SO_4 and 0.1% proline, on 2% glucose and a pH of 5.5 (50 mM succinic acid/TRIS). Growth assays were carried out on synthetic medium containing 1% galactose, YNB without amino acids, K^+ and NH_4^+ and supplemented with various concentrations of $(NH_4)_2SO_4$ or K_2SO_4 as indicated. Plates were incubated at 30 °C for at least 3 d. The K^+ content of agar was determined to be 500 μg g^{-1}, which has been accounted for in the composition of the media.

Statistical analysis

Statistical analysis was performed using SAS software (SAS Institute Inc.,USA, version 9.1).

Results

Uptake and accumulation of NH_4^+ and K^+ in barley

To characterize potential interferences in the uptake and accumulation of NH_4^+ and K^+ in barley, seedlings were cultivated in hydroponic medium, containing NO_3^- but neither NH_4^+ nor K^+. Subsequently, seedlings were transferred for 5 d to medium supplemented with various NH_4^+ and K^+ concentrations. Fresh weight and the soluble NH_4^+ and K^+ content were determined.

Cultivation for 5 d on high levels (3–6 mM) of NH_4^+ without K^+, significantly decreased both root and shoot fresh weight compared with plants grown in similar media but in the absence of NH_4^+ (Figs 1, 2). This growth depression by NH_4^+ was progressively alleviated by the presence of sub-millimolar concentrations of K^+. The weight of seedlings grown in the absence of K^+ and NH_4^+ was only slightly decreased compared with plants that had received K^+, but no NH_4^+ (Fig. 2). Three days on 6 mM NH_4^+ and 0.2 mM K^+, followed by a 2 d K^+-starvation period with unchanged NH_4^+ provision did not produce any significant difference in fresh weight relative to plants with constant K^+ supply.

The soluble NH_4^+ content (Fig. 3A) of plants cultivated in the absence of NH_4^+ was in the range of 0.4–0.7 μmol g^{-1} FW in all tissues, regardless of the K^+ concentration in the medium. A concentration of 0.4–0.7 μmol NH_4^+ g^{-1} FW may therefore reflect the basal NH_4^+ concentration resulting from nitrate reduction and processes such as protein turnover and photorespiration. The presence of NH_4^+ in the nutrient solution led to elevated levels of soluble NH_4^+ in all tissues, with maxima of 5.6 μmol g^{-1} FW in young leaves and 22.9 μmol g^{-1} FW in roots when NH_4^+ was supplied at 6 mM. These amounts were reduced in the presence of K^+, with a higher effect in shoot than in root tissue (Fig. 3A).

The corresponding K^+ content showed values as low as 10 μmol g^{-1} FW in roots and 20 μmol g^{-1} FW in leaves of plants that had never received any K^+ other than their seed reserves (Fig. 3B). In these conditions, increasing levels of NH_4^+ in the growth media had a negative effect on the K^+ concentrations of all the tissues tested, in line with the interpretation that NH_4^+ causes K^+ extrusion (Szczerba *et al.*, 2006). This reduction in K^+ concentration was particularly apparent in roots, where a concentration as low as 3.9 μmol K^+ g^{-1} FW was observed in plants that were not supplied with K^+. When neither K^+ nor NH_4^+ was supplied, the K^+ concentration of roots was more than twice as high (11.5 μmol g^{-1} FW). In the absence of external NH_4^+, the addition of 0.2, 0.4 or 1.2 mM K^+ resulted in comparable levels of K^+, approximately 50 μmol g^{-1} in roots and 140 μmol g^{-1} in leaves. Withholding K^+ during the final 2 d of a treatment with 6 mM NH_4^+ and 0.2 mM K^+ reduced the K^+ concentration in roots, but not in leaves, indicating that barley plants are very efficient in controlling and retaining K^+ in leaves.

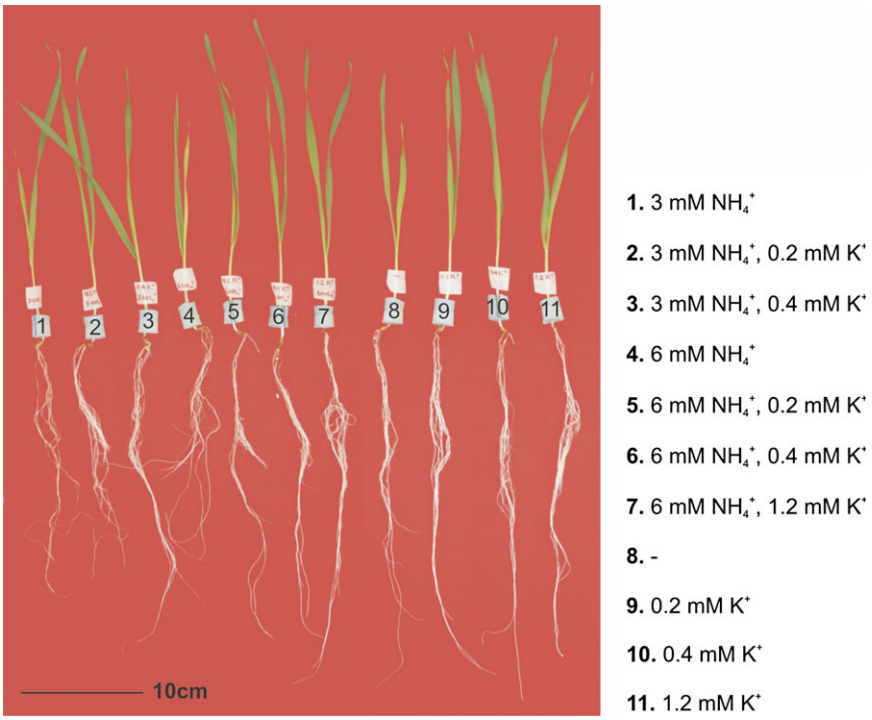

Fig. 1. Barley plants (cv. Antonia) cultivated at various K^+ and NH_4^+ concentrations. Plants were grown in a greenhouse in hydroponic medium with all nutrients including 1.5 mM NO_3^- as nitrogen source but without NH_4^+ or K^+ for 3 d and subsequently transferred for 5 d to various K^+ and NH_4^+ concentrations as indicated.

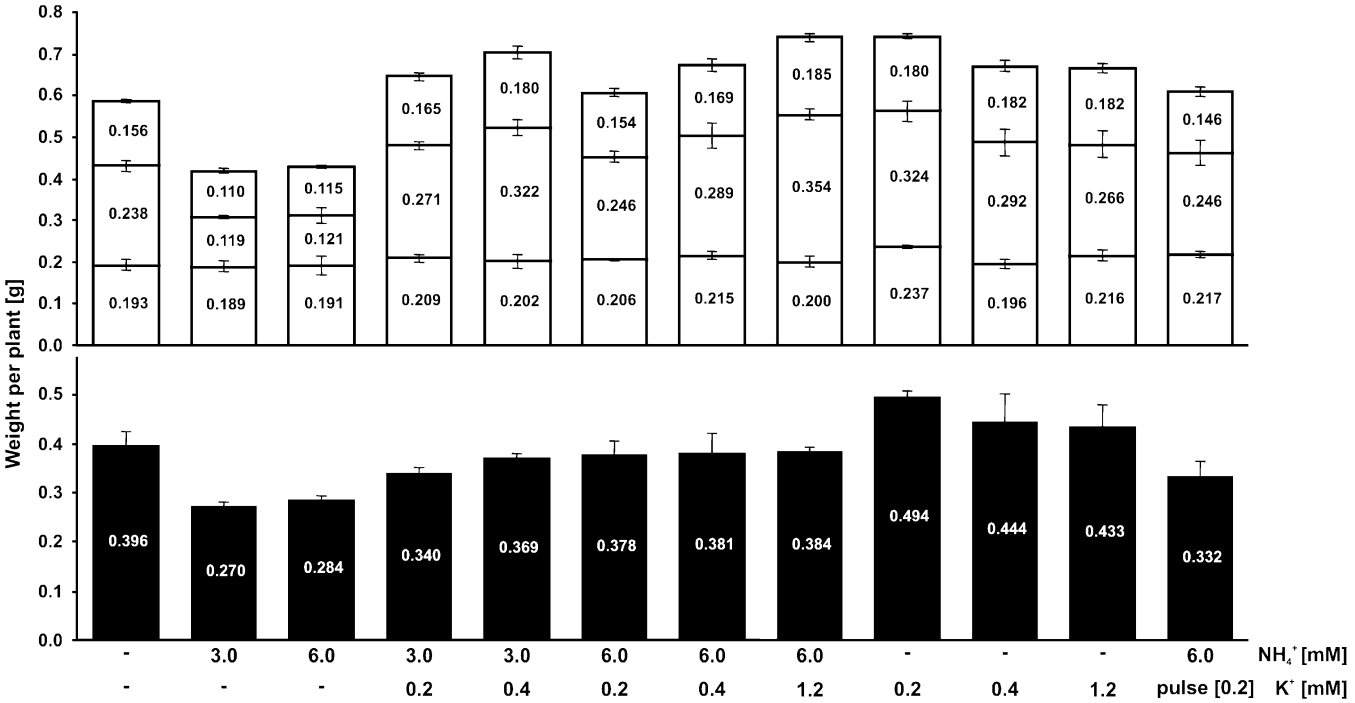

Fig. 2. Fresh weight of barley plants (cv. Antonia) cultivated at various K^+ and NH_4^+ concentrations. Plants were grown in a greenhouse in hydroponic medium with all nutrients including 1.5 mM NO_3^- as nitrogen source but without NH_4^+ or K^+ for 3 d and subsequently transferred for 5 d to various K^+ and NH_4^+ concentrations as indicated. Black bars represent root weight; white bars represent shoot weight, with old leaves (bottom), young leaves (middle), and stem (top). Data represent means ±SE of 12 plants for each measurement.

Besides the smaller size and chlorotic appearance of the young leaves of plants grown on high NH_4^+ concentrations, in the absence of K^+, there were no clear phenotypic differences between plants in different treatments. In the presence of low K^+ concentrations, the negative effects of NH_4^+ provision were ameliorated (Fig. 1).

Blocking K^+ uptake of Arabidopsis *seedlings by pharmacological inhibitors*

An earlier study by Cao *et al.* (1993) reported the dramatic depression of root growth of *Arabidopsis* seedlings caused by the presence of NH_4^+ in the absence of K^+ in the growth media. To study the effect reported by Cao *et al.* (1993) further, *Arabidopsis* seeds were germinated on media containing 6 mM NH_4^+ in the absence of K^+. Seedlings hardly developed roots (Fig. 4). The addition of 0.2 mM K^+ to the 6 mM NH_4^+ medium restored root growth above the level measured when neither NH_4^+ nor K^+ were present, essentially confirming the earlier results of Cao *et al.* (1993). To investigate whether the amelioration of NH_4^+ toxicity by K^+ was due to competition between K^+ and NH_4^+ at the same uptake site, 10 mM tetraethyl ammonium (TEA^+), a known blocker of K^+ channels (Yellen *et al.*, 1991; Lenaeus *et al.*, 2005), was added to the medium (Fig. 4). Addition of TEA^+ generally led to a substantial, significant reduction in root growth, which was most pronounced in the absence of K^+ in the growth medium. This pronounced root growth depression may be due to the inhibition of remobilization of K^+, which had initially derived from seed reserves. However, growth depression by 10 mM TEA^+ in the absence of K^+ and NH_4^+ was less pronounced than growth depression by the application of 6 mM NH_4^+ alone, the latter of which almost completely inhibited root formation. More importantly, in the presence of 10 mM TEA^+ plus 6 mM NH_4^+, growth was not significantly different from growth in the presence of 10 mM TEA^+ alone (Figs 4, 5). In fact, in this case the addition of TEA^+ significantly improved growth and completely abolished the NH_4^+-induced growth depression. These data suggest that K^+ efficiently reduced the uptake of NH_4^+, but that NH_4^+ toxicity was not exclusively the result of competition with K^+. Rather, NH_4^+ toxicity seemed to directly relate to an increased flux of NH_4^+ into the roots. Addition of Gd^{3+}, a known blocker of NSCCs (Demidchik *et al.*, 2002), had a minor effect and did not reverse the effects of NH_4^+ toxicity to the same extent as TEA^+ (data not shown).

Fluxes of NH_4^+ and K^+ under the influence of uptake blockers in Arabidopsis

To characterize more closely the interference between NH_4^+ and K^+ at the site of uptake at the root surface, net NH_4^+, K^+, and H^+ fluxes were determined in the mature root zone of *Arabidopsis* seedlings with microelectrodes using the MIFE® technique (Shabala *et al.*, 1997; Newman, 2001). Roots of seedlings grown on agarose plates without K^+ and NH_4^+ (as described previously), were exposed to various

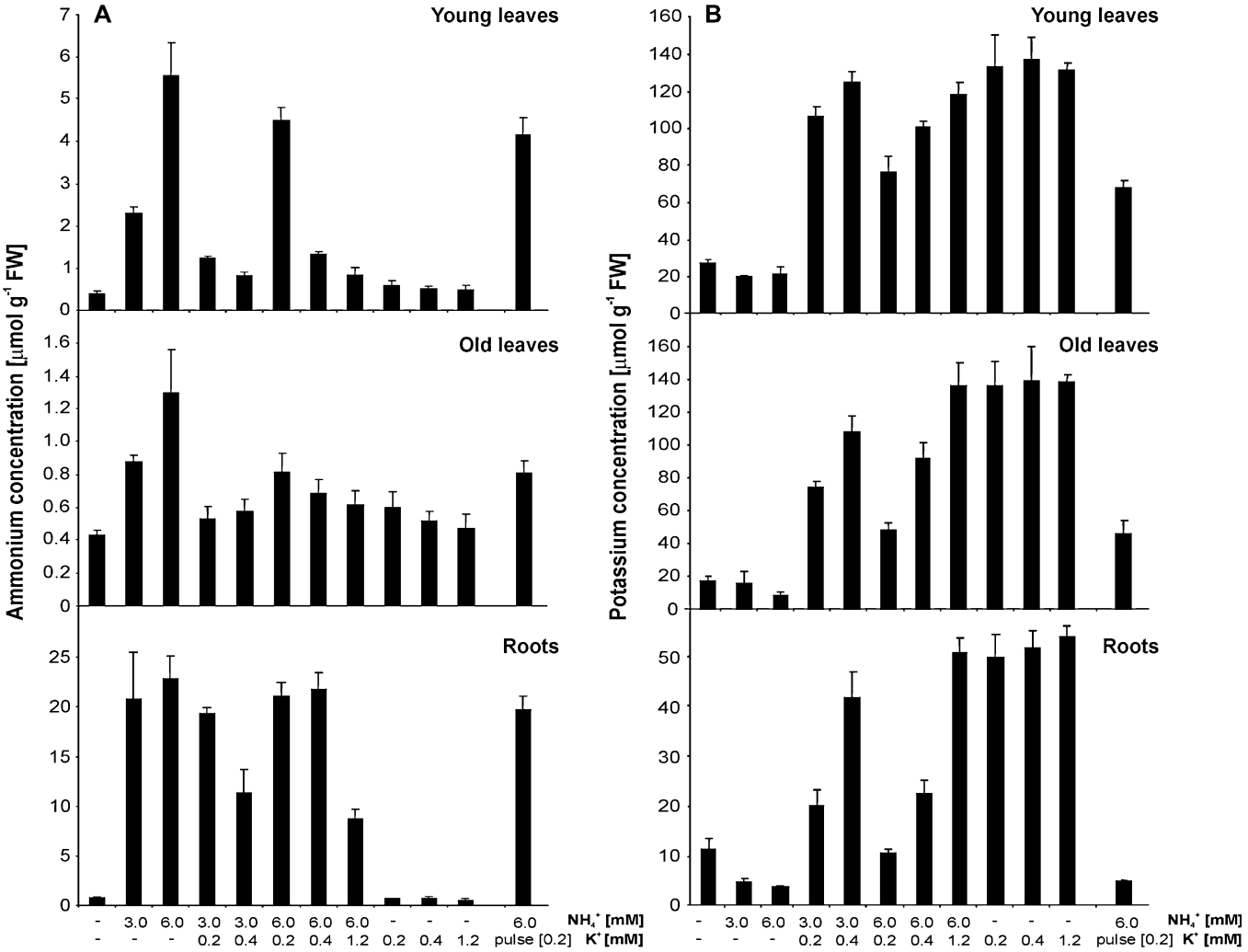

Fig. 3. NH_4^+ (A) and K^+ (B) tissue concentration of barley plants (cv. Antonia) cultivated on different NH_4^+ and K^+ regimes in hydroponic medium. Plants were grown in hydroponic medium including all nutrients and 1.5 mM NO_3^- as nitrogen source but without NH_4^+ or K^+ for 3 d and subsequently transferred to similar medium including various K^+ and NH_4^+ concentrations (as indicated) for an additional 5 d. Data represent means ±SE of three measurements with material from four plants per measurement.

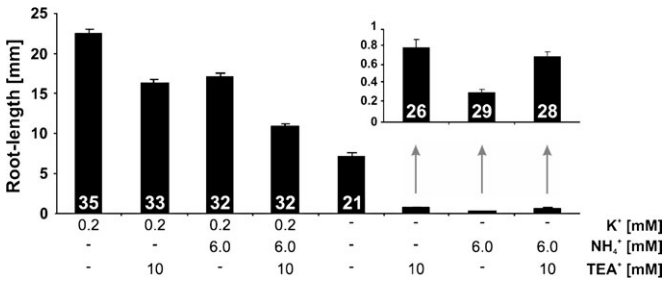

Fig. 4. Primary root length of *Arabidopsis* Col-0 seedlings cultivated for 10 d on plates containing different amounts of NH_4^+, K^+, and TEA^+ as indicated. Inset: Root length at treatments with high levels of NH_4^+ and/or TEA^+ in the absence of K^+. Data represent means ±SE. Between 21 and 35 roots were measured per treatment.

bathing solutions. When fluxes of NH_4^+ and K^+ were measured at constant 0.2 mM K^+ and various NH_4^+ concentrations (Fig. 6), an almost linear inverse correlation of fluxes for the two cations was found. Increasing NH_4^+ in the solution increased the NH_4^+ influx, while reducing the K^+ influx. Addition of NH_4^+ and K^+ separately resulted in increased NH_4^+ and K^+ influxes, respectively (Fig. 7). Interestingly, in the presence of both K^+ and NH_4^+, net influx of NH_4^+ was about double that measured upon sole NH_4^+ exposure. Conversely, net influx of K^+ was reduced to about half of the flux measured under sole K^+ exposure. Thus, K^+ stimulated NH_4^+ influx, while NH_4^+ reduced the influx of K^+. The high net influx of NH_4^+ in the presence of both ions was accompanied by a marked increase in net H^+ efflux across the plasma membrane. The addition of 10 mM TEA^+ and 0.1 mM Gd^{3+} strongly reduced both NH_4^+ and K^+ influxes, supporting the view that stimulation of NH_4^+ influx by K^+ and the inhibition of K^+ influx by NH_4^+ were related to one or several common uptake systems. Application of TEA^+ did not affect H^+ extrusion in the absence of K^+ and NH_4^+, but reduced H^+ effluxes in solution where K^+ and NH_4^+ were present. Net H^+ efflux was less affected by Gd^{3+} than by TEA^+ (Fig. 7).

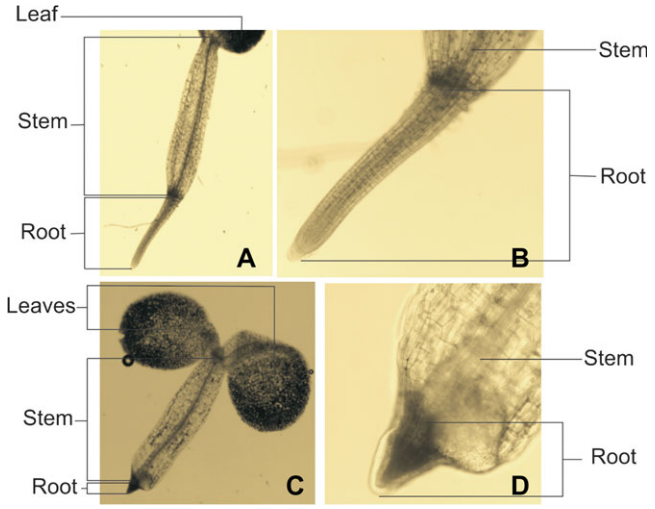

Fig. 5. Roots of *Arabidopsis* Col-0 plants grown on medium without K^+, but in the presence of 6 mM NH_4^+ (C, D) or 6 mM NH_4^+ plus 10 mM TEA^+ (A, B).

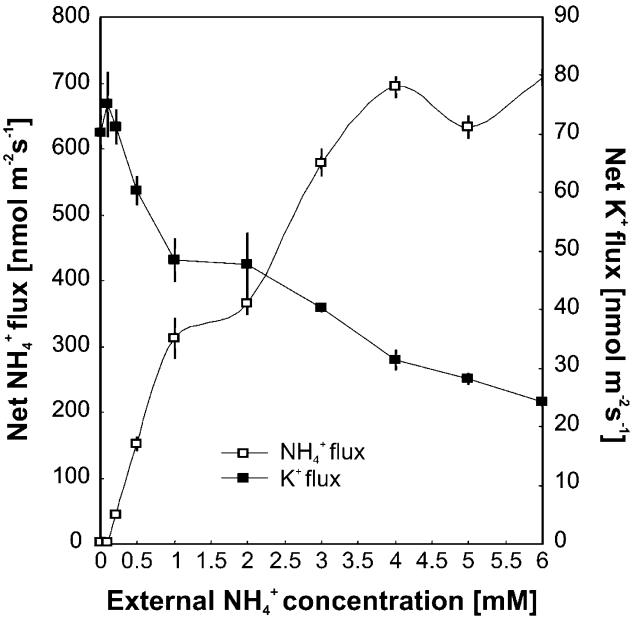

Fig. 6. Net fluxes of NH_4^+ and K^+ at the root surface are dependent on the external concentration of NH_4^+. Arabidopsis Col-0 plants were grown on agarose plates with NO_3^- as nitrogen source and without K^+ and NH_4^+. Measurements were done at constant external K^+ of 0.2 mM in bathing solution (0.1 mM $CaCl_2$).

NH_4^+/K^+ interference via plant transporters expressed in yeast

The results described above suggest that certain plasma membrane transporters or channels in both *Arabidopsis* and barley are capable of transporting NH_4^+ and K^+. To identify putative transporters or channels at the molecular level, several new mutants lacking multiple NH_4^+ and K^+ transporters were generated from single deletion mutants in the yeast BY4741 background (Euroscarf): (i) an NH_4^+ transporter mutant Δ*mep1-3*, devoid of all three NH_4^+ transporter genes *MEP1*, *MEP2*, and *MEP3*, (ii) a K^+ transporter mutant Δ*trk1,2* lacking the two K^+ transporter genes *TRK1* and *TRK2*, and (iii) a 5-fold deletion mutant Δ*mep1-3* Δ*trk1,2* lacking all five genes. The triple mutant Δ*mep1-3*, not expressing the NH_4^+ transporters Mep1p, Mep2p, and Mep3p, did not grow on media containing 10 mM NH_4^+ as the sole nitrogen source after 3 d of incubation at 30 °C (Fig. 8A, empty vector control). The *trk1,2* double mutant did not grow at less than 10 mM K^+ in the medium when proline was the sole nitrogen source (Fig. 8B, empty vector control). Deletion of all five genes in Δ*mep1-3* Δ*trk1,2* thus provided a tool for the identification of transporters that transport both NH_4^+ and K^+ at limiting concentrations.

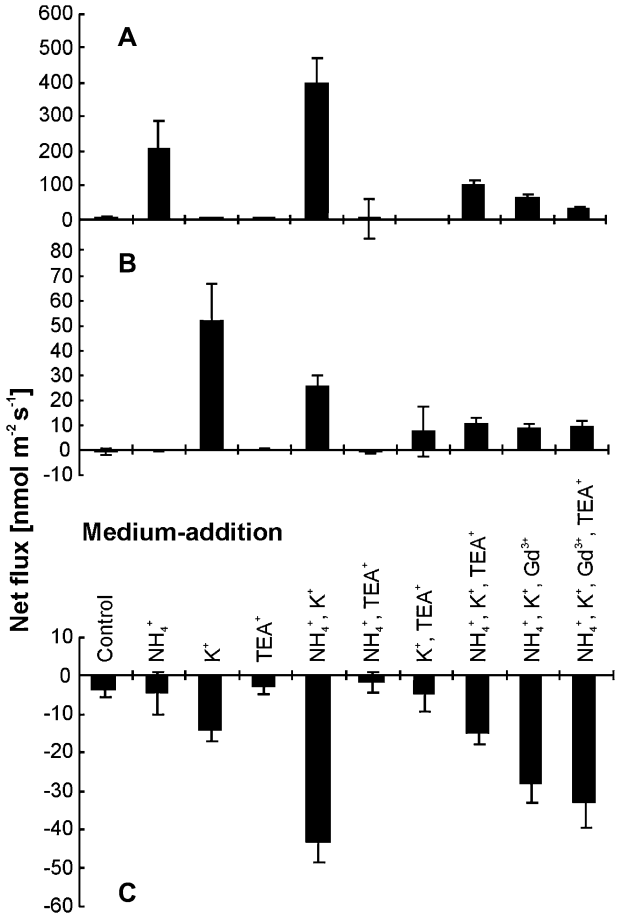

Fig. 7. Net fluxes of NH_4^+ (A), K^+ (B), and H^+ (C). Arabidopsis Col-0 plants were grown on agarose plates with NO_3^- as nitrogen source and without K^+ and NH_4^+. Fluxes were measured for 5 min in bathing solution (0.1 mM $CaCl_2$) 15 min after the addition of various NH_4^+, K^+, TEA^+, and Gd^{3+} as indicated.

The yeast mutant Δ*mep1-3* Δ*trk1,2* was transformed with a cDNA library made from roots of barley seedlings (Pedas *et al.*, 2008). In the initial screen, transformants were grown on media containing six different combinations of pH, $[NH_4^+]$, and $[K^+]$: pH 4.5 with 0.5 mM K^+ and 5 or 10 mM NH_4^+, pH 4.5 with 5 mM K^+ and 5 or 10 mM NH_4^+, pH 5.5 with 1 mM K^+ and 20 mM NH_4^+, and pH 5.5 with 5 mM K^+ and 50 mM NH_4^+. A total of 112 colonies from all

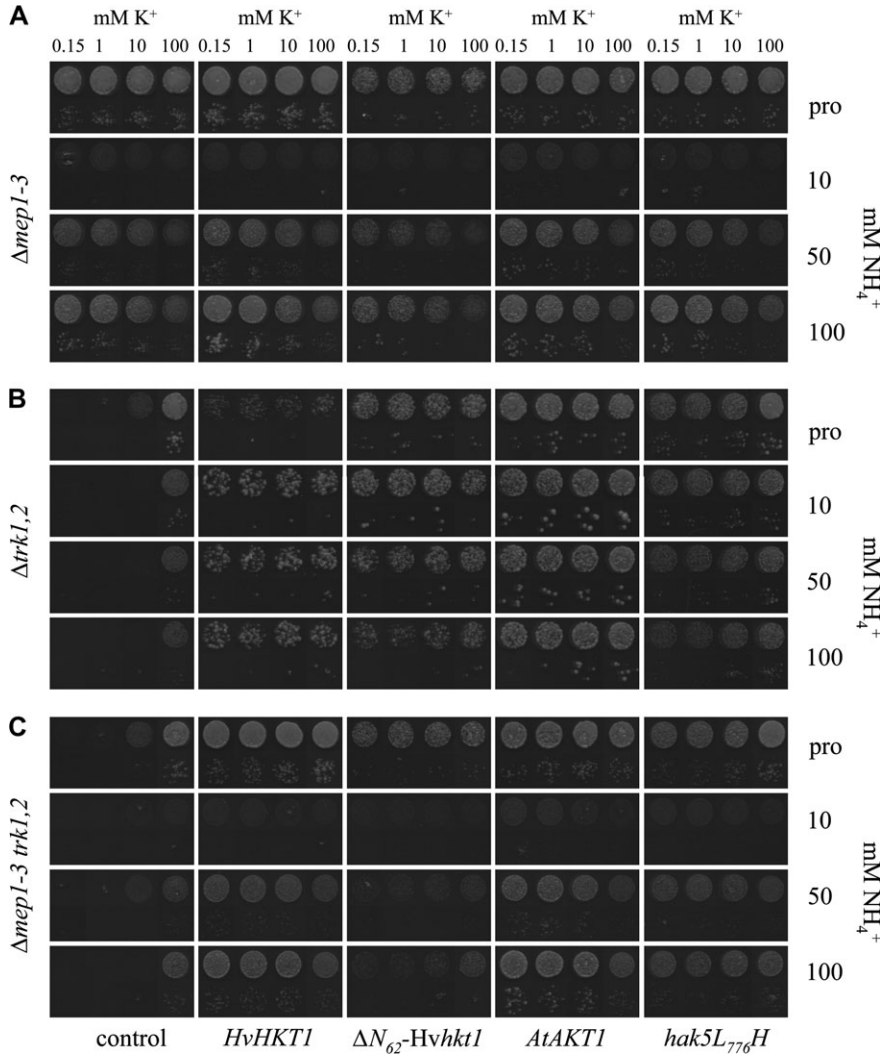

Fig. 8. Growth assays of various yeast strains transformed with cDNA encoding HvHKT2;1, ΔN_{62}-hkt2;1, AtAKT1, or hak5L_{776}H at various K^+ and NH_4^+ concentrations or 0.1% proline instead of NH_4^+ as nitrogen source. The various yeast mutant strains were (A) Δ*mep1-3*, (B) Δ*trk1-2*, and (C) Δ*mep1-3* Δ*trk1-2*. Transformants were pre-grown on SD medium containing 0.1% proline as nitrogen source and supplemented with 100 mM K^+. Yeast suspensions with an OD_{600nm} of 0.01 (upper drop) and 0.0001 (lower drop) were placed onto SG medium (1% galactose; YNB without amino acids, K^+ and NH_4^+) with various supplementations as indicated. Control, empty vector pYES2.

conditions were harvested and plasmids of 24 of these transformants (four of each of the six conditions) were extracted, transformed into *E. coli* and subjected to DNA sequencing. A database search revealed that all 24 coding sequences were identical to *HvHKT2;1*. Strikingly, about half of the plasmids contained a version of *HvHKT2;1* cDNA lacking either 36 or 85 nucleotides in the 5′ end when compared to full-length clones. Twenty nucleotides of this deletion represented a sequence of the 5′-UTR. An in-frame ATG codon, 186 nucleotides downstream of the original ATG start codon, probably served as a translational initiation point resulting in ΔN_{62}-hkt2,1 in both truncated versions. HvHKT2;1 and ΔN_{62}-hkt2;1 as well as hak5L_{776}H and AtAKT1 from *Arabidopsis* were subsequently characterized in the triple mutant Δ*mep1-3*, the double mutant Δ*trk1,2* and the 5-fold mutant Δ*mep1-3* Δ*trk1,2* on media containing various NH_4^+ and K^+ concentrations (Fig. 8). The mutation L_{776}H in the AtHAK5 protein was earlier shown to be important for functional expression of *AtHAK5* in yeast (Rubio *et al.*, 2000).

Full-length *HvHKT2;1* and *AtAKT1* very weakly increased growth of Δ*mep1-3* compared with the empty vector control and other transporter genes at low NH_4^+ (10 mM) and low K^+ (0.15 and 1 mM; Fig. 8A). In all transformants of Δ*mep1-3*, growth was suppressed by increasing K^+ concentration irrespective of the NH_4^+ level, indicating that K^+ competed with NH_4^+ (Fig. 8A) When transformed into Δ*trk1,2*, *AtAKT1*, *HvHKT2;1*, ΔN_{62}-*hkt2;1*, and *hak5*L_{776}*H* clearly complemented the growth deficiency of the mutant at low K^+ concentrations (Fig. 8B). However, increasing NH_4^+ at low K^+ caused growth repression, particularly in Δ*trk1,2* transformed with ΔN_{62}-*hkt2;1*, and *hak5*L_{776}*H* but also, albeit to a smaller extent, in Δ*trk1,2* transformed with *HvHKT2;1* and *AtAKT1* (Fig, 8B) In the

5-fold mutant lacking all yeast endogenous NH_4^+ and K^+ transporters, only *HvHKT2;1*, *AtAKT1* and, less markedly, *hak5L*$_{776}$*H*, complemented the growth deficiency of the mutant (Fig. 8C). Interestingly, when transformed with full-length *HvHKT2;1*, the triple mutant Δ*mep1-3* and the 5-fold mutant Δ*mep1-3* Δ*trk1,2* grew well (Fig. 8C), whereas the double mutant Δ*trk1,2* did not (Fig. 8B). The opposite was the case for its N-terminally truncated counterpart Δ*N*$_{62}$-*hkt2;1*, which complemented growth deficiency on low [K^+] better in Δ*trk1,2* than in Δ*mep1-3* or Δ*mep1-3* Δ*trk1,2*.

Discussion

Changes in cation composition, specifically a reduction in tissue levels of K^+ have been reported in connection with NH_4^+ toxicity in plants (see for example Scherer *et al.*, 1984; Vale *et al.*, 1987, 1988; Finnemann and Schjoerring, 1999; Santa María *et al.*, 2000; Kronzucker *et al.*, 2003; Szczerba *et al.*, 2006). Elevated external concentrations of K^+ can alleviate the effects of NH_4^+ toxicity, and cause a decrease in the tissue concentrations of NH_4^+ (Barker *et al.*, 1967; Cao *et al.*, 1993; Spalding *et al.*, 1999; Szczerba *et al.*, 2008*a*, and references therein). To understand the role of cation transporters and channels in NH_4^+ toxicity better, plant growth, NH_4^+ and K^+ accumulation, and the specific fluxes of NH_4^+, K^+, and H^+ in *Arabidopsis* and barley seedlings were examined. High NH_4^+ and low K^+ concentrations caused chlorosis in leaves and reduced biomass and root growth of barley plants (Figs 1, 2), suggesting that these conditions caused NH_4^+ toxicity. All of these symptoms were alleviated by an elevated provision of K^+.

In barley, plant-soluble NH_4^+ content was low at high external K^+ concentration and *vice versa* (Fig. 3; see also Szczerba *et al.*, 2006, 2008*a*). In young leaves, however, K^+ and NH_4^+ concentrations were relatively constant, with NH_4^+ only reaching high levels when the external NH_4^+ concentration was very high and that of K^+ very low. K^+ levels in young leaves were always above 70–80 μmol g^{-1} FW, unless K^+ was absent from the growth medium. This constant level of K^+ may reflect the important role of K^+ in vital processes such as stomatal opening and closure (Humble and Hsiao, 1969; Cakmak *et al.*, 1994; Dietrich *et al.*, 2001) and protein and starch synthesis (Marschner, 1995). In K^+-deficient barley plants several transport mechanisms may be activated to enable a more efficient translocation from root to shoot, thus sustaining the K^+ concentration of the latter and securing K^+-dependent metabolic processes (Britto and Kronzucker, 2006).

The exact mechanism behind the interaction of K^+ and NH_4^+ is not known, but it has been suggested that membrane passage of NH_4^+ via K^+ channels and NSCCs could play a role (Howitt and Udvardi, 2000; Kronzucker *et al.*, 2001; Szczerba *et al.*, 2008*a*, *b*). Our results show that the growth inhibition by high external NH_4^+ was reduced in the presence of either TEA^+ or Gd^{3+}, which suggests a role for both K^+-selective channels as well as NSCCs in mediating NH_4^+ uptake. Interestingly, alleviation of NH_4^+ induced growth depression by TEA^+ was only seen when the growth medium was supplemented with sucrose (data from experiments without sucrose not shown). This may be explained by an inhibition of internal K^+ transport by TEA^+ causing a negative impact on carbohydrate and energy supply to the roots via the phloem which will be more severe in the absence of external sucrose.

To investigate more closely the transport of NH_4^+ and K^+ at the root surface, flux measurements were undertaken, using the MIFE® technique (Newman, 2001). Net fluxes of NH_4^+, H^+, and K^+ were recorded at the mature root zone of *Arabidopsis* seedlings. At a constant K^+ concentration of 0.2 mM, net NH_4^+ and K^+ fluxes into the roots showed an almost linear inverse correlation with NH_4^+ reducing the uptake of K^+, and K^+ reducing the uptake of NH_4^+ (Fig. 6). This indicates competition for uptake sites between the two ions as also suggested by Scherer *et al.* (1984), Nielsen and Schjoerring (1998), and Szczerba *et al.* (2008*a*).

Although fluxes showed a reverse correlation, co-supplementing plants with K^+ increased NH_4^+ influx (Fig. 7). This could be the result of increased channel- or transporter activity to enable K^+ uptake. Indeed, both Gd^{3+} and TEA^+ strongly reduced the NH_4^+ influx under these conditions with a combination of both blockers giving the largest effect. Very similar results were obtained in experiments using nutrient solution instead of $CaCl_2$ as the bathing medium (data not shown). The inhibition of NH_4^+ influx by uptake blockers suggests that K^+ channels/transporters as well as NSCCs are involved in membrane passage of NH_4^+. This result is in contrast to reports by Szczerba *et al.* (2008*a*) who observed an increased NH_4^+ influx at 0.1 and 40 mM K^+ in the presence of TEA^+, and concluded that the K^+-sensitive influx of NH_4^+ was mainly via the NSCC pathway.

Unlike the short-term K^+ stimulation of NH_4^+ influx in *Arabidopsis*, K^+ provision to barley inhibited NH_4^+ accumulation over time (Fig. 3). The stimulation of NH_4^+ influx by K^+ in our measurements with *Arabidopsis* may be related to the growth conditions: for flux measurement, plants were germinated in the absence of both K^+ and NH_4^+. This would lead to an induction of transporters, such as HAK5, which is repressed by high NH_4^+ (Rubio *et al.*, 2008) but induced by low K^+ supply. It would have been relevant to measure fluxes from plants that were grown in the presence of high NH_4^+ and in the absence of K^+, but such conditions led to root growth inhibition.

Excessive uptake of cations over anions across the plasma membrane in root cells leads to acidification of the rhizosphere (Marschner, 1995), explaining the measured proton efflux with K^+, NH_4^+ or a combination of both ions in the media (Fig. 7C). The highest H^+ efflux was consequently measured in the presence of both NH_4^+ and K^+, with a reduction upon the co-presence of TEA^+ and Gd^{3+}, in line with the reduced uptake of NH_4^+ and K^+. As compared with the H^+ fluxes measured upon the presence of NH_4^+, K^+, and TEA^+, an unexpected large net H^+ efflux was observed in the additional presence of Gd^{3+}, a situation in which the fluxes of NH_4^+ and K^+ were reduced. Gd^{3+} has

been shown to inhibit H^+ influx in the alga *Chara corallina* (McConneaughy and Falk, 1991) although the precise mechanisms behind this inhibition are unknown. A similar mechanism could lead to the external accumulation of H^+ observed in this study.

Competition of NH_4^+ and K^+ at the site of transporters

In *Arabidopsis* roots, AKT1 and particularly HAK5 are the main players in high affinity K^+ uptake (Rubio *et al.*, 2008). AKT1 transports K^+ over a broad range of concentrations and is suggested to facilitate NH_4^+-insensitive K^+ uptake (Hirsch *et al.*, 1998; Angeles Martínez-Cordero *et al.*, 2005). However, a study using insertion lines showed that transport of K^+ by both AKT1 and HAK5 is sensitive to NH_4^+ (Rubio *et al.*, 2008). Our results from heterologous expression of *AKT1* and *HAK5* in the yeast mutant Δ*trk1,2* support this earlier study, although inhibition of K^+ transport by NH_4^+ was more pronounced in yeast expressing *HAK5* relative to *AKT1*. In the presence of NH_4^+, induction of HAK5 is suppressed by low K^+ concentration (Qi *et al.*, 2008; Rubio *et al.*, 2008), suggesting that AKT1 is the main transporter for high affinity K^+ uptake in the presence of NH_4^+ (Rubio *et al.*, 2008) and that HAK5 does not play a role in the inhibition of K^+ uptake by NH_4^+ in *Arabidopsis*. However, in the tomato variety Micro Tom, the gene encoding LeHAK5 is highly expressed independent of whether NH_4^+ is present or not (Nieves-Cordones *et al.*, 2007).

HKT isoforms in various species have been shown to function as K^+/Na^+- or Na^+/Na^+ co-transporters (Rodríguez-Navarro and Rubio, 2006). Our data suggest that *HvHKT2;1* and *AtAKT1*, when expressed in yeast, are able to transport NH_4^+ and K^+. However, the barley cDNA library screen in yeast only retrieved *HvHKT2;1* clones, although transformants were grown on various concentrations of K^+ and NH_4^+. Other isoforms may not have been functionally expressed in our yeast heterologous expression system as was also the case for *AtHAK5* in yeast (Rubio *et al.*, 2000). Lack of functional expression may similarly explain results from a wheat library screen in yeast for the complementation of potassium transport deficiency, which only retrieved *TaHKT2;1* and *TaLCT1* (Schachtman *et al.*, 1997).

The library screen resulted in the identification of both full-length and truncated versions of *HvHKT2;1* with no apparent correlation as to the particular concentrations of NH_4^+ and K^+ and the pH of the media. However, the closer characterization on media with lower galactose concentration and without succinic acid as buffer revealed profound differences between full-length HKT2;1 and ΔN_{62}-hkt2;1. Full length *HvHKT2;1* best supported growth of Δ*mep1-3* and Δ*mep1-3* Δ*trk1,2*, whereas ΔN_{62}-*hkt2;1* complemented better in Δ*trk1,2*, i.e. in the presence of *MEP1-3*. This may suggest a cross-regulation between HKT2;1 and Mep/AMT homologues that deserves further investigation. Haro *et al.* (2005) reported differences in the transport of full-length versus truncated versions of *HvHKT2;1*. A recent investigation by the same group suggested that it is the expression level of *HKT2;1*, rather than its truncation, that is responsible for variation in activity (Bañuelos *et al.*, 2008). These differences in expression levels were due to small ORFs in artificial UTRs resulting from the plasmid. We do not know how truncation in our experiments affected the expression level of the proteins. However, we did not observe ORFs in the polylinker of our plasmid, suggesting that the level of expression of both *HvHKT2;1* and ΔN_{62}-*hkt2;1* was similar.

How could NH_4^+ uptake through K^+ transport proteins and NSCCs result in NH_4^+ toxicity?

Recently, it was suggested that growth depression of barley plants at high NH_4^+ was due to a futile cycling, involving uptake of NH_4^+ followed by active extrusion at the plasma membrane (Britto *et al.*, 2001). While such a mechanism can well explain growth depression, it does not explain why plants would aim to eliminate excess NH_4^+. Extrusion of NH_4^+ should be the cellular response to avoid the accumulation of NH_4^+, which is critical at high concentration. Here, the cost of extrusion would represent a secondary but, potentially, rather serious problem. Alternatively, the cycling of NH_4^+ itself would be an unavoidable process. In such a scenario, futile cycling could indeed be the cause of NH_4^+ toxicity. Yet, such cycling would require uptake and extrusion mechanisms.

It is well accepted that NH_4^+ transporters of the AMT family are highly specific for NH_4^+ over K^+ (Ninnemann *et al.*, 1994). Recent studies on the structure of AmtB have suggested that discrimination of K^+ against NH_4^+ in AMT homologues is due to a de-protonation process of NH_4^+ in the transporter (Khademi *et al.*, 2004). The *E. coli* AmtB binds NH_4^+ with high affinity and, following de-protonation, NH_3, and possibly H^+, is transported through the channel. Thus, AmtB could be considered a catalytic channel that modifies its substrate with the aim of discriminating between NH_4^+ and the highly similar K^+ ion. Although it is a matter of controversy if the transport via AmtB is electrogenic (Javelle *et al.*, 2008), de-protonation at S219 appears to be a prerequisite for transport (Ishikita and Knapp, 2007) and may serve a function to discriminate between NH_4^+ and K^+. Similar roles may apply to plant AMTs which have been shown to perform electrogenic transport (Ludewig *et al.*, 2002). Ion transporters and channels transporting K^+ do not possess such a mechanism of ion discrimination, providing an explanation as to why K^+ transporters and NSCCs do take up NH_4^+ even when NH_4^+ uptake by AMTs is down-regulated. The finding that K^+ transporters and channels as well as NSCCs transport NH_4^+ provides a plausible explanation for the uptake of NH_4^+, at least at low external K^+, being an unavoidable process due to the physico-chemical similarities of both ions.

In plants, plasma membrane transporters for active extrusion of NH_4^+ as postulated by Britto *et al.* (2001) have not been identified. An alternative explanation for an active,

energy-consuming extrusion mechanism could be passive diffusion of ammonia (NH_3) out of the cytoplasm either into the vacuole or across the plasma membrane into the apoplast. The vacuole is likely to be the primary compartment trapping NH_4^+ due to the presence of NH_3 permeable tonoplast intrinsic proteins (Jahn *et al.*, 2004; Loque *et al.*, 2005). Such a role for TIPs in an acid trap mechanism for NH_4^+ has recently been suggested (Ludewig *et al.*, 2007). The vacuolar compartment, however, can only provide a limited volume for buffering of excess cytoplasmic NH_4^+. Once this buffer capacity is exhausted, excess NH_4^+ continuously provided from the external medium could diffuse in the form of uncharged NH_3 along its concentration gradient back across the plasma membrane and into the apoplast. The concentration gradient for NH_3 is maintained by the pH gradient across the plasma membrane. Thus, the continuous uptake of NH_4^+ into the cell versus diffusion of NH_3 out of the cell would result in a futile cycling of H^+ across the plasma membrane, and part of this cycle would be catalysed by the ATP-fuelled plasma membrane H^+-ATPase. Obtaining unambiguous proof for such an hypothesis is challenging, as it is difficult to demonstrate the form in which NH_4^+/NH_3 is transported in and out of the cell. The observation that the addition of TEA^+ inhibited NH_4^+-induced H^+ efflux from *Arabidopsis* roots (Fig. 7) however, supports the interpretation that the activity of the plasma membrane H^+-ATPase represents the energy-consuming step rather than the NH_4^+ extrusion.

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