Journal of Experimental Botany, Vol. 59, No. 2, pp. 303–313, 2008
doi:10.1093/jxb/erm309 Advance Access publication 17 January, 2008
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Alleviation of rapid, futile ammonium cycling at the plasma membrane by potassium reveals K^+-sensitive and -insensitive components of NH_4^+ transport

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Received 8 August 2007; Revised 8 November 2007; Accepted 12 November 2007

Abstract

Futile plasma membrane cycling of ammonium (NH_4^+) is characteristic of low-affinity NH_4^+ transport, and has been proposed to be a critical factor in NH_4^+ toxicity. Using unidirectional flux analysis with the positron-emitting tracer ^{13}N in intact seedlings of barley (*Hordeum vulgare* L.), it is shown that rapid, futile NH_4^+ cycling is alleviated by elevated K^+ supply, and that low-affinity NH_4^+ transport is mediated by a K^+-sensitive component, and by a second component that is independent of K^+. At low external [K^+] (0.1 mM), NH_4^+ influx (at an external [NH_4^+] of 10 mM) of 92 μmol g^{-1} h^{-1} was observed, with an efflux:influx ratio of 0.75, indicative of rapid, futile NH_4^+ cycling. Elevating K^+ supply into the low-affinity K^+ transport range (1.5–40 mM) reduced both influx and efflux of NH_4^+ by as much as 75%, and substantially reduced the efflux: influx ratio. The reduction of NH_4^+ fluxes was achieved rapidly upon exposure to elevated K^+, within 1 min for influx and within 5 min for efflux. The channel inhibitor La^{3+} decreased high-capacity NH_4^+ influx only at low K^+ concentrations, suggesting that the K^+-sensitive component of NH_4^+ influx may be mediated by non-selective cation channels. Using respiratory measurements and current models of ion flux energetics, the energy cost of concomitant NH_4^+ and K^+ transport at the root plasma membrane, and its consequences for plant growth are discussed. The study presents the first demonstration of the parallel operation of K^+-sensitive and -insensitive NH_4^+ flux mechanisms in plants.

Key words: Ammonium, barley, efflux, influx, nitrogen-13, non-selective cation channels, potassium.

Introduction

Ammonium (NH_4^+) is present in many terrestrial ecosystems and over a wide concentration range (Pearson and Stewart, 1993; Miller and Cramer, 2005). At low (micromolar) soil concentrations, NH_4^+ is adequate as a sole N source for many plant species (Kronzucker *et al*., 1997, 1999), but most cannot tolerate millimolar concentrations (Britto and Kronzucker, 2002). In this toxic range, NH_4^+ uptake is mediated by a high-capacity, energetically passive, low-affinity transport system (LATS). However, low-affinity NH_4^+ influx is accompanied by an efflux of NH_4^+ nearly equal in magnitude, resulting in the futile cycling of this ion across the plasma membrane (Britto *et al*., 2001, 2002; Britto and Kronzucker, 2006). The substantial efflux of NH_4^+ under these conditions has been shown to be energetically costly in NH_4^+-sensitive plant species (Kronzucker *et al*., 2001), and it has been postulated that a primary cause of NH_4^+ toxicity in plants is the energy lost due to the active removal of NH_4^+ that has entered root cells at an uncontrolled rate (Britto *et al*., 2001; Kronzucker *et al*., 2001).

NH_4^+ nutrition has been shown to influence the mineral composition of plants dramatically, particularly in the reduction of cation content (Kirkby and Mengel, 1967; Vale *et al*., 1987, 1988*a*; Gerendas *et al*., 1997; Santa-María *et al*., 2000; Szczerba *et al*., 2006*a*). The mechanism underlying this has not been unravelled, but it may be through direct competition between NH_4^+ and other cations for entry through common uptake pathways. In particular, potassium (K^+) channels are considered prime candidates for low-affinity NH_4^+ transport, as NH_4^+ and K^+ are both monovalent cations with similar hydrated atomic radii (Kielland, 1937; Wang *et al*., 1996; White, 1996). However, a hydrated atomic radius may not be

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a critical characteristic for use of a common channel, as the hydrated shell has been shown to be removed as ions pass through the selective filter (Doyle, 2004).

A key relationship between K^+ and NH_4^+ nutrition is that an increase in external K^+ concentration ($[K^+]_{ext}$) protects sensitive plant species from NH_4^+ toxicity (Cao *et al*., 1993; Spalding *et al*., 1999; Santa-María *et al*., 2000; Kronzucker *et al*., 2003*b*; Szczerba *et al*., 2006*a*). This protection is due in part to the restoration of normal K^+ status to the plant, a process that ultimately depends on K^+ fluxes into roots and its subsequent translocation to the shoot (Kronzucker *et al*., 2003*b*; Szczerba *et al*., 2006*a*). In studies using intact barley seedlings, Kronzucker *et al*. (2003*b*) and Szczerba *et al*. (2006*a*) showed that, at low external K^+ concentrations, K^+ fluxes into the root were much lower in seedlings grown with 10 mM NH_4^+ than those grown with 10 mM nitrate (NO_3^-), but at high external K^+, these fluxes were independent of N source. Additionally, the K^+ flux from root to shoot, which was the flux most suppressed by NH_4^+ at low external $[K^+]$, was nearly identical in plants grown with NO_3^- or NH_4^+ at the higher K^+ concentration (Kronzucker *et al*., 2003*b*). These effects, in particular the suppression of K^+ influx at the plasma membrane under low-K^+, high-NH_4^+ conditions, are likely to be due to the inhibitory action of NH_4^+ upon high-affinity KUP/HAK/KT transporters (Spalding *et al*., 1999).

While inhibition of K^+ uptake and modification of K^+ efflux by NH_4^+ has been demonstrated, the reciprocal effect has only been sparsely investigated (Scherer *et al*., 1984; Vale *et al*., 1988*b*; Wang *et al*., 1996; Nielsen and Schjoerring, 1998). Nielsen and Schjoerring (1998) found that 100 mM K^+ reduced the influx of NH_4^+ by 50% in leaf apoplasm of *Brassica napus* L. Other studies have demonstrated moderate suppression of NH_4^+ isotherms by K^+, but never to the same extent as the suppression of K^+ influx by NH_4^+ (Scherer *et al*., 1984; Vale *et al*., 1988*b*; Wang *et al*., 1996). While many of these studies were conducted at low external concentrations of both NH_4^+ and K^+, none considered growth conditions mediated by LATS for either ion.

To investigate how NH_4^+ fluxes are influenced by external $[K^+]$, and how this interaction may underlie potassium's alleviation of ammonium toxicity, NH_4^+ fluxes were examined in intact barley seedlings using the short-lived positron-emitting radiotracer ^{13}N. It was hypothesized that increasing external $[K^+]$ would: (i) decrease unidirectional NH_4^+ fluxes across the plasma membrane; (ii) reduce the high NH_4^+ efflux:influx ratio that is symptomatic (and perhaps a causative agent) of NH_4^+ toxicity; and (iii) lessen the energy burden associated with toxic NH_4^+ fluxes. All three hypotheses were borne out in the study. It is proposed that low-affinity NH_4^+ influx is accomplished by two components, the first responding to K^+ and the second unaffected by it.

Materials and methods

Plant culture

Seeds of barley (*Hordeum vulgare* L. cv. 'Klondike') were surface-sterilized for 10 min in 1% sodium hypochlorite and germinated under acid-washed sand for 3 d prior to placement in 4.0 l vessels containing aerated, 1/4 strength Johnson's solution, at pH 6–6.5, for an additional 4 d. The solution was modified to provide four concentrations of potassium (as K_2SO_4), at 0.1, 1.5, 5, and 40 mM, and NH_4^+ [as $(NH_4)_2SO_4$], at 10 mM. Solutions were exchanged frequently to ensure that plants remained at a nutritional steady state. Plants were cultured in a walk-in growth chamber under fluorescent lights (Philips Econ-o-watt, F96T12), with an irradiation of 200 μmol photons m^{-2} s^{-1} at plant height, for 16 h d^{-1}. Daytime temperature was 20 °C, night-time temperature was 15 °C, and relative humidity was ~70%. On day 6 (1 d prior to experimentation), seedlings were transferred to an experimental radiotracer facility that had similar irradiance and temperature to the growth chamber.

Compartmental analysis

Compartmental analysis by tracer efflux was used to estimate subcellular fluxes and compartmental pool sizes (Lee and Clarkson, 1986; Siddiqi *et al*., 1991; Kronzucker *et al*., 1995). Each replicate consisted of five plants held together at the shoot base by a plastic collar. Intact roots of these plants were labelled for between 30 min and 55 min in solution identical to growth solution but containing the radiotracer ^{13}N ($t_{1/2}$=9.97 min; as $^{13}NH_4^+$) provided by the CAMH cyclotron facility (University of Toronto, Ontario, Canada). Labelled seedlings were attached to efflux funnels and eluted of radioactivity with successive 20 ml aliquots of non-radioactive desorption solution, identical to the growth solution. The desorption series was timed as follows: 15 s (four times), 20 s (three times), 30 s (twice), 40 s (once), 50 s (once), 1 min (five times), 1.25 min (once), 1.5 min (once), 1.75 min (once), and 2 min (eight times). All solutions were mixed using a fine stream of air bubbles. Immediately following elution, roots were detached from shoots and spun in a low-speed centrifuge for 30 s prior to weighing. Radioactivity from eluates, roots, shoots, and centrifugates was counted, and corrected for isotopic decay, using a gamma counter (PerkinElmer Wallac 1480 Wizard 3′′, Turku, Finland). Linear regression of the function $\ln \phi_{co(t)}^* = \ln \phi_{co(i)}^* - kt$ [in which $\phi_{co(t)}^*$ is tracer efflux at elution time t, $\phi_{co(i)}^*$ is initial radioactive tracer efflux, and k is the rate constant describing the exponential decline in radioactive tracer efflux, found from the slope of the tracer release rate; see Fig. 1] was used to resolve the kinetics of the slowest exchanging phase in these experiments, which represents tracer exchange with the cytosolic compartment (Kronzucker *et al*., 1995; Britto and Kronzucker, 2003). Chemical efflux, ϕ_{co}, was determined from $\phi_{co(i)}^*$, divided by the specific activity of the cytosol (SA_{cyt}) at the end of the labelling period; SA_{cyt} was estimated by using external specific activity (SA_o), labelling time t, and the rate constant k, which describes tracer exchange with the cytosol, which are related in the exponential rise function $SA_{cyt} = SA_o(1 - e^{-kt})$ (Kronzucker *et al*., 1995). Net flux, ϕ_{net}, was found using total plant ^{13}N retention after desorption (Kronzucker *et al*., 1995). Influx, ϕ_{oc}, was calculated from the sum of ϕ_{net} and ϕ_{co}. Cytosolic $[NH_4^+]$ ($[NH_4^+]_{cyt}$) was determined using the flux turnover equation, $[NH_4^+]_{cyt} = \Omega \cdot \phi_{oc}/k$, where Ω is a proportionality constant correcting for the cytosolic volume being ~5% of total tissue (Britto and Kronzucker, 2001).

K^+ concentration shift experiments followed a protocol identical to that above except that, upon 12.25 min of elution, subsequent aliquots were no longer identical to the growth and labelling solutions, but contained a new K^+ concentration (0.1 mM or 5 mM).

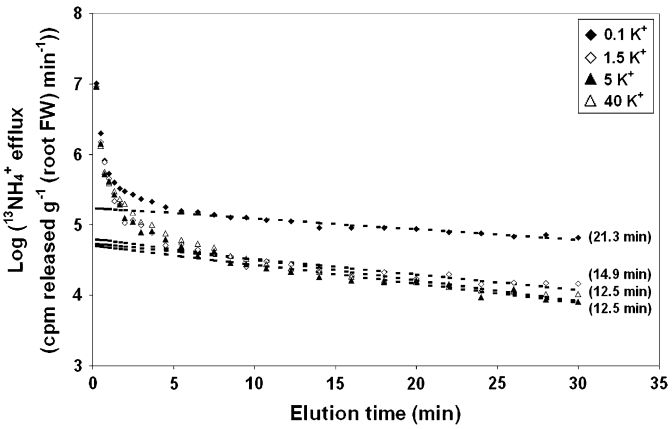

Fig. 1. $^{13}NH_4^+$ efflux from the roots of intact barley seedlings grown with 10 mM NH_4^+ and at four K^+ concentrations (as indicated). Each point is the mean ±SEM of 4–9 replicates (SEM was, on average, 15% of the mean). Cytosolic exchange half-times are listed in parentheses (SEM <8% of the mean).

Direct influx

Influx of NH_4^+ was also determined directly, by short-term labelling with ^{13}N. Seedlings were placed for 5 min in growth solution for equilibration, followed by immersion in labelling solution (containing $^{13}NH_4^+$), for either 1 min or 5 min (the two labelling times were used to investigate the rapidity of NH_4^+ influx response to changing external $[K^+]$). The labelling solution was either identical to the growth solution, for steady-state experiments, or contained a new $[K^+]_{ext}$, for K^+ concentration shift experiments. After labelling, plants were transferred to non-radioactive growth solution for 5 s, to reduce tracer carryover to the desorption solution, which was also identical to growth solution, and in which roots were then desorbed for 5 min. Radioactivity remaining in roots and shoots was quantified by gamma counting. Influx values obtained in this way were very close to those determined using compartmental analysis, indicating that the effect of efflux on the measurement of influx was negligible. K^+ influx was determined as described for NH_4^+, using ^{42}K (provided by the McMaster University Nuclear Reactor, Hamilton, Ontario, Canada), and a single labelling time of 5 min.

Pharmacological agents

Similar to the procedure described above, direct influx measurements by short-term labelling with ^{13}N (or ^{42}K) were conducted, in the presence of the channel inhibitors caesium (Cs^+), lanthanum (La^{3+}), and tetraethylammonium (TEA). Seedlings were placed for 10 min in growth solution for equilibration, containing 10 mM Cs^+, La^{3+}, or TEA. Labelling with ^{13}N (or ^{42}K), and subsequent solution exchanges, were identical to the above procedure except that all solutions contained the appropriate channel inhibitor.

Tissue ammonium determination

To measure tissue NH_4^+ content, barley seedlings were harvested and desorbed for 5 min in 10 mM $CaSO_4$ to remove extracellular NH_4^+. Roots and shoots were then separated and weighed, then transferred to polyethylene plastic vials and frozen in liquid N_2 for storage at –80 °C. Approximately 0.5 g of root or shoot tissue was homogenized under liquid N_2 using a mortar and pestle, followed by the addition of 6 ml of formic acid (10 mM) for the purpose of extracting NH_4^+ (Husted *et al*., 2000). Subsamples (1 ml) of the homogenate were centrifuged at 2.5×10^4 *g* at 2 °C for 10 min. The supernatant was transferred to 2 ml polypropylene tubes with 0.45 μm nylon filters (Costar, Corning Inc., USA) and centrifuged at 5×10^3 *g* (2 °C) for 5 min. The resulting supernatant was analysed by either the indophenol colorimetric (Berthelot) method or the *o*-phthalaldehyde (OPA) method to determine total tissue NH_4^+ content.

Indophenol method: This method has been described in detail elsewhere (Solorzano, 1969; Husted *et al*., 2000). Briefly, three solutions were combined with 1.6 ml of tissue extract: (i) 200 μl of 11 mM phenol in 95% (v/v) ethanol; (ii) 200 μl of 1.7 mM sodium nitroprusside (prepared weekly); and (iii) 500 μl of solution containing 100 ml of 0.68 M trisodium citrate in 0.25 M NaOH with 25 ml of commercial strength (11%) sodium hypochlorite. The colour was allowed to develop for 60 min at room temperature (25 °C) in the dark, and sample absorbance was measured at 640 nm.

OPA method: This method has been described in detail elsewhere for use with spectrophotometry (Goyal *et al*., 1988). Briefly, 100 ml of OPA reagent was prepared by combining 200 mM potassium phosphate buffer (composed of equimolar amounts of potassium dihydrogen phosphate and potassium monohydrogen phosphate), 3.75 mM OPA, and 2 mM 2-mercaptoethanol 1 d before use. Prior to the addition of 2-mercaptoethanol, the solution pH was adjusted to 7 with 1 M NaOH, and filtered through a grade 2 Whatman filter paper. A 10 μl aliquot of tissue extract was combined with 3 ml of OPA reagent, the colour was allowed to develop in the dark for 30 min at room temperature (25 °C), and sample absorbance was measured at 410 nm.

Root respiration and energy cost of transport

Root respiration was determined in intact seedlings using a Hansatech oxygen electrode and Oxygraph control system (Hansatech Instruments, Norfolk, UK). Seedlings were placed in a cuvette with 2.5 ml of air-saturated growth solution. The decline in O_2 concentration was monitored for ~15 min, but only the initial linear decline was used to calculate O_2 depletion rates. The energy costs of ion transport were calculated based upon the following equation:

$$1/U_{\text{theor}} = \left[(H/I_j)\times M_j\right]/\left[(H/P)\times(P/O_2)\right]$$

where $1/U_{\text{theor}}$ is the cost of active ion transport (mol O_2 mol^{-1} ion), H/I_j is the proton/ion stoichiometry, M_j is the number of membranes crossed (one in the present instance), H/P is the number of protons pumped by the hydrolysis of one ATP to ADP, and P/O_2 is the efficiency of oxidative phosphorylation (Kurimoto *et al*., 2004). The application of this equation relies on three major assumptions. (i) The transport of any cation or anion is obligatorily coupled to the export of protons by the plasma membrane ATPase, for charge-balancing purposes, and, additionally, in the case of active transport, to maintain the proton gradient that provides energy for the flux. In the present case, LATS-range NH_4^+ and K^+ transport are assumed to involve an electrogenic uniport that requires charge balancing via the outward pumping of one proton per NH_4^+ or K^+ entering the cell (see Britto and Kronzucker, 2005, 2006). (ii) The stoichiometry of proton export from plant cells via the plasma membrane H^+-ATPase is 1 ATP hydrolysed to 1 H^+ exported. (iii) The phosphorylation ratio that quantitatively links respiratory O_2 consumption to ATP production is ~5 ATP:O_2. Therefore, in the application of this model to the primary unidirectional influx of NH_4^+ or K^+, across the root plasma membrane, $H/I_j=1$, $H/P=1$, and $P/O_2=5$, resulting in $1/U_{\text{theor}}=0.2$ (mol O_2 per mol ion transported). This value of $1/U_{\text{theor}}$

was multiplied by the influx of NH_4^+ or K^+ to determine the theoretical O_2 consumed to sustain the flux. For further details on the application of this model to passive cation influx operating concomitantly with active cation efflux and proton pumping, see Britto and Kronzucker (2006).

Statistical analysis

Statistical analyses were conducted using one-way analysis of variance (ANOVA) with the statistical package SPSS (version 12).

Results

Steady-state NH_4^+ fluxes are strongly affected by K^+

Figure 1 shows the time-dependent efflux of $^{13}NH_4^+$ from roots of 7-d-old intact barley seedlings. The semi-logarithmic plots displayed a compoundly exponential character, and could be precisely resolved into three kinetically distinct linear phases, each representing tracer released from a separate subcellular compartment (Siddiqi *et al*., 1991; Kronzucker *et al*., 1995; Britto and Kronzucker, 2003). Slopes of each linear phase yielded half-times of exchange ($t_{1/2}$) for each compartment. The more rapidly exchanging phases, representing the extracellular surface film and Donnan free space, had $t_{1/2}$ values of 7 s and 59 s, respectively, while the slowest exchanging compartment, identified as the cytosol (Kronzucker *et al*., 1995; Britto and Kronzucker, 2003), had a $t_{1/2}$ of 14 min for the high external K^+ conditions, and 21 min for the lowest $[K^+]_{ext}$ (Fig. 1). Compartment identification was rigorously ascertained in previous studies (Kronzucker *et al*., 1995; Britto and Kronzucker, 2003), and the magnitude of NH_4^+ influx as determined using compartmental analysis (Fig. 2) was confirmed by direct influx measurements (Fig 2, inset; see Szczerba *et al*., 2006*b*).

Root and shoot NH_4^+ tissue content was determined using two independent methods, indophenol and OPA (Table 1). The values obtained by both methods were in excess of what is necessary to account for the $[NH_4^+]_{cyt}$ estimates calculated by compartmental analysis. Following the pattern of changing NH_4^+ activity in the cytosol (which dropped from 240–580 mM to 90–150 mM, depending on activity coefficients used; see Fig. 6), raising the external $[K^+]$ from 0.1 mM to 40 mM dramatically reduced the root tissue content of NH_4^+, from 70 to 14 μmol g^{-1} (root FW).

Net fluxes found with compartmental analysis were similar across treatments, while efflux and influx varied dramatically with external K^+ supply (Fig. 2). At the lowest $[K^+]_{ext}$ condition of 0.1 mM, under which K^+ influx is mediated by a high-affinity transport system (HATS), NH_4^+ influx was significantly greater than under all other conditions, with a rate of 92 μmol g^{-1} h^{-1}. However, when $[K^+]_{ext}$ was elevated into the low-affinity K^+ transport range (≥1.5 mM), NH_4^+ influx declined by as much as 63%, to 34 μmol g^{-1} h^{-1}. Even more dramatic

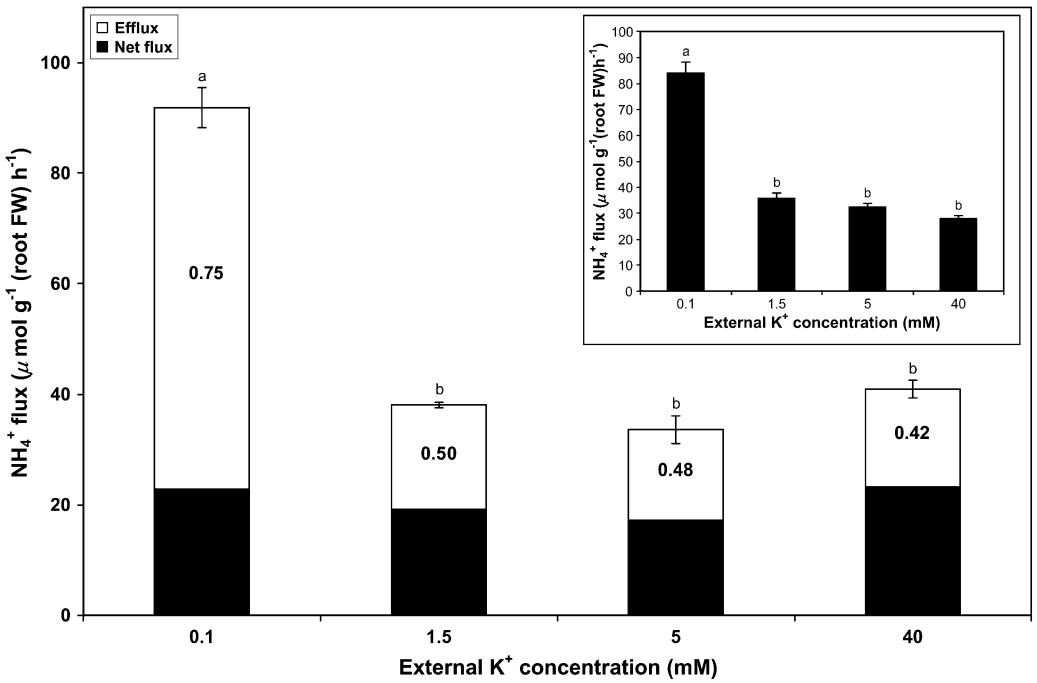

Fig. 2. Steady-state component fluxes of NH_4^+ in roots of barley grown at 10 mM NH_4^+ and at four external K^+ concentrations (as indicated), as determined by compartmental analysis. Bars are divided into net flux (filled segments) and efflux (open segments), which together comprise the influx term. Error bars refer to ±SEM of 4–5 replicates. Different letters refer to significantly different influx means (P <0.05). Inset: direct NH_4^+ influx measurements using short-term labelling for the four K^+ conditions. Error bars refer to ±SEM of 3–18 replicates.

was the effect of elevated K^+ on NH_4^+ efflux, which was reduced by as much as 75%, from 69 μmol g^{-1} h^{-1} to 17 μmol g^{-1} h^{-1}. Because of this differential effect on unidirectional NH_4^+ fluxes, the ratio of efflux to influx declined substantially when $[K^+]_{ext}$ was raised, from 0.75 to as little as 0.42.

Increasing external potassium beyond the LATS threshold value of 1.5 mM did not further reduce NH_4^+ influx or the efflux:influx ratio. Thus, the constant residual flux observed throughout the LATS range is identified as the K^+-insensitive component of NH_4^+ influx.

Table 1. *Tissue NH_4^+ content of roots and shoots of barley seedlings, grown with 10 mM $[NH_4^+]_{ext}$, as determined by the indophenol and OPA methods*

Each value represents the mean ±SEM of 6–7 replicates. Different letters within a column and within a method of NH_4^+ determination refer to significantly different values (P <0.05).

Assay method	$[K^+]_{ext}$ (mM)	Root content [μmol g^{-1} (root FW)]	Shoot content [μmol g^{-1} (root FW)]
Indophenol (Berthelot)	0.1	69.60±2.66 a	43.82±0.72 a
	1.5	22.60±0.49 b	9.47±0.41 b
	40	14.31±0.46 c	9.39±0.39 b
OPA	0.1	71.42±1.00 a	39.80±1.76 a
	1.5	22.93±1.46 b	10.61±0.19 b
	40	15.07±0.32 c	10.72±0.22 b

Elevated K^+ rapidly decreases unidirectional NH_4^+ influx

Figure 3 shows the influx of NH_4^+ into intact barley seedlings, as determined by short-term (5 min) accumulation of $^{13}NH_4^+$. In agreement with compartmental analysis, NH_4^+ fluxes were maximal at 0.1 mM $[K^+]_{ext}$ (reaching a peak value of 84 μmol g^{-1} h^{-1}). However, when low-potassium seedlings were exposed to elevated (5 mM) $[K^+]_{ext}$, NH_4^+ influx was drastically and immediately reduced, by 26% after the first minute of exposure, and by nearly 50% within 5 min (Fig. 3).

A sudden increase in $[K^+]_{ext}$ decreased not only the influx of NH_4^+, but also its efflux (Fig. 4). After introducing an elevated (5 mM) concentration of K^+ midway through an elution protocol, NH_4^+ efflux declined notably within a few minutes. Within 15 min following the shift in $[K^+]_{ext}$, the half-time of cytosolic NH_4^+ exchange appeared to have been re-established to the value seen prior to the shift (Fig. 4). The reverse change in $[K^+]_{ext}$, from high to low, however, did not immediately elevate NH_4^+ efflux (Fig. 4, inset).

La^{3+} application mimics K^+ inhibition of NH_4^+ influx

The channel inhibitors TEA, Cs^+, and La^{3+}, which have been shown to reduce channel-mediated fluxes of K^+ and NH_4^+ (Wegner *et al.*, 1994; Nielsen and Schjoerring, 1998), were used to help identify the mechanisms underlying the K^+-sensitive and -insensitive components of

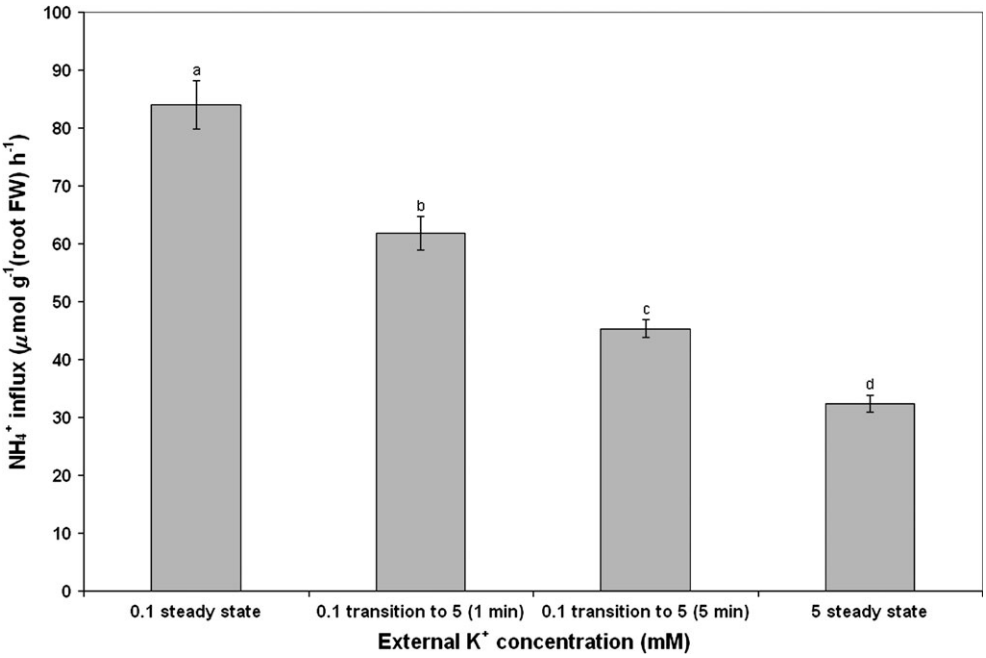

Fig. 3. Effect of K^+ alterations on NH_4^+ influx, directly measured using short-term labelling. Barley seedlings were either grown and tested at steady-state K^+ conditions (0.1 mM or 5 mM $[K^+]_{ext}$), or grown at 0.1 mM $[K^+]_{ext}$ but labelled in solution containing 5 mM $[K^+]_{ext}$, for 1 min or 5 min. All solutions contained 10 mM $[NH_4^+]_{ext}$. Error bars refer to ±SEM of 9–17 replicates. Different letters refer to significantly different means (P <0.05).

NH_4^+ transport (Fig. 5). Interestingly, Cs^+ and TEA both stimulated NH_4^+ influx, under high (40 mM) and low (0.1 mM) $[K^+]_{ext}$, with TEA in particular increasing NH_4^+ influx by nearly 40%. In contrast, La^{3+} application reduced the influx of NH_4^+ by 60% under low $[K^+]_{ext}$ (0.1 mM). At elevated $[K^+]_{ext}$, no reduction in NH_4^+ influx was observed. The effects of La^{3+} upon K^+ influx were also tested, using ^{42}K as a tracer (Fig. 5, inset).

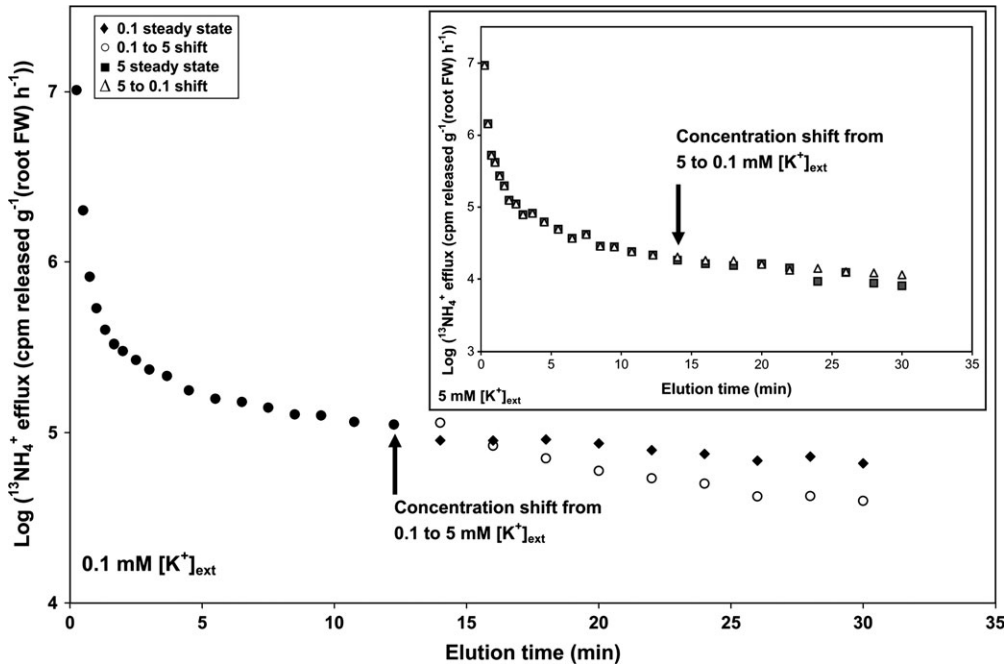

Fig. 4. Effect of K^+ alteration on $^{13}NH_4^+$ efflux from the roots of intact barley seedlings grown with 10 mM $[NH_4^+]_{ext}$ and 0.1 mM $[K^+]_{ext}$. The arrow indicates time of shift in $[K^+]_{ext}$ from 0.1 mM to 5 mM. Each point is the mean ±SEM of 4–9 replicates (SEM was, on average, <9% of the mean), and points prior to the $[K^+]$ shift are pooled. Inset: reverse effect of K^+ alteration on $^{13}NH_4^+$ efflux from the roots of intact barley seedlings grown with 10 mM $[NH_4^+]_{ext}$ and 5 mM $[K^+]_{ext}$. The arrow indicates time of shift in $[K^+]_{ext}$ from 5 mM to 0.1 mM. Each point is the mean ±SEM of 2–6 replicates (SEM was, on average, <17% of the mean).

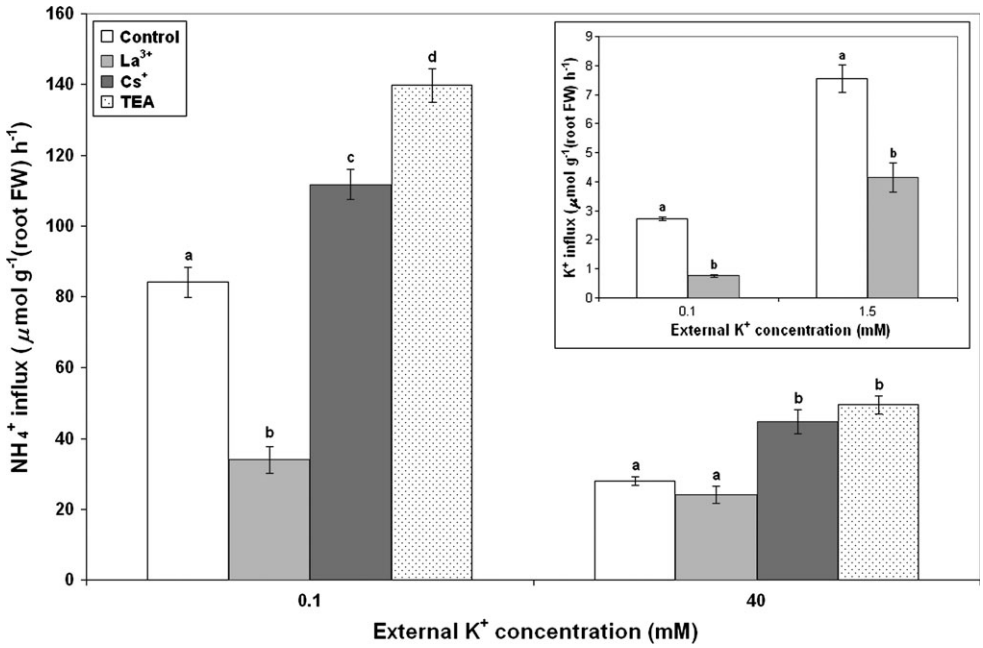

Fig. 5. Effect of channel inhibitors on direct NH_4^+ influx measurements using short-term labelling. Error bars refer to ±SEM of 6–18 replicates. Different letters refer to significantly different means within a $[K^+]_{ext}$ (P <0.05). Inset: direct K^+ influx measurements using short-term labelling, in the presence La^{3+} (legend as in larger figure). Error bars refer to ±SEM of 5–10 replicates.

Surprisingly, K^+ influx was reduced by 70% at the HATS concentration of 0.1 mM, but only by 45% at the LATS concentration of 1.5 mM.

Active NH_4^+ and K^+ fluxes can be energetically costly

The energetics of NH_4^+ transport were analysed using previously reported plasma membrane electrical potentials from the same plant system (Szczerba *et al*., 2006*a*) and cytosolic concentrations of NH_4^+ measured in the present study using compartmental analysis (Fig. 6). The range of activities presented in Fig. 6 were determined using activity coefficients corresponding either to a cytosol dominated by K^+, NH_4^+, and a univalent anion (upper estimate), or to a simple solution of $(NH_4)_2SO_4$ (lower estimate) (Nobel, 1991; Lide, 2007). Although the cytosolic activities of NH_4^+ were significantly different at low and high $[K^+]_{ext}$, the electrochemical potential gradient for NH_4^+ was inwardly directed in both cases. Thus, NH_4^+ influx was determined to occur via facilitated diffusion into the plant cell, and its efflux, in turn, would be energy demanding.

To test the model and relate it to growth and NH_4^+ toxicity, root respiration experiments were conducted (Fig. 7). Specific respiratory costs of the active components of K^+ and NH_4^+ fluxes were determined based on current models of energy usage (Kurimoto *et al*., 2004) and by use of K^+ fluxes determined previously (Szczerba *et al*., 2006*a*). In all conditions tested, the respiratory costs not associated with NH_4^+ and K^+ fluxes were similar. The two conditions with the lowest energy requirement for NH_4^+ and K^+ transport (1.5 mM and 5 mM K^+) had the lowest overall root respiration, but the largest root and shoot masses (Fig. 7).

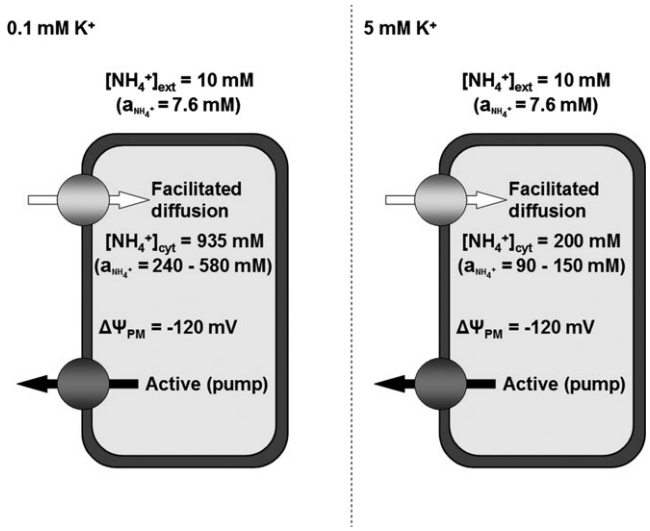

Fig. 6. Model of NH_4^+ unidirectional plasma membrane fluxes mediated by a low-affinity transport system, and at K^+ concentrations representing two K^+ influx mechanisms (HATS and LATS). Listed are the directions of active and passive NH_4^+ fluxes and measured plasma membrane electrical potentials ($\Delta\psi$, from Szczerba *et al*., 2006*a*). Also included are measured cytosolic NH_4^+ concentrations and ranges of NH_4^+ activities calculated using two estimates for the ionic strength (*I*) of the cytosol: (i) *I* based on K^+, NH_4^+, and a monovalent anion—upper value; or (ii) *I* based on a simple solution of $(NH_4)_2SO_4$—lower value.

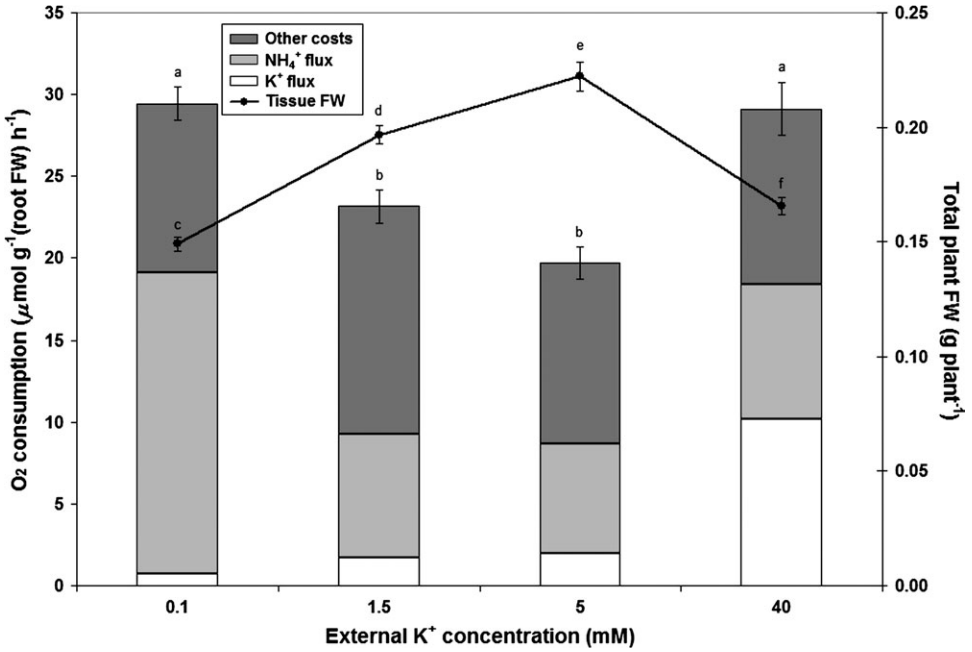

Fig. 7. Measured rate of oxygen uptake and whole-plant fresh weights for individual barley seedlings. Each bar (oxygen uptake) has been divided into the amount of respiration necessary to drive K^+ or NH_4^+ influx, and for maintenance and growth. Error bars refer to ±SEM of 12–14 replicates. Each point (whole plant FW) refers to the sum of roots+shoots. Error bars refer to ±SEM of 90–270 individual seedlings. Different letters refer to significantly different means: a, b (oxygen uptake); c–f (whole-plant FW) (P <0.05).

Discussion

Rapid, futile NH_4^+ cycling

Efflux of ions from plant cells into the external environment can be monitored using several techniques, but none is as comprehensive as compartmental analysis by tracer efflux, which facilitates the simultaneous measurement of unidirectional fluxes, subcellular concentrations, and compartmental exchange rates of labelled ions (Lee and Clarkson, 1986; Siddiqi *et al*., 1991; Britto *et al*., 2001; Kronzucker *et al*., 2003*b*; Szczerba *et al*., 2006*a*). This technique initially led to the discovery of rapid, futile cellular NH_4^+ cycling in a number of plant systems (Britto *et al*., 2001, 2002; Kronzucker *et al*., 2003*a*; Britto and Kronzucker, 2006), and to the close association of this phenomenon with NH_4^+ toxicity (Kronzucker *et al*., 2001). In the present study, the high unidirectional fluxes of NH_4^+ in both directions across the plasma membrane of barley root cells were confirmed, as well as the high ratio of efflux to influx, both of which are key characteristics of the futile cycling condition (Fig. 2).

Because NH_4^+ toxicity in plants can be relieved by increasing potassium availability (Cao *et al*., 1993; Spalding *et al*., 1999; Santa-María *et al*., 2000; Kronzucker *et al*., 2003*b*; Szczerba *et al*., 2006*a*), it was hypothesized that changes in external $[K^+]$ would alter the magnitude of NH_4^+ fluxes and the degree of futile cycling in NH_4^+-susceptible barley plants. As shown in Fig. 2, this hypothesis was borne out on both counts. Changing the steady-state K^+ supply from 0.1 mM to 1.5 mM significantly reduced both influx and efflux of NH_4^+, and substantially decreased the ratio of efflux to influx. Increasing $[K^+]_{ext}$ to higher values (5 mM and 40 mM) had no further effect on these flux parameters. Importantly, this shift is also associated with relief from NH_4^+ toxicity (Fig. 7). Increasing $[K^+]_{ext}$ from 0.1 mM to 1.5 mM or above caused a reduction in both root tissue and cytosolic NH_4^+ of ~70%, showing that the amelioration of NH_4^+ toxicity by increasing $[K^+]_{ext}$ was paralleled by a reduction in NH_4^+ tissue content (Fig. 6, Table 1). The $[NH_4^+]_{cyt}$ values for the toxic condition, while high, are in agreement with values reported in a separate study on NH_4^+ toxicity in barley seedlings (Britto *et al*., 2001). Moreover, the tissue NH_4^+ values are well in excess of what is required to account for the $[NH_4^+]_{cyt}$ estimates, demonstrating that a substantial NH_4^+ pool is also present in the vacuole. While vacuolar pools were not directly measured here, since the focus of the study was the futile cycling of NH_4^+ at the plasma membrane, subtraction of cytosolic content from whole-root content of NH_4^+ yields estimates of vacuolar pools that range between 2.4 and 25 μmol g^{-1} (FW) (depending on N and K status; not shown), in good agreement with prior studies (Lee and Ratcliffe, 1991; Wang *et al*., 1993).

While the dramatic differences in key flux parameters shown in Fig. 2 were determined under steady-state nutritional conditions, it was of further interest to examine the time scale over which K^+-induced alterations in NH_4^+ transport occur. Figures 3 and 4 show that these potent effects become manifest within 1–5 min of increased K^+ supply, for both influx and efflux of NH_4^+. The rapidity of this response suggests that K^+ regulates NH_4^+ fluxes possibly by acting allosterically on an ammonium transporter, or by competing directly with NH_4^+ for a common transport mechanism. Such short-term changes are likely to precede longer-term changes that involve alterations in gene expression, that would bring about the further lowering of NH_4^+ influx seen in the rightmost column in Fig. 3. The lack of an immediate stimulatory effect on NH_4^+ influx by a reduction in external $[K^+]$ may indicate that the shift from the high-K^+ condition to the low-K^+ condition entails the up-regulation of NH_4^+-sensitive transporters, which would occur over a longer time scale. Alternatively or in addition, it may indicate that the release of K^+ from inhibitory binding sites on NH_4^+ transporters also occurs over a longer time scale.

K^+-sensitive NH_4^+ influx pathway

It is not yet fully resolved how NH_4^+ enters the plant cell, particularly in the low-affinity range. The present study provides new insight into possible candidates and characteristics of low-affinity NH_4^+ transport. The dramatic reduction of NH_4^+ fluxes and cycling brought about by elevating $[K^+]_{ext}$ from 0.1 mM to 1.5 mM or higher (Fig. 2) is strong evidence that at least two pathways of NH_4^+ influx operate simultaneously, one sensitive and the other insensitive to $[K^+]_{ext}$. Because NH_4^+ influx under LATS conditions is high capacity and energetically passive (Fig. 6; also see section on energetics below), it is very likely that ion channels are responsible for catalysing both components of the flux.

More specifically, the present evidence suggests that the K^+-sensitive pathway, which catalyses the greater amount of NH_4^+ influx, involves the operation of either non-selective cation channels (NSCCs) or inward rectifying K^+ channels, such as AKT1. NSCCs comprise a large group of relatively uncharacterized transporters that have been shown to transport a variety of ions, including Na^+, Ca^{2+}, K^+, and NH_4^+ (Demidchik *et al*., 2002*b*). NSCCs are inhibited by lanthanides, but tend to be insensitive to traditional K^+ channel blockers, particularly Cs^+ and TEA (Tyerman and Skerrett, 1999). In the present study, NH_4^+ influx at 0.1 mM $[K^+]_{ext}$ was strongly inhibited by the relatively broad-spectrum channel blocker La^{3+}, but no La^{3+} effect was observed on the K^+-suppressed flux at 40 mM $[K^+]_{ext}$ (Fig. 5). This is consistent with the idea that, at this supply level, K^+ much more effectively competes for a La^{3+}-sensitive NSCC pathway, making it unavailable for NH_4^+ transport. Evidence that K^+ is indeed also transported through the La^{3+}-sensitive pathway is seen in the inhibitory effect of

La^{3+} on K^+ influx (Fig. 5, inset; the inhibitory effect on the low-K^+ control is most likely to be due to the suppression of high-affinity KUP/HAK/KT transporters by NH_4^+; see Spalding *et al*., 1999, and Introduction). The lack of inhibition of NH_4^+ influx by Cs^+ and TEA is also consistent with permeation through NSCCs. Indeed, it is tempting to attribute K^+-sensitive, La^{3+}-sensitive NH_4^+ transport to the activity of the weakly voltage-dependent NSCC described by White and Lemtiri-Chlieh (1995), White (1996), and Davenport and Tester (2002), because this channel displays several physiological attributes strongly reminiscent of the characteristics defined here: transport of both NH_4^+ and K^+ as competing substrates; relative insensitivity to TEA and Cs^+; and strong inhibition by the lanthanide Gd^{3+} as well as by La^{3+} itself. The observation that, in the present study, TEA and Cs^+ actually stimulated NH_4^+ influx at both K^+ conditions tested (Fig. 5) is perhaps surprising, but supports the finding that both of these agents can increase Na^+ influx (Wang *et al*., 2006), and TEA has also been shown to increase the influx of both Ca^{2+} (Demidchik *et al*., 2002a) and Cs^+ (Hampton *et al*., 2004) in roots of *Arabidopsis*. In addition, both of the latter studies postulated that the transporters involved were NSCCs, and showed that Gd^{3+} was effective in inhibiting the TEA-stimulated fluxes, a result very similar to the La^{3+} suppression of TEA-stimulated NH_4^+ influx observed in the present study.

Many studies, in a variety of organisms including bacteria, yeast, animals, and plants, have suggested that NH_4^+ enters the cell through K^+-specific channels (Wang *et al*., 1996; Nielsen and Schjoerring, 1998; Hess *et al*., 2006), and the K^+ suppression of NH_4^+ influx (Fig. 2) supports the idea that K^+ channels are responsible for the K^+-sensitive component of low-affinity NH_4^+ uptake, instead of, or in addition to, NSCCs. At higher $[K^+]_{ext}$, these channels would be occupied by K^+, limiting NH_4^+ influx to NH_4^+-specific pathways. Consistent with this observation was the finding that La^{3+} (known to block K^+ channels as well as NSCCs; Wegner *et al*., 1994) blocked NH_4^+ influx at low $[K^+]_{ext}$, in addition to reducing K^+ influx at both high and low $[K^+]_{ext}$ (Fig. 5). However, the stimulation of NH_4^+ influx by the K^+-channel blockers Cs^+ and TEA does not support NH_4^+ permeation through K^+ channels. Thus, the proposal that NSCCs are responsible for the K^+-sensitive component of root NH_4^+ influx in the LATS range is more congruent with the present data. Low-affinity fluxes in shoots may be mediated by a different mechanism, as was shown by Nielsen and Schjoerring (1998), who observed in leaves of *B. napus* a 30% and 47% reduction in NH_4^+ influx with La^{3+} and Cs^+ treatments, respectively.

K^+-insensitive NH_4^+ influx pathway

In addition to K^+-sensitive NH_4^+ conductance, a substantial portion of low-affinity NH_4^+ entry into barley root cells is mediated by a K^+-insensitive mechanism (Fig. 2). This mechanism is resistant to increases in $[K^+]_{ext}$ from 1.5 mM to 40 mM, suggesting that, because of the lack of a competitive effect, the transporter involved is neither a K^+-specific channel nor an NSCC. Several other possibilities arise as to its molecular identity. One is that it is a high-affinity NH_4^+ transporter, such as AMT1 (Ninnemann *et al*., 1994; Rawat *et al*., 1999), which may have some dual-affinity character, such as has been seen for nitrate and potassium transporters (Fu and Luan, 1998; Liu *et al*., 1999). However, AMT1-mediated NH_4^+ transport is down-regulated by high NH_4^+, both genetically and functionally (Rawat *et al*., 1999), which eliminates its likelihood as a candidate for NH_4^+ influx under the NH_4^+ supply (10 mM) used in the present study. Another possibility is that NH_4^+ permeates via aquaporins. Several recent studies have shown that in addition to water, *Xenopus* oocytes expressing *Arabidopsis* TIP genes (encoding aquaporins) could mediate the transport of small molecules such as CO_2, glycerol, urea, NH_4^+, and NH_3 (Uehlein *et al*., 2007). However, Detmers *et al*. (2006) found that TEA effectively inhibited aquaporin-mediated water transport, while in the present study TEA failed to inhibit NH_4^+ influx, casting doubt on the role of aquaporins in low-affinity NH_4^+ transport. The elimination of these two candidates suggests that NH_4^+ enters root cells under high-K^+, high-NH_4^+ conditions via NH_4^+-specific channels, the molecular identity of which remains to be determined.

Energetics of NH_4^+ and K^+ unidirectional fluxes

It is instructive to examine, from an energetics perspective, the unidirectional NH_4^+ fluxes observed here. The thermodynamic analysis shows that, under all experimental conditions, NH_4^+ exchange across the plasma membrane takes the form of a 'leak–pump' scenario, i.e. with passive NH_4^+ influx coupled to active NH_4^+ efflux (Fig. 6). In this respect, bidirectional NH_4^+ transport follows a pattern that has been observed in the low-affinity exchange of other major cations, such as K^+ (Szczerba *et al*., 2006*a*) and Na^+ (Wang *et al*., 2006; Kronzucker *et al*., 2006). Based upon current models of ion transport (Kurimoto *et al*., 2004; Britto and Kronzucker, 2006), which consider the coupling and stoichiometry of ion fluxes in relation to proton fluxes and ATP hydrolysis, the respiratory costs of NH_4^+ and K^+ transport weres estimated, and they were compared with measured respiration rates and growth (Fig. 7). In agreement with estimates indicating that, under certain conditions, as much as 70% of total root respiration can be invested in the transport of the NO_3^- anion (Scheurwater *et al*., 1999), it was found that as much as 64% of the measured respiration rates could be accounted for by the combined plasma membrane fluxes of the two cations NH_4^+ and K^+. The plants that displayed rapid, futile cycling of NH_4^+ or K^+ not only showed the highest respiration, but had significant reductions in total plant biomass (Fig. 7). This effect is

attributed to the differential allocation of carbohydrate supply in the various treatments, with a greater proportion directed towards the wasteful process of futile ion cycling, in the case of the growth-compromised plants.

Concluding remarks

This study provides the first demonstration of the parallel operation of K^+-sensitive and -insensitive root NH_4^+ fluxes in the low-affinity transport range, and offers insight into the mechanism by which K^+ is able to alleviate NH_4^+ toxicity. Elevated K^+ eliminates a major fraction of low-affinity NH_4^+ influx, and substantially reduces the amount of futile cycling of this toxic ion. Intriguingly, this effect contrasts sharply with the effect of NH_4^+ on K^+ transport, where high-affinity influx is diminished by NH_4^+, but low-affinity influx remains unaffected (Spalding *et al*., 1999; Kronzucker *et al*., 2003*b*; Szczerba *et al*., 2006*a*). It is proposed that low-affinity NH_4^+ transport may be mediated by the dual operation of non-selective, K^+-sensitive cation channels on the one hand, and K^+-insensitive, NH_4^+-specific channels on the other. However, it should be pointed out that the present study does not rule out the existence of other mechanisms of low-affinity NH_4^+ transport, in addition to the two proposed here. The shift between low and high external [K^+] steady states may entail the expression of a genetically and mechanistically distinct complement of transporters, a possibility that only extensive new genetic analyses can unravel. Nevertheless, the physiological observations presented here, that: (i) the NH_4^+ fluxes under both high- and low-K^+ conditions show virtually the same degree of La^{3+} resistance (Fig. 5); and (ii) NH_4^+ influx is rapidly suppressed, when the low-K^+ condition is suddenly altered to a high-K^+ condition, almost to the same extent as observed at a high-K^+ steady-state, strongly suggest that the La^{3+}- and K^+-insensitive component of low-affinity NH_4^+ influx is operative under all K^+ conditions. This study demonstrates how pivotal a role K^+ plays in the regulation of NH_4^+ toxicity, reducing the energy burden of toxic NH_4^+ fluxes, and substantially improving growth under a high-NH_4^+ nutritional regime.

Acknowledgements

We thank Dr A Wilson and the staff at the Centre for Addiction and Mental Health (CAMH) in Toronto, Ontario, Canada, for supplying the $^{13}NH_4^+$, and M Butler and staff at McMaster University in Hamilton, Ontario, Canada, for supplying the ^{42}K required to conduct these experiments. We also would like to thank S Ebrahimi and AB Vesterberg for assistance with experiments. The work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canada Research Chair (CRC) program, Canada's National Green Crop Network (NSERC), and the International Plant Nutrition Institute [formerly the Potash & Phosphate Institute (PPI)].

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